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Effect Of Chemical Modification Of Cysteinyl, Lysyl And Tyrosyl Residues On The Catalytic And Regulatory Properties Of Glutamate Dehydrogenase.

Mikio Nishida
University of Alabama at Birmingham

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EFFECT OF CHEMICAL MODIFICATION OF CYSTEINYL, LYSYL AND TYROSYL RESIDUES ON THE CATALYTIC AND REGULATORY PROPERTIES OF GLUTAMATE DEHYDROGENASE

by

Mikio Nishida

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Dissertation Committee:

R. Lamont Gilbert Chairman
Gerald L. Carlson
John M. McKibbin
David Dawdin
Robert E. Taylor

Director of Graduate Program John M. McKibbin

Dean, UAB Graduate School S B Barker

Date 20 May 1971

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ABBREVIATIONS

N-Acetylimidazole	=	NAIm
Adenosine Diphosphate	=	ADP
Alpha Ketoglutarate	=	αKG
N-Bromosuccinimide	=	NBS
Curie	=	Ci
Diethylstilbestrol	=	DES
Dimethyl Sulfoxide	=	DMSO
2,5-Diphenyloxazole	=	PPO
Ethylenediamine Tetraacetic Acid	=	EDTA
1-Fluoro-2, 4-Dinitrobenzene	=	FDNB
Glutamate Dehydrogenase	=	GDH
Glutathione	=	GSH
Guanosine Triphosphate	=	GTP
Nicotinamide Adenine Dinucleotide	=	NADH
Tetranitromethane	=	TNM
Trinitrobenzene Sulfonate	=	TNBS
Tris (hydroxymethyl) aminomethane	=	Tris

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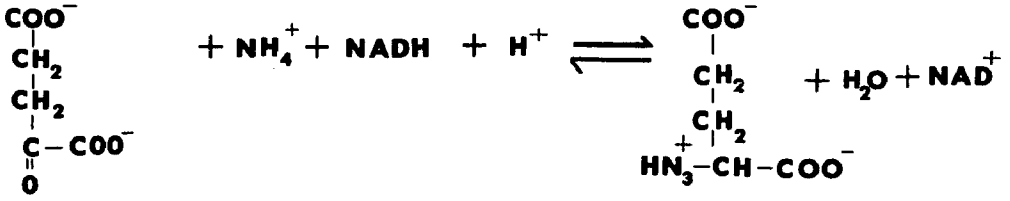
INTRODUCTION

The major goal of this dissertation is to provide information on the mechanisms for regulating enzyme catalytic activity through studying specific modification of amino acid side chains in relation to the catalytic and the regulatory properties of glutamate dehydrogenase (GDH).

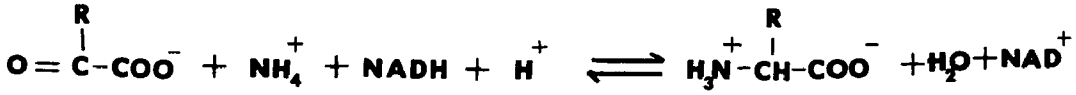
Enzymes are macromolecules capable of forming specific covalent or non-covalent complexes with substrates, and with a variety of reagents which serve to modify the catalytic activity. Thus, at least two types of "active sites" exist on enzymes: one, where the catalytic action takes place, is reactive toward the substrates and is called the substrate site; the others, called regulatory sites, bind specific chemical modifiers resulting in regulation of the catalytic function by means of conformational changes. Such control involving one or more ligands as modifiers at specific sites with resulting change in structure and catalytic properties has been proposed as a major means of enzyme regulation (7,26,28,29,74,79,80,82,83) and has been termed "allosteric regulation" (42). One enzyme may possess more than one such regulatory site as illustrated by the fact that glutamate dehydrogenase is regulated by at least five different classes of reagents (16,29).

GDH can catalyze two types of reactions according to the following schemes, and the specificity of the enzyme for different substrates is a function of its conformation, which can be altered by allosteric modifiers.

Type I. (for dicarboxylic substrates)



Type II. (for monocarboxylic substrates)



Where $R = -CH_3$, $-CH_2-CH_3$

Previous studies (74,77,82) have proven that GDH is a good model for a study of the relationship between the structure of a multichain enzyme and its catalytic activity and substrate specificity, and for studies of the regulatory effects of 'allosteric' modifiers on catalytic activity and the enzyme conformation. The tentative amino acid sequence of GDH subunits which was published also makes it possible to make detailed studies of structure-function relationships in this, and other dehydrogenases which may be related (61).

Many studies of the kinetics (16,22,26-28), regulation (2,7-9,70-72,74) and physico-chemical properties of GDH (11,17,20,23, 50,51,58) have been done. The enzyme is composed of immunologically identical subunits with a molecular weight of 53,500 (21), and

exists in various conformational forms (7-9,29,72,74). These subunits are organized into aggregates of several sizes by non-covalent bonds. The smallest aggregated state which is catalytically active, comprised of six subunits, is called "monomer." These monomers aggregate at higher protein concentrations to form "polymers" having various molecular weights of over one million. This concentration dependent monomer-polymer equilibrium reflects changes in subunit conformation which correlate with changes in catalytic activity. Various allosteric regulatory reagents such as guanosine triphosphate (GTP) (15,20,27), Zn^{++} (50), and steroid analogues (73,80,85), in the presence of the coenzyme, reduced nicotinamine adenine dinucleotide (NADH), can produce changes in conformation such that the monomer-polymer equilibrium is shifted toward the monomer and the L-glutamate activity is drastically diminished (74). The catalytic activity toward the corresponding monocarboxylic acid reaction is increased; however adensine diphosphate (ADP) (27,28,76), NAD^{+} (16,26,27) and leucine (81) can reverse this modifier-induced change in conformation (20,72,75,76,82). For the over-all state of the enzyme, the following model has been proposed by Yielding et al (72,82) and by Frieden (29), to explain the relationship between enzyme structure and catalytic activity.

$$\text{Polymer} \rightleftharpoons \text{Monomer } x \rightleftharpoons \text{Monomer } y$$

where monomer x has L-glutamate activity and monomer y has monocarboxylic acid substrate activity. x and y may represent conformationally different states of the monomer. Regulators.

such as GTP can shift the equilibrium toward the right. This scheme may be an oversimplification of the actual mechanism, but has been a useful model for considering the relationship between structure and regulation.

Chemical modifications of specific amino acid residues in GDH have resulted in changes in its sensitivity to allosteric reagents as well as its physical and catalytic properties. Reagents studied with GDH in the past are listed in Table I, as are the presumed amino acid residues they modify.

Hellerman et al (36,52,55,56) reported that among different types of organic mercurials, a compound of the type R-Hg-X reacts readily with sulfhydryl residues of cysteine and stiumlates the enzyme catalyzed L-glutamate dehydrogenase reaction. Bitensky et al (8,9) have studied further the effects of organic mercurials on GDH and found that this type of organic mercurial (R-Hg-S) shows reciprocal effects on the glutamate and the corresponding monocarboxylic acid substrates. In addition, it was also reported (8,9) that there is a drastic decrease in the response of the mercurial treated enzyme to the effect of regulatory allosteric reagents. These changes in properties of GDH by allosteric regulators have been rationalized on the basis of a shift in equilibrium in the scheme shown on page 3 and are brought about by a change in conformation of the enzyme. The details of such a mechanism have not been elucidated.

It is quite certain, however, that the findings by Bitensky et al (8,9) represent the discovery of a useful reagent to regulate allosteric effects of various compounds on the enzyme

TABLE I

CHEMICAL MODIFIERS FOR PROTEINS AND THEIR CORRESPONDING AMINO ACID SPECIFICITY

Reagents Used	Amino Acid Modified
Various organic Mercurials (8,9,36,45,55,56) Silver ion (36,45,54), Iodoacetimide (4), Mercaptoethaneol (8) N-ethylamaleimide (8)	-SH group
Trinitrobenzene sulfonate (12,24,32), Pyridonal 5'-phosphate (1,48) 1-Fluoro-2,4-dinitrobenzene, (17), Acetic anhydride (14,79), N-acetyl imidazole (49), A-acetyl-4-sulphamoylphenyl maleimide (37)	lysyl group (or amino group)
Tetranitromethane (49), N-acetylimidazole (49)	tyrosyl group
N-bromosuccinimide (66)	tryptophanyl group (?)

and provide a tool for promoting the understanding of the relationship between the ligand evoked conformational changes of the protein and the catalytic activity.

The present studies are intended to extend our understanding of the relationship between specific structural features of the enzyme and its catalytic and regulatory properties. Therefore, radioactive $*CH_3HgI$ was synthesized and used for the following purposes:

(1) Confirmation of the specificity of the reagent CH_3HgI for -SH groups;

(2) The determination of the exact stoichiometry between GDH and the mercurial on modification, and a study of results of such modification on enzyme properties;

(3) A study of the enzyme modified with more than one reagent to examine the relationships among various amino acid residues related to regulation;

(4) Integration of these findings to devise a model of the regulatory mechanism. It should be emphasized that the knowledge obtained during this research is not directly applied to an in vivo system, which obviously does not contain a mercurial attached to the enzyme, but should provide insight into the fundamental nature of the enzyme. Thus, a multistep regulatory mechanism involving the functions of other amino acid residues with the -SH groups in the enzyme, will be discussed.

MATERIALS AND METHODS

Chemicals and Enzyme:

C^{14} methyl iodide with specific activity of 51.5 mCi/mM was purchased from Amershum Searls. All the other reagents used were commercial products of the highest purity available, and used without further purification. These chemicals and their sources are listed in Table II.

Solvents for chromatography were distilled at least once prior to use.

Glutamate dehydrogenase from bovine liver in crystalline purity was obtained from Sigma or Boehringer Ltd. as a suspension in ammonium sulfate or as a solution in 50% glycerol.

