The Role of the Sulfhydryl Groups of Lactic Dehydrogenases*

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Inhibition of enzymatic activity of beef heart lactic dehydrogenase, chicken heart lactic dehydrogenase, and chicken muscle lactic dehydrogenase due to binding of *p*-mercuribenzoate and mercuric chloride to the sulfhydryl groups of the enzymes has been studied. Some 90–97% of the enzymatic activity is lost upon binding of 4 moles of mercurial per mole of enzyme of molecular weight 135,000. Reduced 3-acetylpyridine adenine dinucleotide protects the enzymes from binding of *p*-mercuribenzoate; less but definite protection is shown by oxidized nicotinamide adenine dinucleotide, reduced nicotinamide adenine dinucleotide, and oxidized 3-acetylpyridine adenine dinucleotide; pyruvate and lactate do not show any protection. The optical rotation parameters, fluorescence, sedimentation constant, and immunologic properties of the enzymes bound to mercurials are the same as for the native enzymes. Inhibition is also obtained with methyl mercuric iodide. These data provide evidence for the involvement of the sulfhydryl groups of lactic dehydrogenases in the mechanism of action of these enzymes and for the fact that the subunits of the enzymes can operate independently from each other.

Inhibition of lactic dehydrogenase activity caused by binding of sulfhydryl reagents has been demonstrated by several authors for the enzyme from beef heart (Nielands, 1954; Takenaka and Schwert, 1956; Nygaard, 1956), as well as from other sources (Pfleiderer *et al.*, 1959). However, a simple relationship between the amount of reagent bound and inhibition of enzymatic activity has not been demonstrated, nor has the effect of coenzymes on the binding of sulfhydryl reagents to the enzymes been fully studied. Moreover, no evidence has been provided that binding of the reagents to the enzyme is not accompanied by structural modifications of the protein which could be responsible for the loss in enzymatic activity.

The present paper deals with these features of the inhibition by SH reagents of lactic dehydrogenases in relation to the mechanism of action of these enzymes.

Experimental Procedure

BHLDH[1] was purchased from Worthington Biochemical Co. and further purified through a DEAE-cellulose column. CHLDH and CMLDH were prepared according to Pesce *et al.* (in preparation). The molecular weight of all three enzymes was assumed to be 135,000; the adopted ϵ_{max} at 280 mμ were 2.0×10^5 for BHLDH and CMLDH and 1.8×10^5 for CHLDH (Pesce *et al.*, in preparation). The enzymes studied showed a single band on electrophoresis; they were kept in 50% saturated ammonium sulfate, and before use were dialyzed against the buffer of incubation. *p*-Mercuribenzoate was purchased from Sigma Chemical Co. Methyl mercuric iodide was a product of Metalsalts Co., Hawthorne, N.J. Cysteine HCl and mercuric chloride were obtained from Fisher Scientific Co. NAD and NADH were purchased from California Corporation for Biochemical Research. AcPyAD, AcPyADH, and NHXDH were prepared as described by Kaplan and Stolzenbach (1957). Unless otherwise indicated, the enzymes were incubated at a concentration of 6×10^{-6} M, in 0.1 M sodium phosphate buffer, *p*H 6.9, with 1- to 10-fold molar excess *p*-mercuribenzoate; the temperature of incubation was 20° for the experiments with BHLDH and 25° for the experiments with CHLDH. The amount of *p*-mercuribenzoate bound to the enzyme was determined according to the method of Boyer (1954). An ϵ_{max} of 7.6×10^3 was applied for the increase in optical density at 250 mμ due to mercaptide formation. In the experiments in which the enzymatic activity was measured, a small aliquot was pipetted from the incubation mixture at suitable intervals of time and quickly diluted in 0.1 M ice cold sodium phosphate buffer, *p*H 6.9; 0.03 ml was taken from this solution and used to assay enzymatic activity. The activity of the enzymes was determined spectrophotometrically at 340 mμ. For reduction of pyruvate to lactate, the additions to a 3.0-ml cuvet were made in the following order: 2.9 ml of 0.1 M sodium phosphate buffer, *p*H 6.9, 0.03 ml of 0.02 M sodium pyruvate, and 0.03 ml of 0.014 M NADH; for oxidation of lactate to pyruvate: 2.9 ml of 0.1 M glycine-NaOH buffer, *p*H 10.0, 0.04 ml of 0.2 M lithium L-lactate, and 0.04 ml of 0.02 M NAD. In both cases, the reaction was started by addition of enzyme; the optical density was measured for 1 minute at 15-second intervals. All spectrophotometric measurements were made with a Zeiss spectrophotometer, model PMQ 11. In the experiments at high concentrations of NAD and AcPyAD (cf. Table III) the light path was reduced to 0.5 mm by means of a quartz insert in order to decrease the initial absorbance. Fluorescence measurements were made with an Aminco - Bowman spectrofluorophotometer using a quartz cell having a 1-cm light path; the light source was a xenon lamp. Optical rotatory dispersion was measured with a Rudolph model 80 photoelectric spectropolarimeter equipped with a d-c xenon lamp; about fifteen readings were taken between 589 and 350 mμ in a 2-dm cell; the enzyme concentration was 0.30–0.35 g/100 ml ($2.2–2.6 \times 10^{-5}$ M)λ_c values were calculated from the slopes of plots of $[\alpha]$ vs. $[\alpha]\lambda^2$. Sedimentation coefficients were determined with a Spinco Model E analytical ultracentrifuge; 12-mm cells were used and the concentration of the enzyme was about 6 mg/ml. All immunologic assays were performed by

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[1] Abbreviations: BHLDH: beef heart lactic dehydrogenase. CHLDH: chicken heart lactic dehydrogenase. CMLDH: chicken muscle lactic dehydrogenase. NAD and NADH: oxidized and reduced nicotinamide adenine dinucleotide. AcPyAD and AcPyADH: oxidized and reduced 3-acetylpyridine adenine dinucleotide. NHXDH: reduced nicotinamide hypoxantine dinucleotide. DEAE: diethylaminoethyl cellulose.

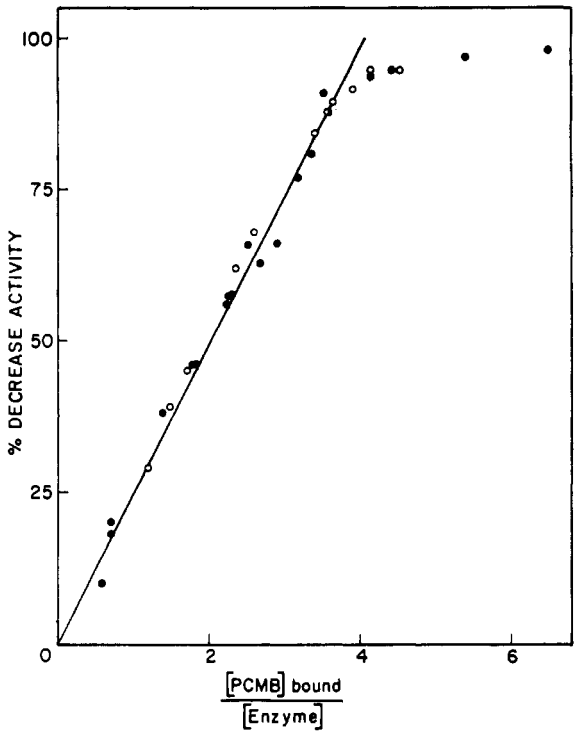

FIG. 1.—Decrease in enzymatic activity of BHLDH caused by binding of *p*-mercuribenzoate (PCMB). Line calculated on the basis of 25% loss of enzymatic activity per mole of *p*-mercuribenzoate bound; ● = activity measured by reduction of pyruvate to lactate; ○ = activity measured by oxidation of lactate to pyruvate. Incubation in 0.1 M sodium phosphate buffer, *p*H 6.9, 20°.

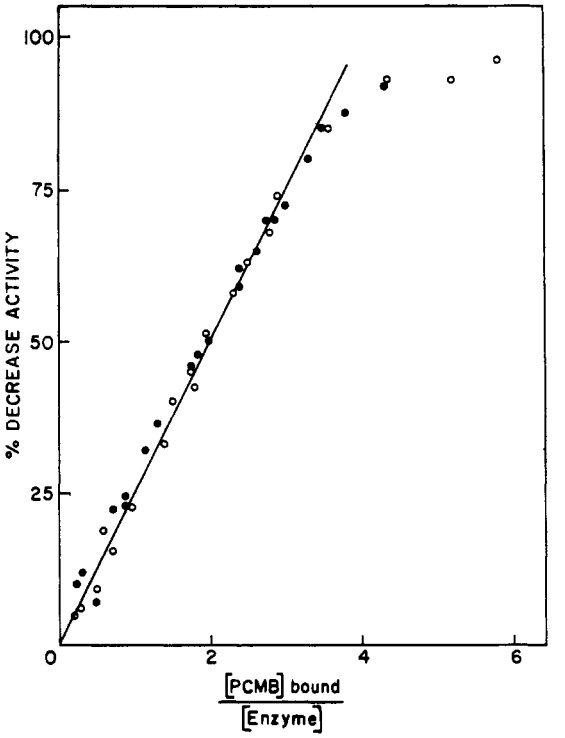

FIG. 2.—Decrease in enzymatic activity of CHLDH caused by binding of *p*-mercuribenzoate (PCMB). Line calculated on the basis of 25% loss of enzymatic activity per mole of *p*-mercuribenzoate bound. Symbols are the same as in Fig. 1. Incubation in 0.1 M sodium phosphate buffer, *p*H 6.9, 25°.

the quantitative microcomplement fixation method of Wasserman and Levine (1961).