Enzymatic assay:

The reaction mixture for assay of enzyme catalytic activity contained the following components unless stated otherwise: 1×10^{-3} M NADH, 1×10^{-1} M NH_4Cl, 1×10^{-3} M EDTA, and 2×10^{-3} M α-keto glutarate in 2.5 ml of 0.05 Tris buffer (pH 8.6). The rate of the enzyme reaction was measured in the Gilford model 2000 spectrophotometer by following the decrease in NADH absorption at 340 mμ.

TABLE II

SOURCE OF CHEMICALS USED

Source	Chemicals
Aldrich Chemical Company	NBS, TNM
J. T. Bakers Chemicals	NH_4Cl, $HgCl_2$, Hg, DMSO Na_2HPO_4, NaH_2PO_4 H_20, PPO, Toluene
Beckman Instrument Company	BIO-SOLV
P-L Biochemicals Incorporated	GTP
CYCLO Chemical Company	S-Methyl Cysteine
Dupont	Acetic Acid
Eastman Organic Chemicals	EDTA, CH_3I, CH_3CH_2I, TNBS
Fisher Scientific Company	I_2
Mann Research Laboratory	DES, Ninhydrin
Merck Laboratory Chemicals	$ZnCl_2$, $AgNO_3$
Sigma Chemical Company	NADH, αKG, ADP, GSH L-Amino acids, Tris
U. S. Industrial Chemical Company	CH_3CH_2OH

Radioactivity Measurement:

The radioactivity of C^{14} labeled material was measured in the Beckman DPM-100 Liquid Scintillation system with an efficiency of 86% - 87%. Samples were mixed with 2 volumes of BIO-SOLV, added to 10 ml of a toluene and PPO (0.5% w/v) mixture, and counted for 5 to 10 minutes with recycling.

Sedimentation Study:

Sedimentation studies were performed in the Beckman Model E Analytical Centrifuge with a rotor type AN-E at 50,740 rpm or a rotor type AN-D at 59,780 rpm for 20 minutes at 20^{o}.

Treatment of GDH with various chemicals:

Details of the incubation of the GDH with various chemicals will be found together with experimental data in the results section. Enzyme concentrations were determined at 280 mμ using a molar extinction coefficient for the enzyme of 5.25×10^{4} $M^{-1}cm^{-1}$ based on a molecular weight of 53,500 (21). Enzyme treated with excess amounts of the mercurial became visibly turbid, however and the Lowry method was also used when absorbancy measurements could not be applied.

Preparation of Radioactive Methylmercuric Iodide:

C^{14}-methylmercuric iodide ($^{*}CH_3HgI$) was synthesized according to a modification of the procedure by Baldoni and

Miyashiro (5), using about one eightieth of the amounts of reactants reported previously. The tips of the ampoules containing the radioactive methyl iodide ($^*CH_3I$, 0.5 mCi in each of two ampoules) were kept in a dry ice-acetone bath overnight to condense the $^*CH_3I$. As soon as the ampoules were opened, 1.0 ml of cooled non-radioactive CH_3I was added to each ampoule, and the solutions were combined in a 5 ml glass-stoppered round bottom flask that had been cooled. Each ampoule was then rinsed into the flask with an additional 0.5 ml aliquot of CH_3I. The experiment was done in a walk-in cold room, to minimize the loss of radioactive methyl iodide. After 0.10 g of I_2 and 1.23 g (0.09 ml) of Hg were added to the flask it was placed in a thermostated water bath and attached to a reflux system, consisting of a long reflux cooler with circulating ice water and a silica gel tube on top.

To provide a source for the photoreaction, a 150 Watt reflector lamp (Westinghouse) with hood was placed as close as possible to the reaction flask (about 15 cm) and the entire apparatus was enclosed in aluminum foil. The reaction mixture was subjected to vigorous agitation with a magnetic stirrer at 42^o-45^o for about 3 hours. As the reaction proceeded, the color turned from purple to dark green and finally to yellow. The crystalline yellow product was collected on a Buchner funnel, and recrystallized from hot ethyl alcohol. The final yield was 1.619 g of a slightly yellowish, plate-like crystalline product with a melting point of 143^o-145^o. The product had a

slightly unpleasant odor and a tendency to sublimate.

The specific radioactivity determined at an efficiency of 86% - 87%, was 4.45×10^{10} cpm/M. The compound, which gradually decomposed at room temperature was stored dry at 5^{o} in a brown bottle.

Attempts to decrease the solvent volume in order to increase the specific radioactivity resulted in side reactions and a poorer yield. The pure synthesized compound was dissolved in 0.05 M Tris buffer (pH 7.5) by heating, and was then stored in the refrigerator at a concentration as high as 1×10^{-3} M; at 2×10^{-3} M, recrystalization occurred during storage in the cold.

Little loss of radioactivity was observed over a period of more than six months during storage in Tris buffer. The non-radioactive CH_3HgI and CH_3CH_2HgI used in these studies were also synthesized in the same way. The melting point of CH_3CH_2HgI was 178^{o}, as compared to a range of 172^{o} - 193^{o} reported previously (6).

The CH_3CH_2HgI which was sparingly soluble was dissolved both in 0.05 M Tris buffer (pH 7.5) at 5.2×10^{-5} M and in DMSO at 2×10^{-3} M. DMSO itself did not have any significant effect on the catalytic activity of GDH in the final concentrations used (up to 8% v/v.)

RESULTS

PART I: GLUTAMATE DEHYDROGENASE MODIFIED WITH THE ORGANIC MERCURIAL TYPE R-Hg-I REACTION OF METHYLMERCURIC IODIDE WITH MODEL COMPOUNDS AND GLUTAMATE DEHYDROGENASE

Paper chromatography of sulfhydryl compounds in the presence of methylmercuric iodide

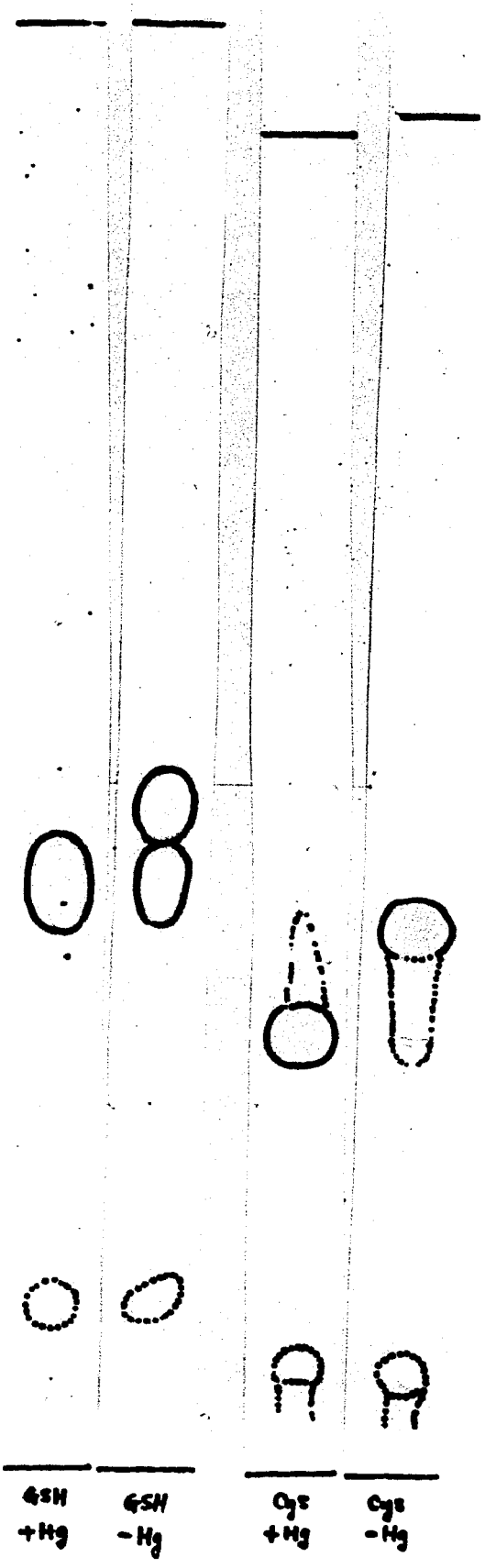
In order to confirm that the mercurial could bind only to the free -SH group of cysteine, experiments were done in which the radioactive reagent was co-chromatographed with serine, threonine, cysteine, C-methylcysteine, histidine, and glutathione. Of those tested, only cysteine and glutathione showed altered chromatographic behavior (Figure 1).

Effects of Various Amino Acids and Glutathione on the Volatility of Radioactive Methylmercuric Iodide

The solution of $*CH_3HgI$ in Tris buffer is fairly stable, but when it is dried under vacuum, the radioactivity is lost. When the mercurial reacts covalently with the specific amino acid in solution, the radioactivity should remain with the amino acid after the solution is dried. In order to test the specificity of the mercurial reaction with amino acids, mixtures of various amino acids with the mercurial were subjected to drying under vacuum and the residues tested for radioactivity. As shown in Table III, only cysteine and glutathione prevented

FIGURE 1

The Effects of Methylmercuric Iodide on Paper Chromatography of Sulfhydryl Compounds. Amino acids and glutathione were prepared as 1 x 10^{-2} M solutions in water. 50 λ of each amino acid solution and 25 λ of glutathione were spotted on chromatographic paper (Whatman 3MM) and resolved by decending chromatography in butanol - water - acetic acid: 500:800:100, containing 1 x 10^{-2} M non-radioactive CH_3HgI. Development at room temperature required about 17 hours for amino acids, and 20 hours for glutathione. The control was chromatographed in the same way but without CH_3HgI. Spots were located by their color reaction with ninhydrin. The solid lines indicate clear spots and the dotted lines a trace of color. Cys and GSH represent cysteine and glutathione respectively and the presence (+) or absence (-) of the mercurial (Hg) is indicated.