RESULTS

Number of SH Groups in Lactic Dehydrogenases.—The total number of SH groups, determined by amperometric titration with $HgCl_2$ in the presence of 4.0 M guanidine HCl, is 17 for BHLDH, 27 for CHLDH, and 24 for CMLDH. The same amount of cysteic acid has been found for the three enzymes by amino acid analysis after treatment with performic acid, and therefore none of the enzymes seems to contain S—S bridges. Details of the SH composition of a number of lactic dehydrogenases are presented elsewhere (Di Sabato *et al.*, in press).

Effect of Binding of p-Mercuribenzoate on the Decrease of Enzymatic Activity.—The relationship between moles of *p*-mercuribenzoate bound per mole of enzyme and loss in enzymatic activity is shown in Figures 1 and 2 for BHLDH and CHLDH, respectively. The binding of *p*-mercuribenzoate to BHLDH and CHLDH (6×10^{-6} M) is accompanied by a loss in enzymatic activity assayed both by reduction of pyruvate to lactate and by oxidation of lactate to pyruvate; the loss in enzymatic activity is practically complete (90–97%) when 4 moles of *p*-mercuribenzoate are bound per mole of enzyme, and is linear in respect to *p*-mercuribenzoate concentration. The same dependence of the enzymatic activity on the amount of *p*-mercuribenzoate bound was shown by CHLDH 5×10^{-5} M. In some experiments, cysteine (about 200- to 500-fold molar excess over *p*-mercuribenzoate present in the incubation mixture) was added to the *p*-mercuribenzoate-bound enzyme, and after a few minutes the enzymatic activity was measured. The results of these experiments (Table I) show that 1 mole of enzyme can bind up to 4 moles of *p*-mercuribenzoate and still completely recover its catalytic activity upon addition of cysteine.

Effect of Binding of Other Sulfhydryl Reagents on the Decrease in Enzymatic Activity.—Incubation of BHLDH CHLDH, and CMLDH with 1-, 2-, 3-, and 4-fold molar excess $HgCl_2$ caused a decrease in activity of all three enzymes; the decreases in activity were reached within a few minutes of incubation at room temperature and remained practically constant after 60–90 minutes. Amperometric titration showed rapid and complete binding of 4–5 moles of $HgCl_2$ per mole of enzyme. Table II gives the percentage inhibition of activity due to binding of $HgCl_2$ and shows that each mole of $HgCl_2$ bound causes a loss of about one-fourth of the original enzymatic activity. Addition of cys-

TABLE I
RECOVERY OF ENZYMATIC ACTIVITY OF *p*-MERCURIBENZOATE-BOUND ENZYME UPON CYSTEINE ADDITION

Enzyme	Moles *p*-Mercuribenzoate Bound/Mole Enzyme	Activity Present[a] (%)	Activity Present After Addition of Cysteine[a] (%)
BHLDH	2.5	34	103
	3.1	16	99
	4.5	5	92
	4.2	6[b]	96[b]
CHLDH	2.4	42	91
	3.6	19[b]	92[b]

[a] Measured from reduction of pyruvate to lactate, unless otherwise indicated. [b] Measured from oxidation of lactate to pyruvate.

TABLE II
DECREASE IN ENZYMATIC ACTIVITY OF BHLDH, CHLDH, AND CMLDH
6×10^{-6} M in the Presence of $HgCl_2$

$HgCl_2$ ($M \times 10^5$)	% Decrease Enzymatic Activity		
	BHLDH[a]	CHLDH[a]	CMLDH[b]
0.6	28	22	23
1.2	52	51	48
1.8	76	74	72
2.4	95	96	95

[a] Incubation in 0.1 M Tris-acetate buffer, *p*H 7.0, at 23–24°. [b] Incubation in 0.1 M Tris-HCl buffer, *p*H 7.0, at 23–24°.

teine to the enzymes inactivated by 4 moles of $HgCl_2$ bound resulted in complete recovery of the enzymatic activity.

In a few experiments, the action of methyl mercuric iodide on the activity of CHLDH was investigated. This compound seems to react very slowly at room temperature with CHLDH in 0.1 M Tris-acetate buffer, *p*H 7.0, containing 10% dimethylformamide in order to solubilize methyl mercuric iodide. After 24 hours of incubation at room temperature with molar ratios of methyl mercuric iodide to enzyme of 2 and 4, about 40% and 70%, respectively, of the enzymatic activity was lost. The control, without methyl mercuric iodide, did not show appreciable loss in activity over the same period of time.

Effect of Coenzymes and Substrates on Binding of p-Mercuribenzoate to the Enzymes.—The results of these experiments are reported in Table III and expressed as moles of *p*-mercuribenzoate bound per mole of BHLDH and CHLDH after 30 and 120 minutes of incubation, respectively. Among the compounds tested, AcPyADH gave the best protection of both enzymes from binding of *p*-mercuribenzoate; with this coenzyme maximal protection was obtained with a molar ratio of coenzyme to enzyme of 4; a further increase in coenzyme concentration did not appreciably improve the protection. NADH, NAD, and AcPyAD gave less but definite protection. The oxidized coenzymes, because of their large dissociation constants (Takenaka and Schwert, 1956; Velick, 1958), have been used at concentrations 20- to 200-fold greater than those of the reduced coenzymes. Table III also shows that no detectable protection was given by pyruvate and lactate. As an example of the protection given by coenzymes from binding of *p*-mercuribenzoate to the enzymes, Figure 3 shows the rate of binding of *p*-mercuribenzoate to BHLDH alone and in the presence of NADH and AcPyADH. Similar results were obtained with CHLDH. However, as shown in Figure 3, CHLDH binds *p*-mercuribenzoate approximately four times more slowly than CMLDH does. This could be due to the fact that the SH groups of CHLDH are less reactive and/or more hindered than

TABLE III
EFFECT OF SUBSTRATES AND COENZYMES ON THE BINDING OF *p*-MERCURIBENZOATE TO BHLDH AND CHLDH
Enzymes 6×10^{-6} M; *p*-mercuribenzoate 6×10^{-5} M, unless otherwise indicated. Incubation in 0.1 M sodium phosphate buffer, *p*H 6.9; temperature: 20° for BHLDH, 25° for CHLDH.

Compound Tested (M)		Moles *p*-Mercuribenzoate Bound/Mole Enzyme After:	
		30 min. BHLDH	120 min. CHLDH
None		3.3	3.0
AcPyADH	1.2×10^{-5}	2.3	2.1
	1.8×10^{-5}	1.7	1.5
	2.4×10^{-5}	1.2	1.0
	5.0×10^{-5}		1.0
	6.0×10^{-5}	1.1	
NADH	1.2×10^{-5}	3.1	2.4
	1.8×10^{-5}	2.9	2.1
	2.4×10^{-5}	2.8	2.0
	4.6×10^{-5}		2.1
	6.0×10^{-5}	2.6	
Pyruvate	1.2×10^{-3}	3.4	
	9.0×10^{-4}		3.2
Lactate	1.0×10^{-3}	3.5	
	9.0×10^{-4}		3.1
None		3.8[a]	4.9[b]
AcPyAD	1.0×10^{-3}	2.6[a]	3.4[b]
NAD	2.0×10^{-3}	2.9[a]	3.6[b]

[a] Enzyme 2.1×10^{-5} M, *p*-mercuribenzoate 2.0×10^{-4} M. [b] Enzyme 3.6×10^{-5} M, *p*-mercuribenzoate 3.6×10^{-4} M.

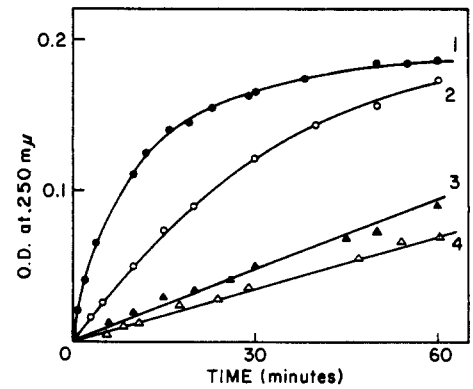

FIG. 3.—Kinetics of binding of *p*-mercuribenzoate (PCMB) to BHLDH alone and in the presence of NADH and AcPyADH, and to CHLDH. (1) BHLDH, 6×10^{-6} M; *p*-mercuribenzoate, 6×10^{-5} M; (2) BHLDH, 6×10^{-6} M; *p*-mercuribenzoate, 6×10^{-5} M; NADH, 6×10^{-5} M; (3) BHLDH, 6×10^{-6} M; *p*-mercuribenzoate, 6×10^{-5} M; AcPyADH, 6×10^{-5} M; (4) CHLDH, 5.2×10^{-6} M; *p*-mercuribenzoate, 5.2×10^{-5} M. Incubation medium was the same as in Fig. 1.