GSH
+Hg
GSH
-Hg
Cys
+Hg
Cys
-Hg

volatilization of the radioactive mercurial. The results were consistent with those using chromatography and suggest a specificity for cysteine as reported previously (8,9), although the possibility (36,60) of weak binding to another amino acid residue could not be completely ruled out.

Effect of $^{*}CH_3HgI$ on Glutamate Dehydrogenase

The catalytic activity of glutamate dehydrogenase was stimulated by the addition of $^{*}CH_3HgI$ (Figure 2) as reported previously for other methyl mercurial derivatives (8,9,36). The maximum stimulation was obtained at a molar ratio of about 1:1 of the compound to enzyme, based on the enzyme chain molecular weight of 53,500, and the activity decreased gradually at higher concentrations of $^{*}CH_3HgI$ (Figure 3a, Curve I). As seen in Figure 2, the stimulatory effect did not follow a simple saturation curve at mole ratios of added reagent of less than 0.4:1.

Stoichiometry of $^{*}CH_3HgI$ Binding in Relation to Catalytic Activity

Comparisons were made of the catalytic activity of samples of the enzyme subjected to reaction with specific ratios of mercurial. In addition, catalytic activity and the amount of bound mercurial were determined for each enzyme sample after dialysis. These results are shown in Figure 3a. Curve I shows the relationship between catalytic activity and added reagent, while Curve II shows the relationship to binding. A maximum binding ratio for the mercurial compound of 3.6 was obtained with a 10-fold

TABLE III

Effects of Amino Acids and Glutathione on the Volatility of Radioactive Methylmercuric Iodide. Amino acids and glutathione at concentrations of 1×10^{-2} M were dissolved in 0.05 M Tris buffer (ph 7.5), except for tyrosine which was less soluble and required the addition of several drops of ammonium hydroxide. 0.1 ml of each sample was incubated in a 1 ml vial containing 2×10^{-3} M radioactive $^{*}CH_3HgI$ solution (85,000 cpm), and placed in a desiccator containing silica gel which was connected to a water aspirator via a trap, and bottom-cooled with dry ice-acetone. After all the samples were completely dried under vacuum, 1.0 ml of water was added to each sample and the remaining radioactivity was counted by the method described previously. Each sample is reported as the average of duplicate determinations. Efficiency of counting did not vary significantly from sample to sample (86% - 87%). The background is the count of 1.0 ml of water, and 0.1 ml of the buffer mixed with 1.0 ml of $^{*}CH_3HgI$ served as the control.

TABLE III

SAMPLE	COUNTS (CPM)
Background	51.1
Control	337.0
Cysteine	77,333.3
Glutathione	67,524.9
Tyrosine	347.5
Histidine	361.0
Serine	395.0

FIGURE 2

Effect of the Concentration of Methylmercuric Iodide on the Catalytic Activity of Glutamate Dehydrogenase. The enzyme (5 mg/ml) was incubated for 5 minutes at room temperature in 0.05 M phosphate buffer (pH 7.4) with methylmercuric iodide at the molar ratios indicated. For assay of catalytic activity, the incubated enzyme was diluted 10 times with buffer, and 5 μg added to 2.5 ml of the assay medium described under materials and methods. The enzymatic activity was plotted as a relative activity which is the ratio of the catalytic activities in the presence and the absence of the mercurial.

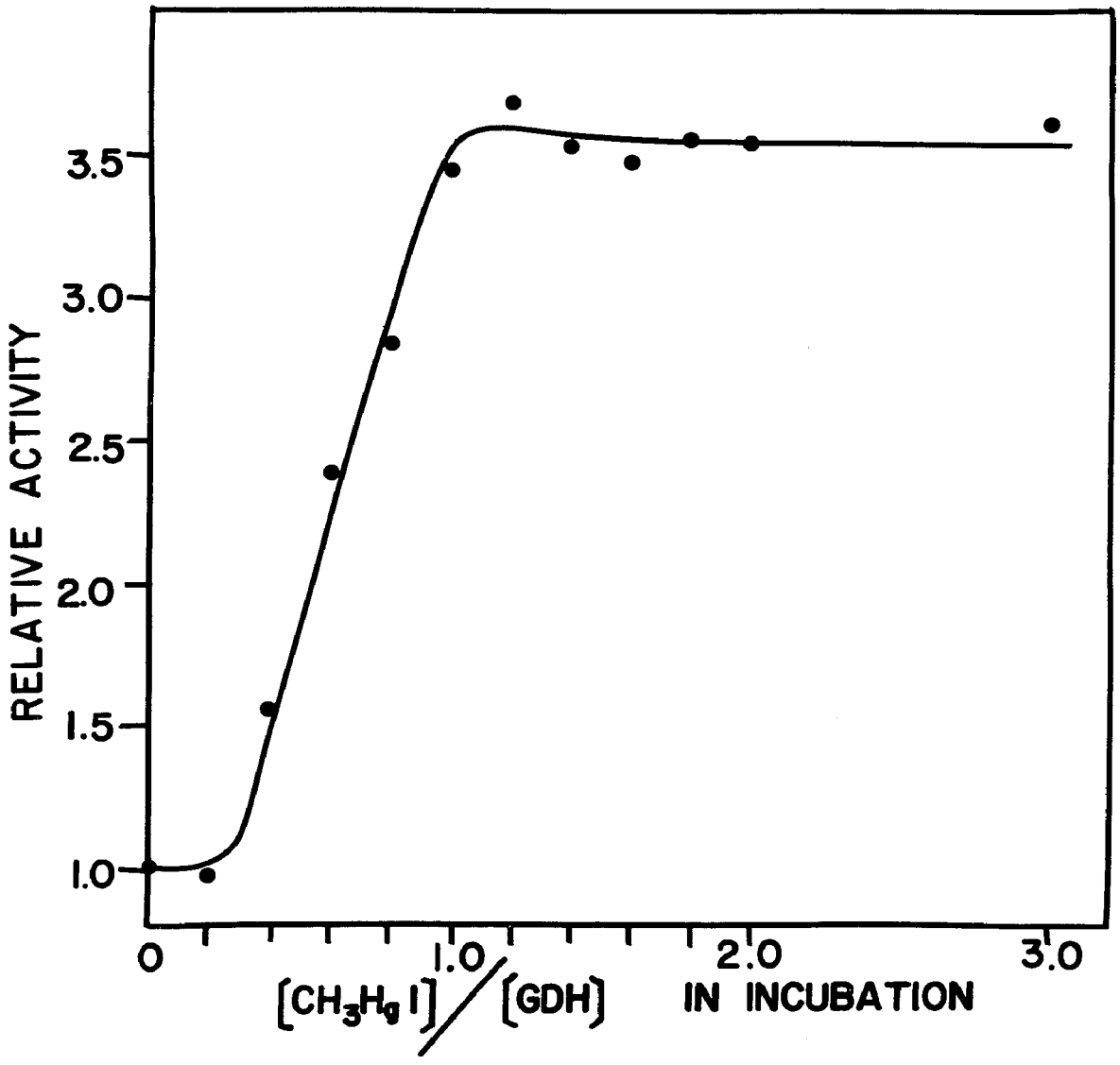
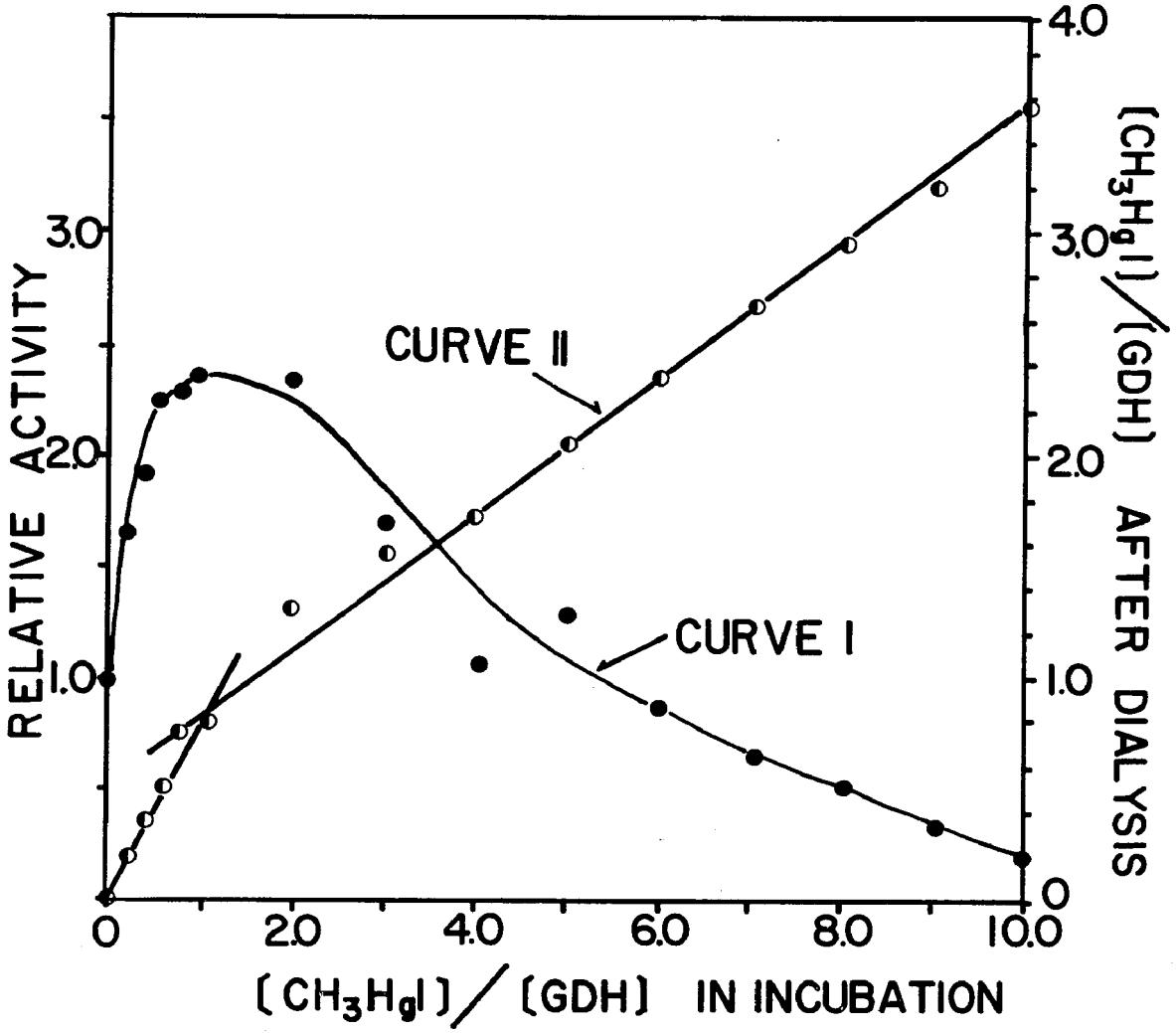