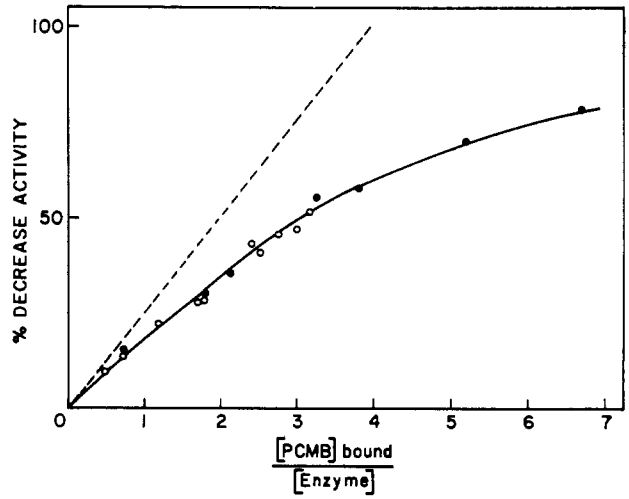

FIG. 4.—Decrease in enzymatic activity of BHLDH caused by binding of *p*-mercuribenzoate (PCMB) in the presence of AcPyADH. Activity measured by reduction of pyruvate to lactate. Solid line: BHLDH, 6×10^{-6} M; *p*-mercuribenzoate, 6×10^{-5} M; O = AcPyADH, 2.4×10^{-5} M; ● = AcPyADH, 4.8×10^{-5} M. Broken line: theoretical loss in activity in the absence of coenzyme (cf. Fig. 1). Incubation medium was the same as in Fig. 1.

TABLE IV
SOME PHYSICOCHEMICAL CHARACTERISTICS OF LACTIC DEHYDROGENASES BOUND TO SULFHYDRYL REAGENTS

Enzyme	Optical Rotation $-[\alpha]^{25}_D$	Optical Rotation λ_c	Complement Fixation (% reactivity with homologous antibody)	Fluorometry[a] (in arbitrary units)	Ultracentrifugation $s_{20,w}$
Control BHLDH[b]	51	259		62	7.3
p-Mercuribenzoate-bound BHLDH[b,e]	48	259		62	7.3
Control CHLDH[b]	33	271	100	70	7.2
p-Mercuribenzoate-bound CHLDH[b,e]	34	268	100	72	7.3
Control CHLDH[d]				31	7.0
$HgCl_2$-bound CHLDH[c,d]				29	7.1
Control CMLDH[e]				53	6.9
$HgCl_2$-bound CMLDH[c,e]				51	7.0

[a] Excitation at 280 mμ, emission at 340 mμ. All samples had an optical density at 280 mμ of 0.08–0.1. [b] In 0.1 M sodium phosphate buffer, *p*H 6.9. [c] 3–4 moles of sulfhydryl reagent bound per mole of enzyme. [d] In 0.1 M Tris-acetate buffer, *p*H 7.0. [e] In 0.1 M Tris-HCl buffer, *p*H 7.0.

those of the beef enzyme. In order to rule out any interaction between *p*-mercuribenzoate and NADH or AcPyADH resulting in modifications of the optical density at 250 mμ, *p*-mercuribenzoate, 6×10^{-5} M, was incubated with equimolar amounts of NADH or AcPyADH. The optical density at 250 mμ of the mixture was, within experimental error, the summation of the optical densities of the individual components, and no modifications of the initial values were detected after 2 hours of incubation at room temperature.

If the binding of *p*-mercuribenzoate to the enzymes is allowed to proceed in the presence of 4- to 8-fold mloar excess NADH or AcPyADH, not only are fewer *p*-mercuribenzoate molecules bound but also bound *p*-mercuribenzoate causes less decrease in enzymatic activity than in the experiments without coenzymes. This is shown in Figure 4, where the decrease in activity of BHLDH bound to *p*-mercuribenzoate in the presence of AcPyADH (solid line) is compared to the loss in activity one would expect if the coenzyme were not present (broken line).

No attempt has been made to investigate the protection by the coenzymes from binding of $HgCl_2$ because of the known interactions of NADH and AcPyADH with $HgCl_2$ (Kaplan, 1960).