RELATIVE ACTIVITY
3.5
3.0
2.5
2.0
1.5
1.0
0
1.0
2.0
3.0
[CH3Hg I] / [GDH] IN INCUBATION

FIGURE 3a

Comparison of the Effect of Methylmercuric Iodide Added at Different Molar Ratios on the Catalytic Activity of Glutamate Dehydrogenase and the Resulting Ratio of Bound Mercurial to Enzyme. Incubations for a minimum of 5 minutes were carried out by mixing 0.25 ml of enzyme in $(NH_4)_2SO_4$ (20 mg/ml) with 1 ml of 0.05 M phosphate buffer pH 7.4 containing the mercurial in the ratios given in the abscissa, followed by transfer to dialysis tubing.

The removal of all samples took about 90 minutes. The enzyme mixtures were then dialyzed for about 17 hours in the cold against 3 changes of 200 ml of 0.05 M phosphate buffer (pH 7.4).

After dialysis, the enzyme samples were transferred quantitatively to volumetric flasks and made up to 5.0 ml with phosphate buffer. Aliquots of 1 ml were used for radioactivity measurements and enzyme catalytic activity was measured as in Figure 2 using 4.0 µg of the enzyme. Symbols: Curve 1 (●-●) = catalytic activity of dialyzed enzyme. The left ordinate expresses activity relative to the untreated dialyzed control. Curve II (◐-◐) = Bound ratio after dialysis of mercurial per enzyme chain of 53,500 molecular weight as expressed on right ordinate.


RELATIVE ACTIVITY
1.0
2.0
3.0
CURVE II
CURVE I
0
2.0
4.0
6.0
8.0
10.0
[CH3HgI] / [GDH] IN INCUBATION
4.0
3.0
2.0
1.0
0
[CH3HgI] / [GDH] AFTER DIALYSIS

excess of reagent under the condition applied, Smith et al have recently reported a total of 6 -SH groups for the enzyme (61). The maximum enhancement of the catalytic activity was observed at a bound ratio of 1:1 and loss of catalytic activity resulted from additional binding of $*CH_3HgI$. It was also apparent that the slope of the binding curve was considerably steeper at bound ratios up to 1, and then became less steep. From these results, it was concluded that one specific -SH group per enzyme chain was clearly distinguishable from the others. To determine whether the effect of the bound mercurial is cooperative or not, the data for Curve I in Figure 3a were replotted to show activity as a function of the mercurial bound (Figure 3b). Although the large extend of cooperativity seen in Figure 3a is eliminated by expressing the data in terms of bound reagent, it would still appear that the bound mercurial exerts a cooperative effect since binding to the extent of 0.5/chain produces more than 50% of the maximum effect.

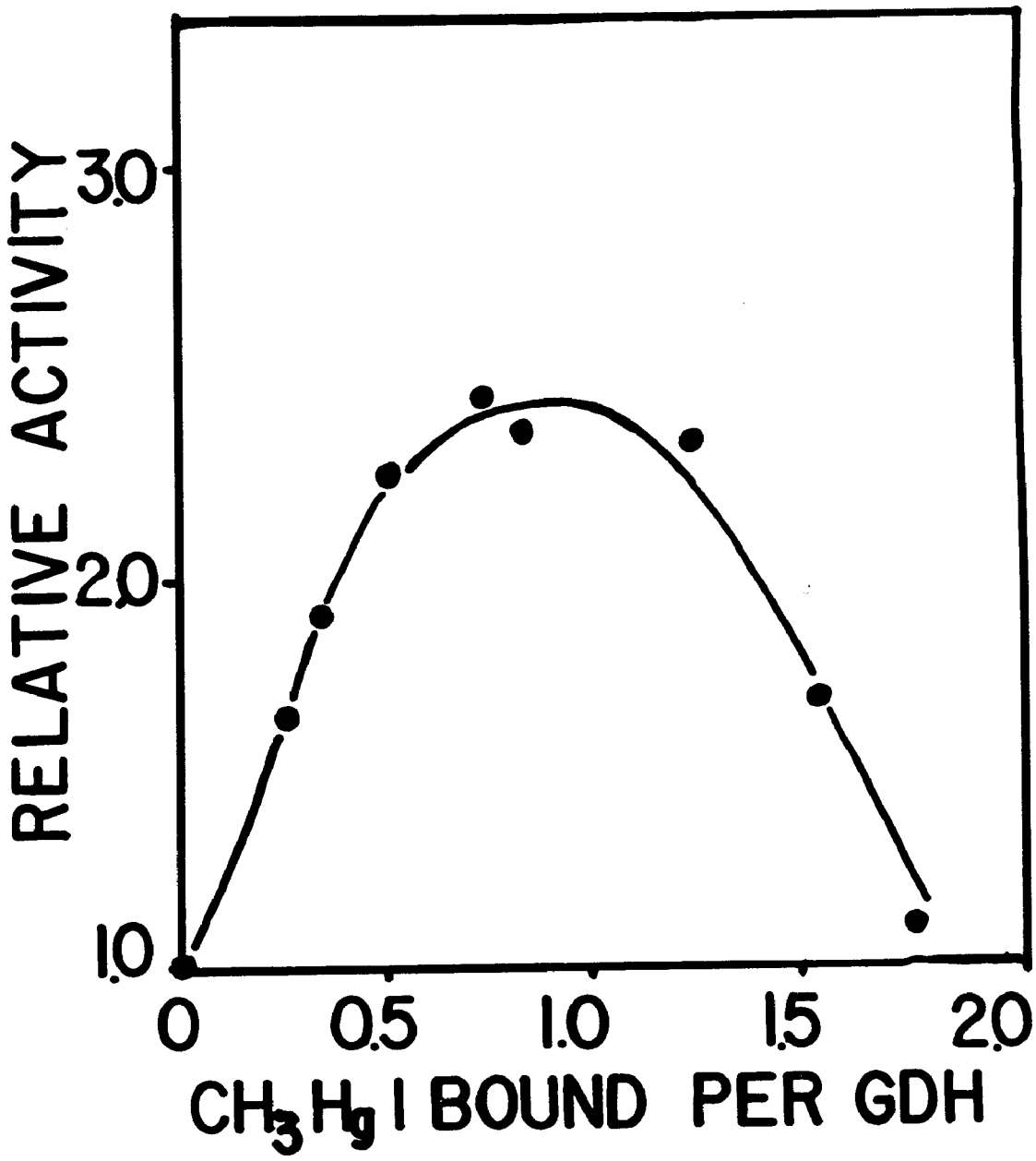
In order to confirm that the effects observed were not due to mercury itself, the enzyme was also incubated with the corresponding concentration of $HgCl_2$ and dialyzed. The enzyme treated with inorganic mercury precipitated during dialysis and lost all its catalytic activity.

Effects of pH on the Stability of $*CH_3HgI$ bound to GDH

In order to gain more information on the properties of the mercurial binding site, the stability of the bound reagent was studied as a function of pH. For this purpose, the enzyme

FIGURE 3b

Effect of Bound Methylmercuric Iodide on the Catalytic Activity of Glutamate Dehydrogenase. The data shown for relative activity as curve I in Figure 2, were replotted as a function of moles of methylmercuric iodide bound per mole of glutamate dehydrogenase after dialysis. The experimental procedures were described in Figure 2.


RELATIVE ACTIVITY
3.0
2.0
1.0
0
0.5
1.0
1.5
2.0
CH3HgI BOUND PER GDH

was incubated with a 3-fold excess of *CH_3HgI as described before, and dialyzed at pH 3.5, 7.5 and 9.2. There was no significant difference of bound radioactivity between the three groups (Table IV). It was concluded, therefore, that the bound mercurial is quite stable over the pH range 3.5 - 9.2.

Effects of "Allosteric" Reagents on the Properties of GDH Substituted with One Mercurial per Enzyme Chain

Previous studies have shown that treatment of the enzyme with low concentrations of various organic mercurials caused substantial reduction in its sensitivity to regulatory ligands such as ADP, GTP, diethylstilbestrol and Zn^{++} (8,9). In the present experiments the effects of such reagents were re-examined on preparations of the enzyme in which the stoichiometry for mercurial binding had been established directly as described above. These results are shown in Figures 4,5,6 and 7.

From these experiments, it became clear that the reduced sensitivity of the enzyme to regulation after mercurial treatment resulted from binding of only one mercurial molecule to each enzyme chain.