Physicochemical and Enzymatic Characteristics of the Enzymes Bound to p-Mercuribenzoate and $HgCl_2$.—It is known that denaturation results in a decrease in the protein fluorescence of a number of lactic dehydrogenases (McKay and Kaplan, 1961; Brand *et al.*, 1962). However, as shown in Table IV, *p*-mercuribenzoate and $HgCl_2$ binding does not affect the fluorescence yields of lactic dehydrogenases. There is also no significant detectable change in sedimentation coefficients and optical rotation parameters when 3 or 4 moles of *p*-mercuribenzoate or $HgCl_2$ are bound. Levine (1962) and Gerstein *et al.* (in preparation) have presented evidence indicating that alterations in protein structure may result in changes in immunologic properties. Moreover, certain amino acid reagents have been found to promote modifications of the structure of lactic dehydrogenases as detected by changes in immunologic properties (Kaplan and White, 1962; Robinson *et al.*, 1963). However, as indicated in Table IV, there is no shift in the complement-fixation characteristics of the *p*-mercuribenzoate-bound CHLDH when compared to the native dehydrogenase.

It is known that small differences between lactic dehydrogenases (Kaplan *et al.*, 1960; Kaplan and Ciotti, 1961; Cahn *et al.*, 1962) or small modifications of these molecules (Kaplan and White, 1962) cause changes in the enzymes' sensitivity toward inhibition by high substrate concentration with pyridine nucleotide coenzymes and their analogs. With this in mind, the activity of the enzymes bound to *p*-mercuribenzoate or $HgCl_2$ has been assayed at three different concentrations of pyruvate in the presence of NADH or NHXDH. The results of these experiments are given in Table V and show that, under the conditions reported, there are no significant differences between the controls and the enzymes bound to *p*-mercuribenzoate or $HgCl_2$.

TABLE V
EFFECT OF MERCURIALS ON CATALYTIC PROPERTIES OF LACTIC DEHYDROGENASES

The enzymatic assays were run in 0.1 M potassium phosphate buffer, *p*H 7.0, at 23° in the presence of NADH, 1.4×10^{-4} M, or NHXDH, 2.3×10^{-4} M. Subscripts 1, 2, and 3 indicate that 3.0×10^{-4} M, 3.0×10^{-3} M, and 1.0×10^{-2} M pyruvate, respectively, were used in the assay.

	$NADH_1$	$NADH_2$	$NADH_3$
	$NHXDH_1$	$NHXDH_2$	$NHXDH_3$
Control BHLDH	0.99	0.62	0.55
p-Mercuribenzoate-bound BHLDH[a]	0.97	0.65	0.53
Control CHLDH	1.04	0.67	0.47
p-Mercuribenzoate-bound CHLDH[a]	1.03	0.63	0.55
$HgCl_2$-bound CHLDH[a]	1.06	0.64	0.50
Control CMLDH	1.85	1.34	1.04
$HgCl_2$-bound CMLDH[a]	1.82	1.18	1.06

[a] About 3 moles of mercurial bound per mole of enzyme.

DISCUSSION

Our results indicate that the SH groups of BHLDH, CHLDH, and CMLDH are involved in the mechanism of action of these enzymes. The main points in support of this thesis are the following:

1. *Loss in Enzymatic Activity Caused by Binding of p-Mercuribenzoate.*—The reaction of *p*-mercuribenzoate or $HgCl_2$ with the three enzymes causes a loss of about one-fourth of the enzymatic activity for each mole of sulfhydryl reagent bound per mole of enzyme. It appears, therefore, that the "active" SH groups are among the first four reacting fast and homogeneously with sulfhydryl reagents.

2. *Protection by Coenzymes from Binding of p-Mercuribenzoate to Enzymes and from Their Inactivation.*—NADH, NAD, and their acetylpyridine analogs protect BHLDH and CHLDH from binding of *p*-mercuribenzoate. This protection is more evident

with the reduced than with the oxidized coenzymes and more evident with the acetylpyridine analogs than with the natural coenzymes; these differences are probably related to differences in dissociation constants of these coenzymes to lactic dehydrogenases (Takenaka and Schwert, 1956; Velick, 1958). Pyruvate and lactate do not protect the enzymes from binding of *p*-mercuribenzoate, in accordance with the fact that substrates do not bind directly to these enzymes (Takenaka and Schwert, 1956). When the enzymes are protected by AcPyADH or NADH, *p*-mercuribenzoate seems to bind also to other SH groups besides the "active" ones, as indicated by the fact that, in the presence of the reduced coenzymes, more SH groups must be bound in order to achieve a certain degree of inactivation (Fig. 4). Possibly the coenzymes protect the "active" SH groups to such an extent that their speed of reaction with *p*-mercuribenzoate becomes comparable with that of the other SH groups which are not at the active site.