Sedimentation Properties of GDH Modified with One Mole of *CH_3HgI per Chain

It has also been reported that mercurial treatment favors aggregation of the enzyme monomers and antagonizes the effects of GTP and other reagents which cause disaggregation. These previous studies however, gave no consideration of the stoichiometry of mercurial binding. Therefore, the enzyme with

TABLE IV

Effect of pH on the Binding of Methylmercuric Iodide to Glutamate Dehydrogenase. 0.75 ml of GDH in $(NH_4)_2SO_4$ suspension (20 mg/ml) was centrifuged in the Sorvall refrigerated centrifuge at 4,200 x g for 10 minutes. The pellet was dissolved in 1.5 ml of 0.05 M phosphate buffer (pH 7.5) and 1.8 ml of 1×10^{-3} M of $*CH_3HgI$ added to the enzyme solution. After 5 minutes the samples were divided into three groups of 1 ml each followed by dialysis at 4^{o} overnight against 500 ml of: (1) 0.05 M citrate-phosphate buffer pH 3.5, (2) 0.05 M phosphate buffer pH 7.5, and (3) 0.05 M Tris buffer pH 9.2. Dialysates were replaced once. After dialysis, the samples were made up to 2.5 ml with their respective buffers. 1 ml of the enzyme solution was used for radioactivity counts. Efficiency of radioactivity counts was determined in each buffer. The results are expressed as dpm per ml of dialysate. Total recovery of radioactivity was 96.2%.

TABLE IV

pH →	← dpm/ml of dialysate
3.5 →	← 4,305
7.5 →	← 3,260
9.2 →	← 4,400

FIGURE 4

Effect of Adenosine Diphosphate on Native and Methylmercuric Iodide Treated Glutamate Dehydrogenase. The enzyme was incubated with methylmercuric iodide at a molar ratio of 1:1 for 5 minutes and dialyzed against the phosphate buffer. Native enzyme was treated in the same way except for the mercurial. The assay was done as described in the text with varying concentrations of ADP. The medium contained 1.3 μg enzyme/ml, respectively. Symbol: (●-●) = native enzyme, (◐-◐) = mercurial treated enzyme.

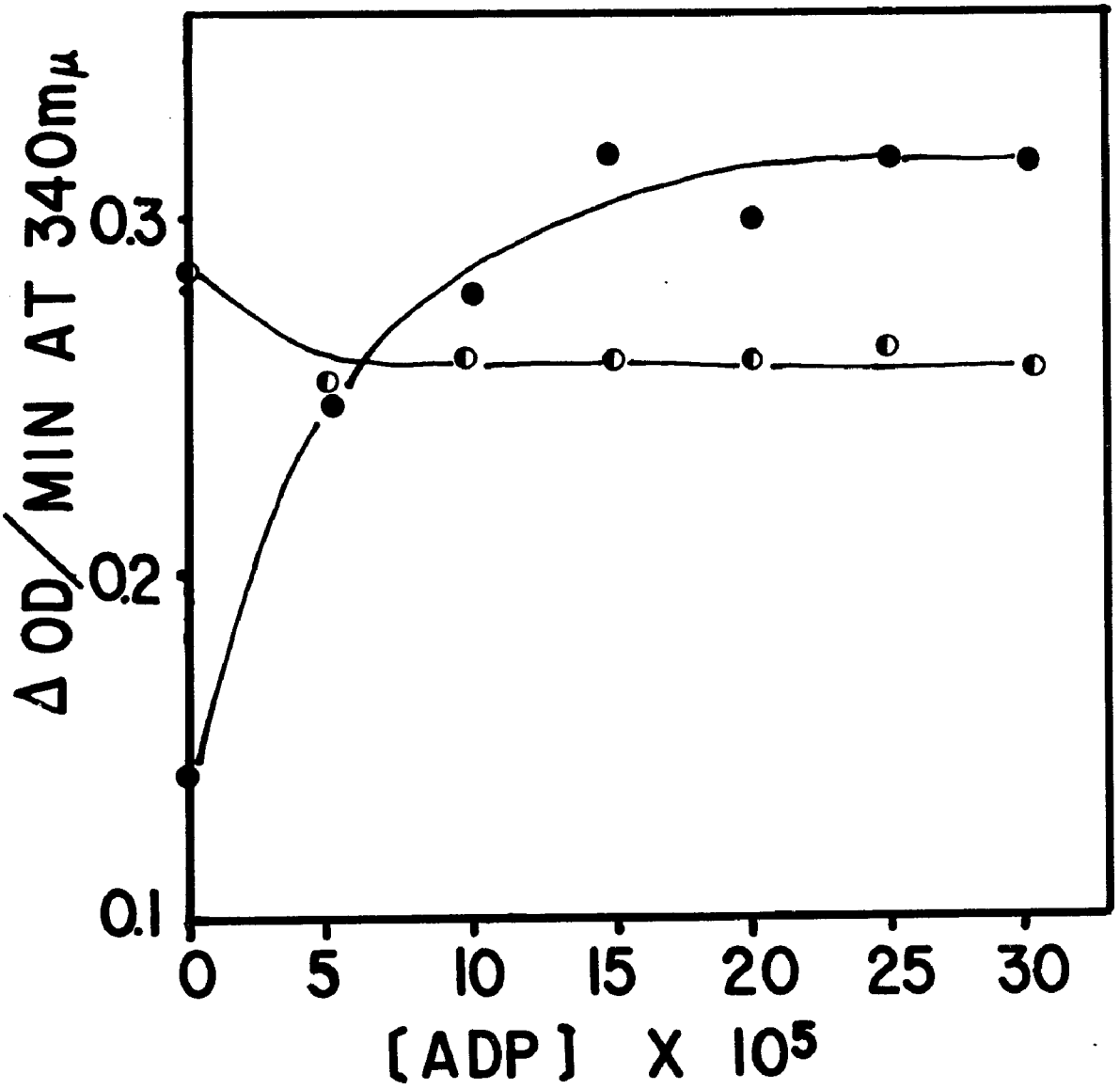

Δ OD/MIN AT 340mμ
0.3
0.2
0.1
0
5
10
15
20
25
30
[ADP] X 10⁵

FIGURE 5

Effect of Diethylstibestrol on Native and Methylmercuric Iodide Treated Glutamate Dehydrogenase. The enzyme was incubated with methylmercuric iodide for 5 minutes at a molar ratio of 1:1 and followed by dialysis. The enzyme was assayed as described in the text with varying amounts of diethylstilbestrol in 50% propyleneglycol at an enzyme concentration of 0.40 µg/ml of medium. (◐ - ◐) = native enzyme; (◑ - ◑) = methylmercurial treated enzyme.

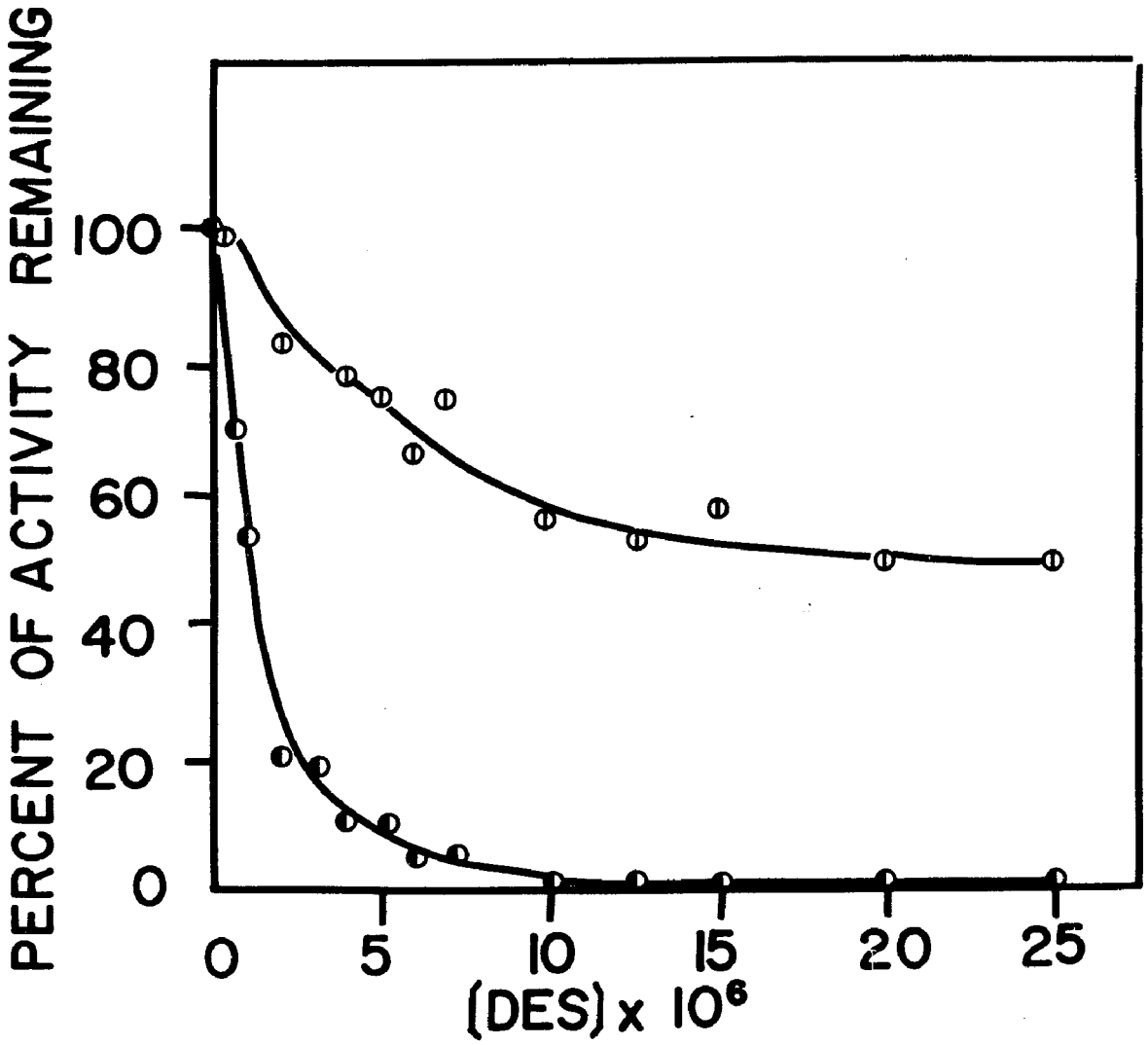

PERCENT OF ACTIVITY REMAINING
100
80
60
40
20
0
0
5
10
15
20
25
(DES) x 10⁶

FIGURE 6

Effect of Guanosine Triphosphate on Native and Methylmercuric Iodide Treated Glutamate Dehydrogenase. The enzyme was incubated with methylmercuric iodide for 5 minutes at a molar ratio of 1:1 and followed by dialysis. The enzyme was assayed as described in the text with varying concentrations of GTP at an enzyme concentration of 4.0 μg/ml of medium. Symbols: (◐ - ◐) = native enzyme; (◑ - ◑) = mercurial treated enzyme.

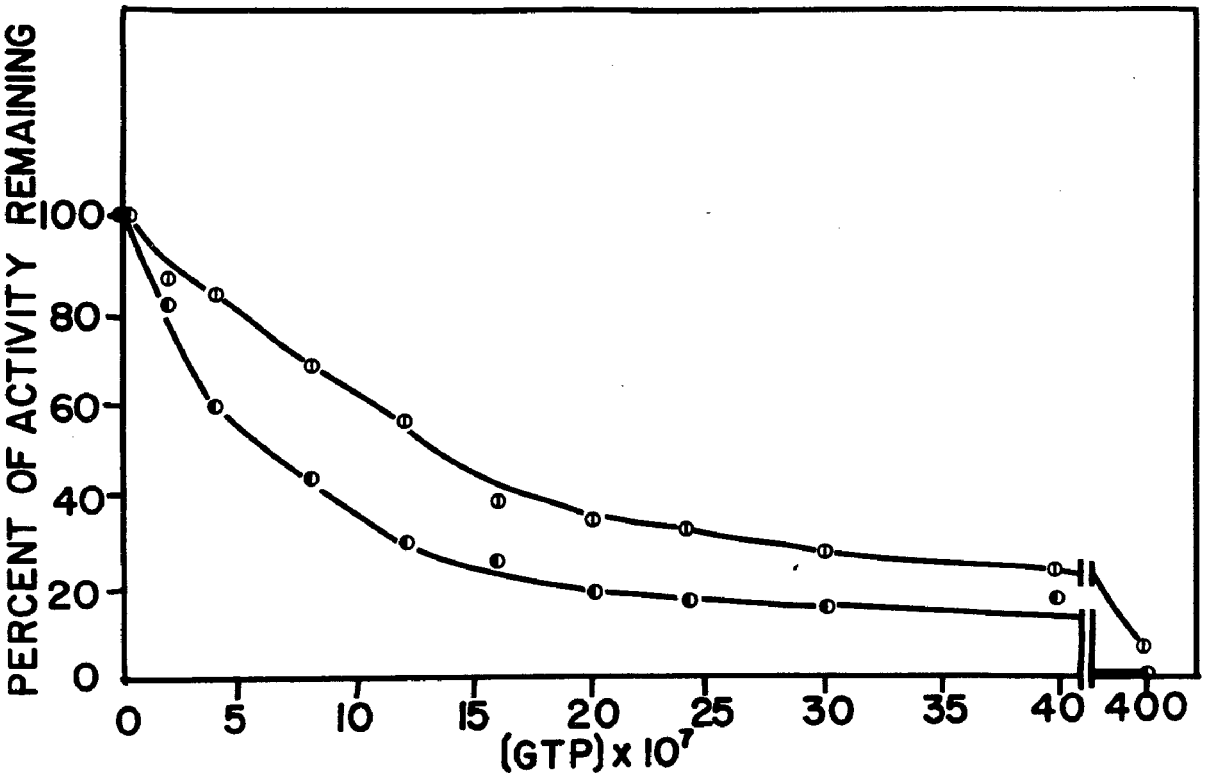
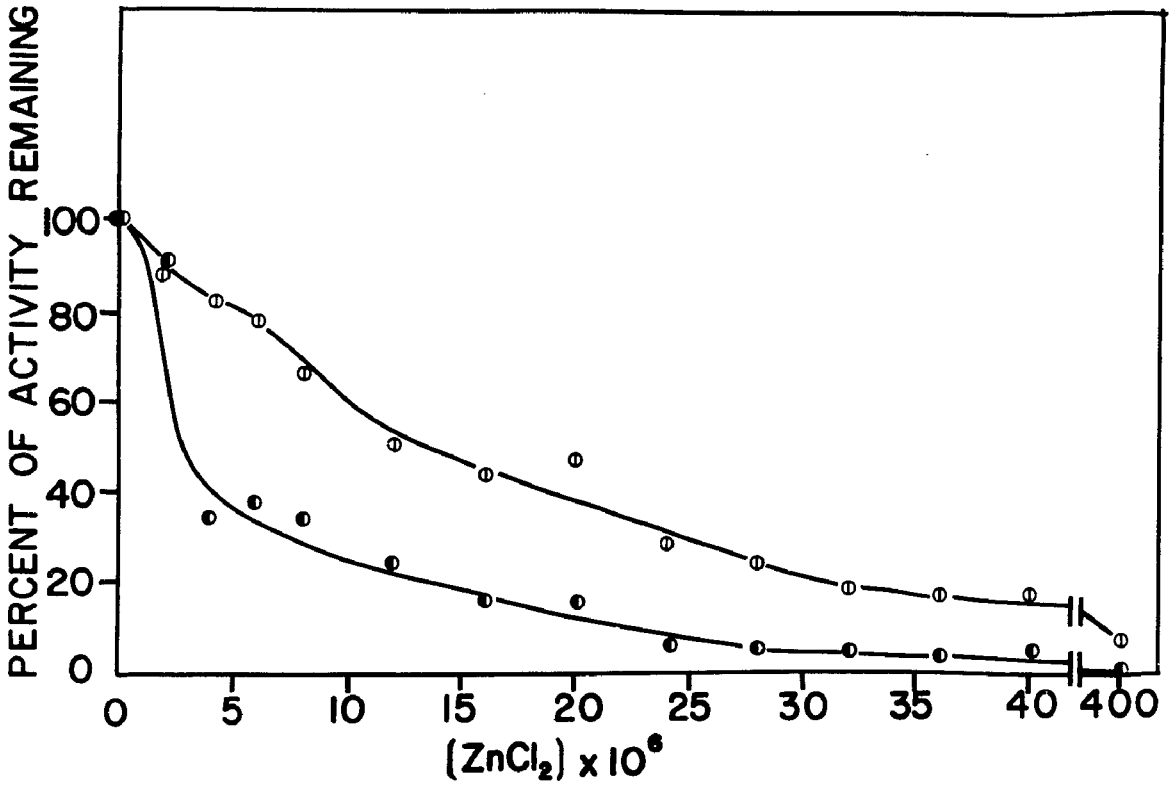

PERCENT OF ACTIVITY REMAINING
100
80
60
40
20
0
0
5
10
15
20
25
30
35
40
400
(GTP) x 10⁷

FIGURE 7

Effect of Zinc Chloride on Native and Methylmercuric Iodide Treated Glutamate Dehydrogenase. The enzyme was incubated with methylmercuric iodide for 5 minutes at a molar ratio of 1:1 and followed by dialysis. The enzyme was assayed as described in the text with varying concentrations of $ZnCl_2$. EDTA was omitted from the assay system, which contained 1.3 µg enzyme/ml medium. Symbols: (◐-◐) = native enzyme; (◑-◑) = mercurial treated enzyme.


PERCENT OF ACTIVITY REMAINING
100
80
60
40
20
0
0
5
10
15
20
25
30
35
40
400
(ZnCl2) x 10^6

one mercurial bound per chain was examined in the analytical centrifuge. These studies showed that the treated enzyme tended to remain aggregated in the presence of GTP and NADH as reported previously (8,9), contrary to the results with untreated enzyme with GTP (Figure 8). Thus, the substitution of one amino acid residue of one GDH chain with one mercurial appears to be responsible for this change in aggregation properties.

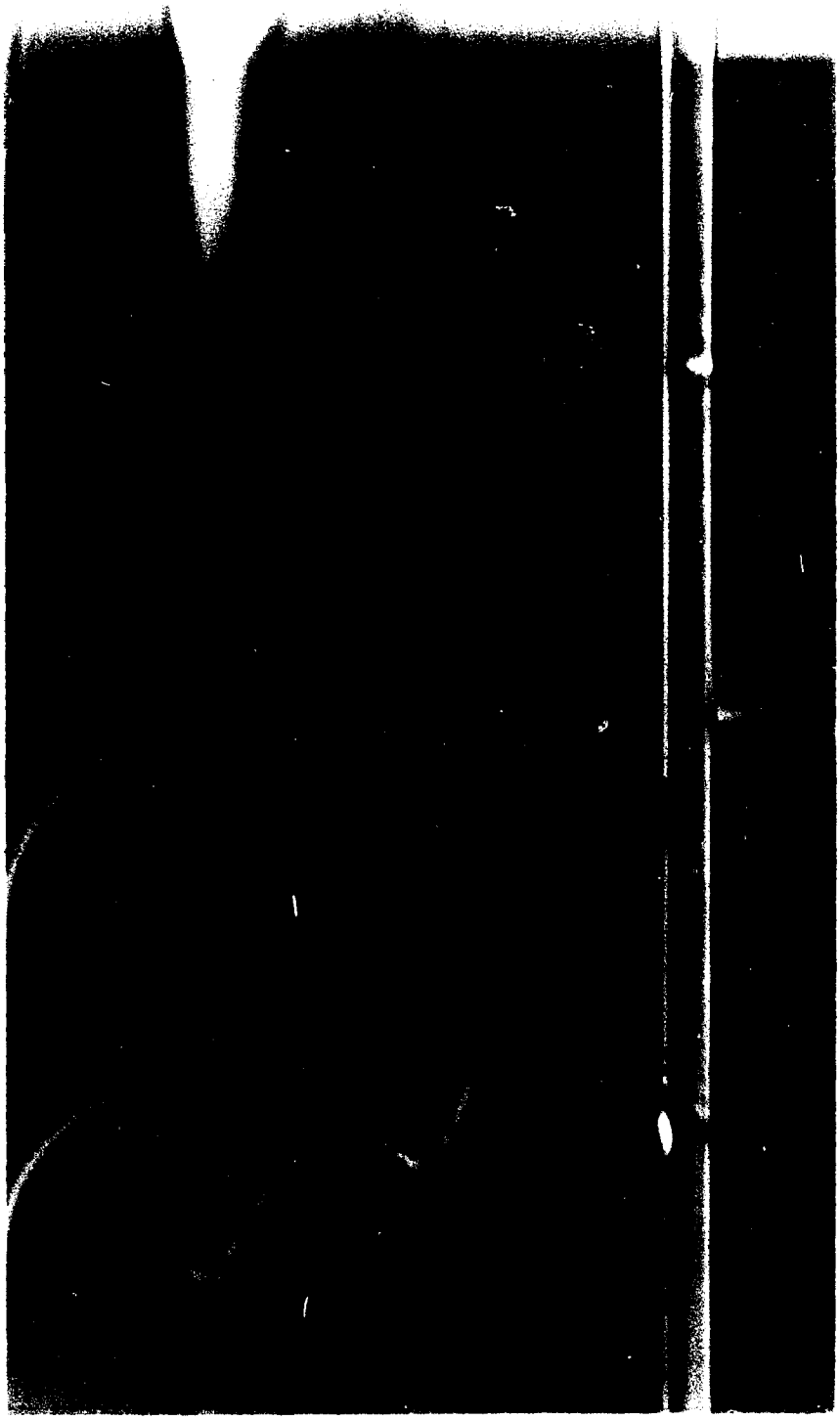
The Effect of Methylmercuric Iodide on the Stability of GDH to Heat