3. *Recovery of Enzymatic Activity upon Addition of Cysteine and Physicochemical and Enzymatic Characteristics of the Enzymes Bound to Sulfhydryl Reagents.*— The practically complete recovery of activity of the enzymes bound to 4–5 moles of *p*-mercuribenzoate or $HgCl_2$ and treated with cysteine demonstrates by itself only the reversibility of the inhibition by mercurials; the possibility of denaturation of the enzymes induced by the bound mercurial and reversed upon release of it by cysteine is not excluded. However, the fact that the optical rotation characteristics, the fluorescence, the sedimentation coefficient, and the immunologic properties are not modified in the mercurial-bound enzymes, compared to the native enzymes, makes it very unlikely that gross alterations of the conformation have taken place in the enzymes bound to the mercurials. No significant differences have been found in the ratios of enzymatic activity at increasing substrate concentrations between native enzymes and enzymes bound to sulfhydryl reagents. This is also an indication of the fact that *p*-mercuribenzoate and $HgCl_2$ do not exert appreciable structural modifications of the enzymes to which they are bound. However, some structural modifications of the enzyme may occur when more than 4 moles of $HgCl_2$ are bound, as indicated by changes in the catalytic characteristics.

The possibility still remains that *p*-mercuribenzoate and $HgCl_2$ inhibit enzymatic activity because they bind to SH groups *close* to the active sites but not directly involved in the mechanism of action of the enzymes, in this way hindering some step of the enzymatic mechanism. In this connection the following facts may be considered: BHLDH, CHLDH, and CMLDH have about 1200 amino acid residues per molecule, of which 17 in BHLDH, 27 in CHLDH, and 24 in CMLDH are cysteine residues (Di Sabato *et al.*, in press); i.e., one out of 70 residues in BHLDH, one out of 44 residues in CHLDH, and one out of 50 residues in CMLDH is a cysteine residue. It seems, therefore, very unlikely that in all three enzymes one cysteine is close to each of the active sites just by "random distribution," without playing any actual role in the mechanism of action of the enzyme.

These results do not provide conclusive evidence about the intimate role played by the SH groups in the mechanism of action of lactic dehydrogenases; however, the protection given by the coenzymes from binding of *p*-mercuribenzoate, together with the lack of protection by pyruvate and lactate, suggest an involvement of the SH groups in the enzyme-coenzyme bond. Moreover, the facts that the enzymes are almost completely inactivated when about four SH groups are blocked per mole of enzyme of molecular weight 135,000, and that 4 moles of AcPyADH give maximal protection are consistent with the findings of previous authors that 1 mole of BHLDH binds about 4 moles of coenzyme (Takenaka and Schwert, 1956; Velick, 1958; Pfleiderer *et al.*, 1959).

Evidence has been given recently for the existence of four equal subunits in lactic dehydrogenases (Cahn *et al.*, 1962; Appella and Markert, 1961); one is therefore tempted to postulate the existence of one active site (containing one SH group) for each subunit. Since the block of each one of the first four SH groups causes a loss of about one-fourth of the enzymatic activity, it appears reasonable that the four subunits can function quite independently from each other.

Finally, mention must be made of the fact that the sedimentation constants for CHLDH and CMLDH bound to $HgCl_2$ are unchanged. This is evidence against the formation of dimers of the type protein-S-Hg-S-protein which have been demonstrated in other proteins treated with $HgCl_2$ (Edelhoch *et al.*, 1953). Mercury may bind intramolecularly to two SH groups belonging to different subunits. In this case, on the basis of the evidence provided by the experiments with *p*-mercuribenzoate, mercury should bind to one "active" SH in one subunit and one "inactive" SH in another subunit in order to explain the fact that 1 mole of mercury bound per mole of enzyme causes the loss of one-fourth of the enzymatic activity. The possibility remains that mercury behaves in this instance as a monofunctional cation, giving a compound of the type protein-S-HgCl, as demonstrated by Kolthoff *et al.* (1957) with serum albumin. However, it has been shown by Di Sabato *et al.* (in press) that in the presence of 4.0 M guanidine 1 atom of mercury binds to two SH groups.