A possible explanation for the effect of the mercurial is that it stabilizes the enzyme conformation so that ligand perturbation of the structure is prevented. It was of considerable interest, therefore, to determine whether the mercurial treated enzyme is stable to heat, and whether the modified enzyme is protected against conformational changes normally induced by heat. Aliquots of enzyme alone, and combined with three different amounts of $*CH_3HgI$ of 0.4, 1.0 and 5.0 molecules per enzyme chain were examined for catalytic activity after identical exposure to temperature of 30°, 40°, 50°, and 60° for 5 minutes (Figure 9).

The enzyme stability was not affected by heat as long as the ratio of mercurial bound did not exceed one molecule/chain. With an initial mercurial to enzyme ratio of 5/1 some degree of inactivation occurred almost immediately and was considerable at 50°. This experiment seems to indicate that the presence of large amounts of mercurial destablizes the enzyme such that

FIGURE 8

Effect of Methylmercuric Iodide on the Sedimentation Velocity of Guanosine Triphosphate Treated Glutamate Dehydrogenase. Each sample contained 5 mg/ml of enzyme, 1.2×10^{-4} M GTP, 1.8×10^{-4} M NADH and 1×10^{-3} M EDTA in 0.05 M Tris buffer (pH 7.4). The upper curve shows the sedimentation pattern of GDH after treatment with $*CH_3HgI$ for 5 minutes at a mole ratio of 1:1, immediately prior to loading in the centrifuge cell. The sedimentation of a control sample not treated with mercurial is shown in the lower curve. Sedimentation (from left to right) was carried out (at 50, 740 rpm for 20 minutes at 20^{o}) in a Beckman Model E. Analytical Ultracentrifuge using a rotor type AN-E.



it is more heat sensitive.

Effects of CH_3CH_2HgI on the GDH Catalytic Activity

The modification of an -SH group with CH_3HgI introduces a longer non-polar residue in place of the -SH. This increases the catalytic activity, reduces the sensitivities to allosteric reagents, and causes structural changes. Therefore, it is reasonable to consider that the methylmercuric moiety of the organic mercurial is playing a part in the stabilization of the enzyme. It is of interest to determine whether a longer aliphatic moiety of the mercurial compound affects the catalytic activity of GDH. Therefore, CH_3CH_2HgI was synthesized and tested with GDH. As seen in Figure 10 (a and b), L-glutamate activity of GDH was stimulated by the addition of CH_3CH_2HgI at a concentration as high as 8.7×10^{-3}M. The concentration of the mercurial which had the maximum stimulatory effect on GDH could not be determined due to limitations in its solubility.

2×10^{-1}M of ethylmercuric iodide was prepared in DMSO in order to determine the concentration at which GDH activity is maximally stimulated, but the compound crystallized out in the assay system at a concentration of 8.7×10^{-3}M.

At low concentrations of CH_3CH_2HgI, there was no appreciable difference in its effect on GDH as compared with CH_3HgI (Figure 11). Moreover it appears that both mercurials attached the enzyme at the same region, since $*CH_3HgI$ bound enzyme at a mole ratio of 1:1 was not affected any more by CH_3CH_2HgI (Figure 12), and the enzyme preincubated with CH_3CH_2HgI

FIGURE 9

Effect of Treatment with Methylmercuric Iodide on the Stability of Glutamate Dehydrogenase to Heat. For incubation 0.25 ml of GDH (20 mg/ml in $(NH_4)_2SO_4$) was diluted with 0.05 M phosphate buffer (pH 7.4) and made up to 1.0 ml by the addition of a calculated volume of methylmercuric iodide solution to give molar ratios of 0/1, 0.4/1, 1:1, and 5:1. After heating for 5 minutes at the temperature shown, an aliquot of each enzyme preparation was removed, diluted, and 5 µg used for catalytic assay at 25°. The activities for each enzyme preparation at each temperature are expressed relative to those on identical samples maintained at 25°. Under these experimental conditions binding to enzyme was virtually complete for mercurial to enzyme ratios of 0.4/1 and 1/1. But, as discussed previously, when a ratio higher than one was used, not all the mercurial is bound to the enzyme. In this instance, when the ratio was 5/1, only 3.2 molecules of mercurial were bound per chain. Symbols: Control enzyme (0/1) = (●-●); 0.4/1 mercurial treated enzyme = (◐-◐); 1/1 mercurial treated enzyme = (■-■); 5/1 mercurial treated enzyme = (◑-◑).

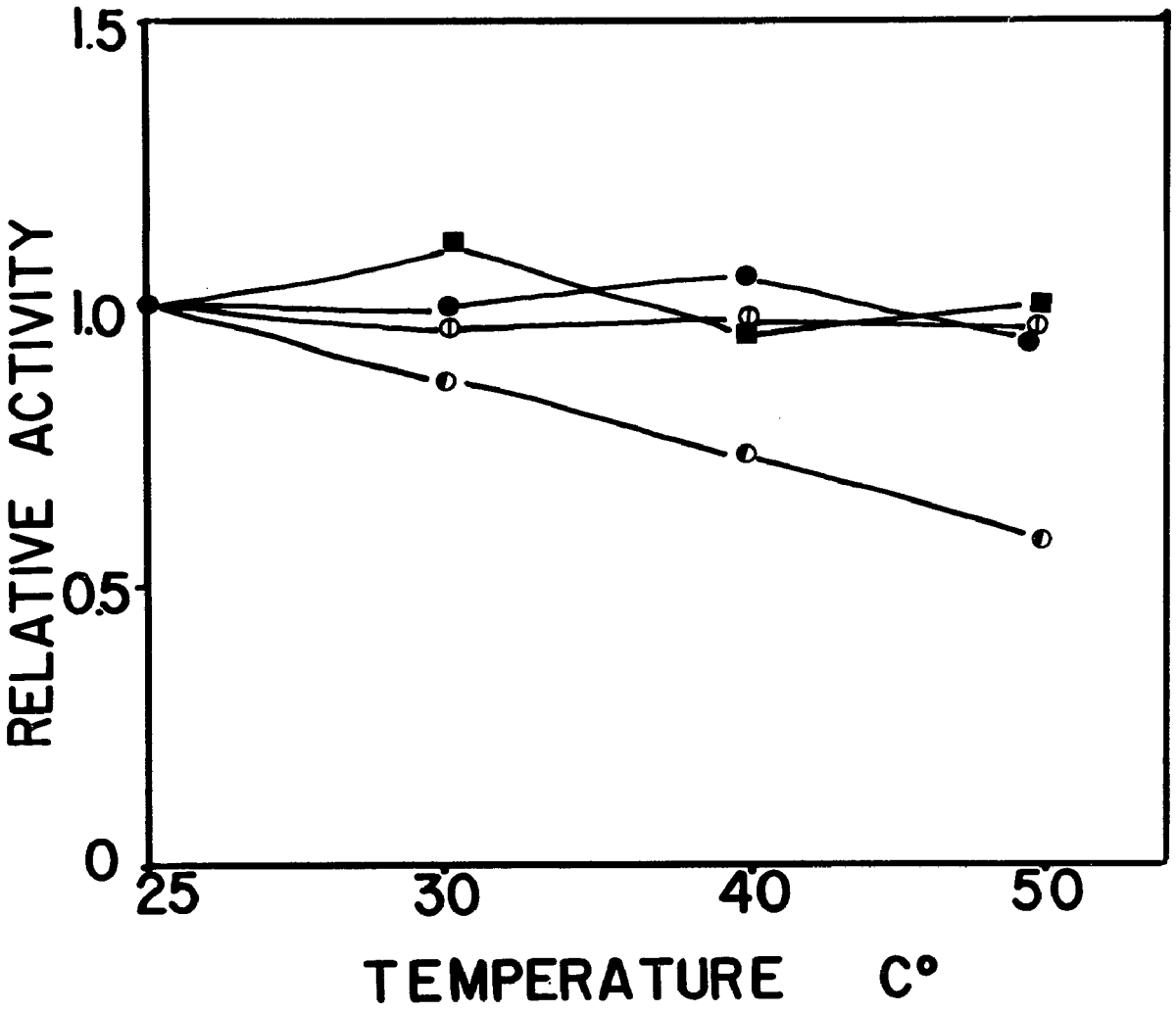
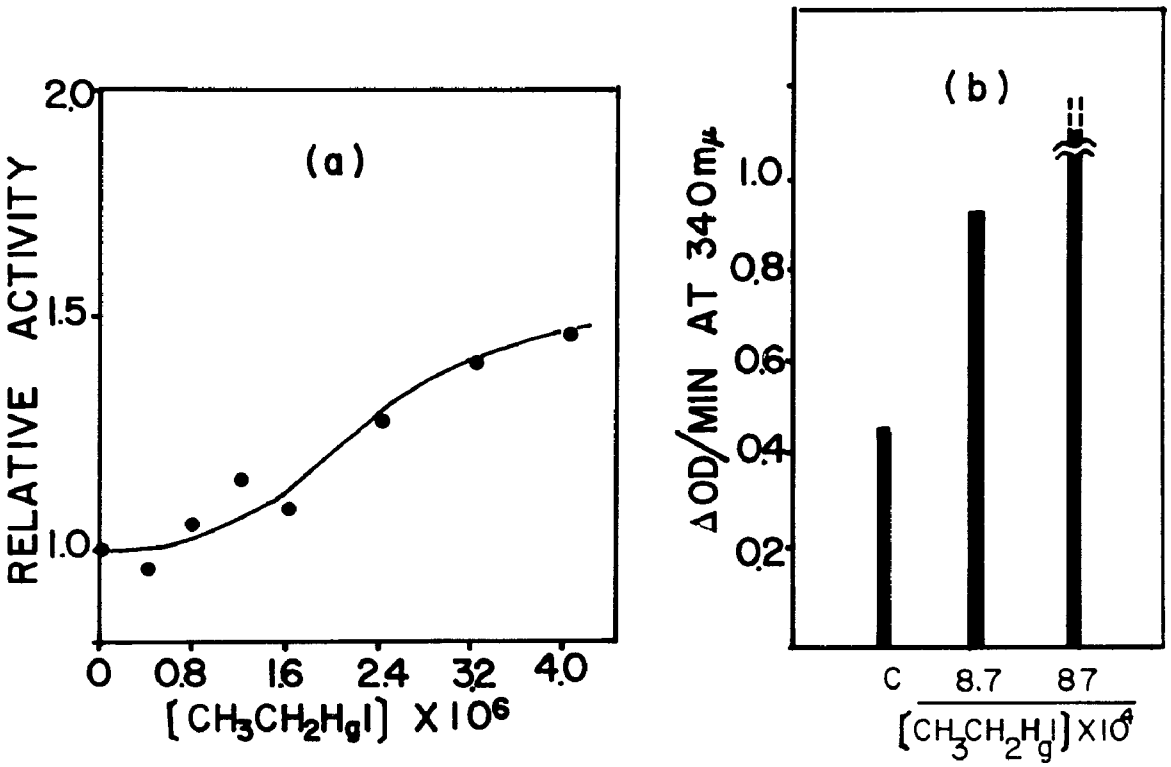