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The Effect of Dioxane on the Dissociation and Activity of Glutamic Dehydrogenase*

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Dioxane has been found to cause dissociation of the glutamic dehydrogenase molecule without interfering with the activity of the enzyme. For a 1% protein solution the apparent molecular weight of glutamic dehydrogenase is 990×10^3 ($s_{20,w} = 24$ S) in buffer, and decreases to 550×10^3 ($s_{20,w} = 16.5$ S) in 5% dioxane, and to 300×10^3 ($s_{20,w} = 13$ S) in 10% dioxane. There is no change in rotatory dispersion associated with the dissociation, and several properties of the subunits (absorption, fluorescence, DPNH binding, and enzymatic activity) appear to be unaffected by dioxane. Glutamic dehydrogenase is known to dissociate on dilution, and it has been found that regardless of the nature of any dissociating or associating reagents added (DPN, DPNH, diethylstilbestrol, dioxane), the extrapolated value of $s_{20,w}$ at infinite dilution is 13 S. It is therefore concluded that the active form of glutamic dehydrogenase *in vitro* must be the low (300×10^3) molecular weight unit.

The dissociation of beef liver glutamic dehydrogenase into subunits of different size can be affected by a number of reagents, and the current picture of the various forms of the enzyme is a rather complex one. At high concentration the apparent molecular weight of glutamic dehydrogenase (GDH)[1] is 10^6 g/mole, but, as was first shown by Olson and Anfinsen (1952), this high molecular weight molecule appears to dissociate on dilution. A number of workers have investigated the dissociation of the 10^6 mw GDH, and found that dissociation into 250×10^3 mw "subunits" can be caused by steroid hormones (Yielding and Tomkins, 1960; Tomkins *et al.*, 1961), by high concentration ($> 10^{-4}$ M) of DPNH (Frieden, 1959a, 1959b), by thyroxine (Wolff, 1961a, 1961b), by simple inorganic and organic anions (Frieden, 1962; Wolff, 1961a), and by extremes of *p*H (Frieden, 1962). ADP, DPN, and low levels of DPNH appear to stabilize the high molecular weight unit (Frieden, 1959a, 1959b). In all these cases the association - dissociation is reversible, and since the conditions favoring dissociation also seem to cause inhibition of the enzyme and since association is promoted by the coenzymes, several attempts have been made to correlate dissociation and loss of enzyme activity. Such correlations have recently been questioned (Mildvan and Greville, 1962). A number of reagents have also been found to cause irreversible dissociation of GDH beyond the 250×10^3 units. Thus urea and dodecylsulfate (Wolff, 1961a; Jirgensons, 1961) cause dissociation into units of 43×10^3 mw, and, at *p*H above 10, GDH dissociates into some 18 subunits (Fisher *et al.*, 1962).

In the present paper, we report the reversible dissociation of GDH into apparent 500×10^3 and 250×10^3 units in dioxane, without any loss in biological activity. The evidence presented favors the hypothesis that no unit larger than 250×10^3 mw can exist under the conditions of the *in vitro* enzyme assay, and any direct correlation between the dissociation process and enzyme activity thus appears very unlikely.

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[1] Abbreviations used in this paper: GDH, glutamic dehydrogenase.

EXPERIMENTAL

Materials.—Crystalline bovine liver GDH was obtained from Calbiochem (Lots 501180, 502398, and 502991 of Boehringer preparations) as ammonium sulfate suspensions. The enzyme was centrifuged, dissolved in 0.05 M sodium phosphate buffer *p*H 7.2, and dialyzed against the same buffer for at least 10 hours before use. Highest purity grade DPN, DPNH, and substrates were obtained from Sigma Chemical Company and from Calbiochem. Dioxane was reagent grade, was purified by reflux with KOH followed by distillation, and was stored in sealed, dark bottles.

Methods.—All the sedimentation experiments were performed with the Spinco Model E ultracentrituge at constant temperatures in the range from 4° to 9°. The experimental sedimentation coefficients were normalized to water at 20° by correcting for viscosity and density in the normal manner. The temperature-viscosity relationships for buffer, 5% dioxane-buffer, and 10% dioxane-buffer were determined directly with Ostwald-Fenske viscometers and are given in Table I. For the density corrections, the contribution of dioxane to the density was disregarded and only the temperature corrections were considered. $\bar{V}$ was assumed to be constant and equal to 0.75 ml/g. The protein concentration was determined from the 280 mμ absorption using the extinction coefficient reported by Olson and Anfinsen (1952). Moving boundary electrophoresis was carried out in the Spinco Model H electrophoresis-diffusion apparatus. Absorption spectra were recorded with the Bausch and Lomb Spectronic 505 spectrophotometer, and measurements of fluorescence spectra and fluorescence polarization were carried out with the Aminco-Bowman fluorescence spectrophotometer sup-