1.5
1.0
0.5
0
RELATIVE ACTIVITY
25
30
40
50
TEMPERATURE C°

FIGURE 10

Effect of Ethylmercuric Iodide on the Catalytic Activity of Glutamate Dehydrogenase. a. Relative catalytic activity expressed as a function of ethylmercuric iodide addition. The enzyme was assayed as described in the text using an enzyme concentration of 4 μg/ml. b. Effects of higher concentrations of ethylmercuric iodide added from a stock solution in dimethylsulfoxide, to the limit of solubility.


(a)
RELATIVE ACTIVITY
2.0
1.5
1.0
0
0.8
1.6
2.4
3.2
4.0
[CH3CH2Hg1] X 10^6
(b)
ΔOD/MIN AT 340mμ
1.0
0.8
0.6
0.4
0.2
C
8.7
87
[CH3CH2Hg1] X 10^6

at a mole ratio of 1:1 did not show any further stimulation by the addition of *CH_3HgI (Figure 13). This result indicated that a modification of a second -SH group of the enzyme does not stimulate the catalytic activity of GDH any further.

Effect of CH_3CH_2HgI on the Stability of GDH to Heat

GDH in glycerol was incubated with CH_3CH_2HgI at the molar ratio of 1:1, and its stability against heat effect tested. The general procedure was the same as described previously with *CH_3HgI. The results show that the modified enzyme could maintain its integrity as well as the control enzyme up to 50° (Figure 14). These experiments show that the ethyl and methyl mercurials have the same effects on the enzyme.

Summary (Part I)

^{14}C-labeled methylmercuric iodide was synthesized, and used for studying the regulatory effects of an organic mercurial on glutamate dehydrogenase, and its specificity for -SH group containing compounds was confirmed.

When the methylmercuric iodide was bound to the enzyme at a mole ratio of 1:1, based on the enzyme molecular weight of 53,500, there was maximum stimulation for the glutamate ⇌ α-ketoglutarate reaction and the enzyme became relatively insensitive to the effect of various allosteric effectors.

When the binding ratio did not exceed 1:1, the enzyme remained stable in solution. An organic mercurial having a longer aliphatic chain like CH_3CH_2HgI had a similar effect on GDH as CH_3HgI although the stoichiometry could not be determined due to

FIGURE 11

Effects of Methylmercuric Iodide and Ethylmercuric Iodide on Glutamate Dehydrogenase. Each mercurial was dissolved at a concentration of 2×10^{-3} M in DMSO. Identical concentrations of each mercurial were added to the assay system. The assay procedure is described in the text. The enzyme concentration was 0.8 µg/ml. Symbols: (●-●) = GDH activity in the presence of methylmercuric iodide; (◐-◐) = activity in the presence of ethylmercuric iodide.

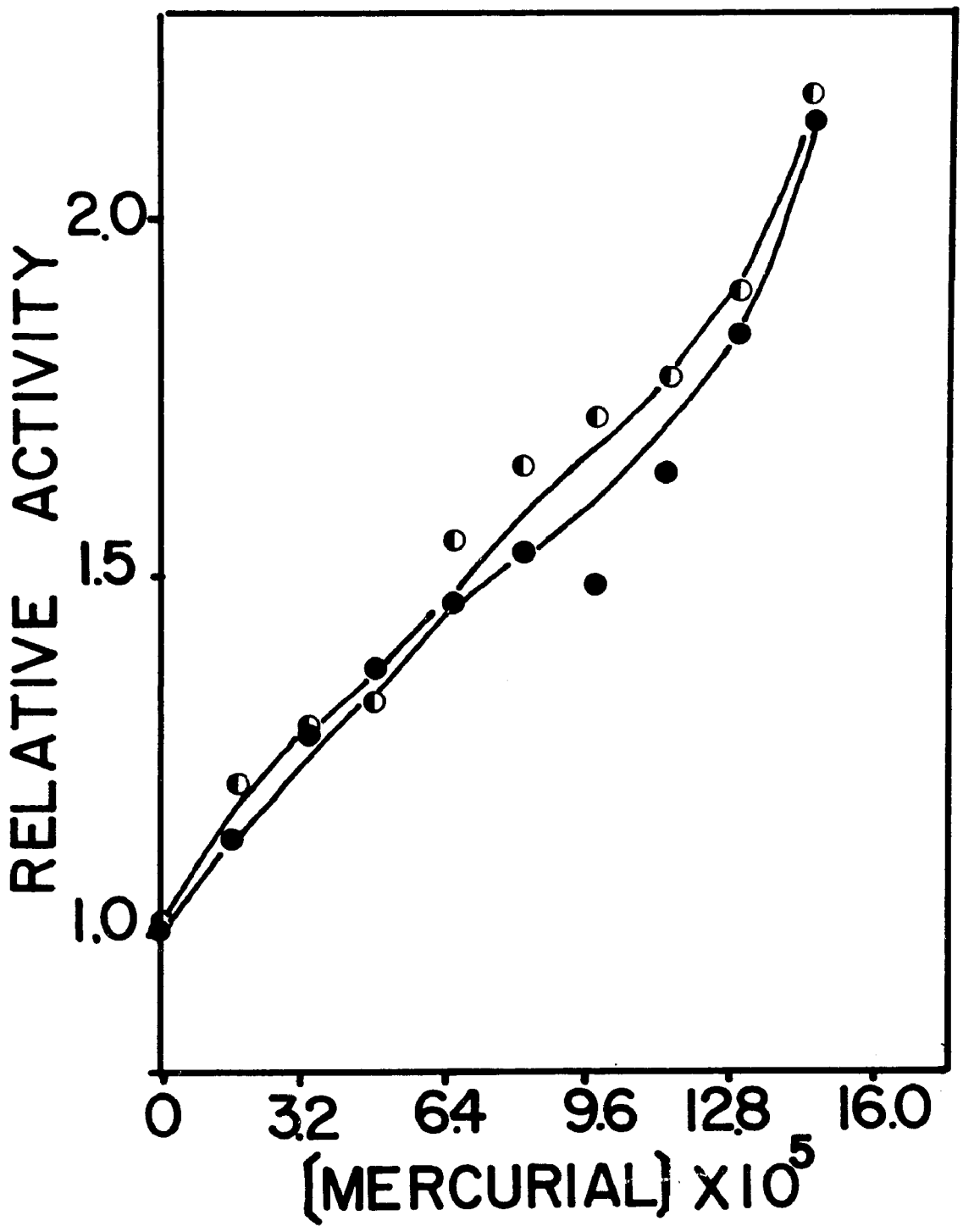

RELATIVE ACTIVITY
2.0
1.5
1.0
0
3.2
6.4
9.6
12.8
16.0
[MERCURIAL] X10^5

FIGURE 12

Effect of Ethylmercuric Iodide on the Catalytic Activity of the Native and of the Methylmercuric Iodide Modified Glutamate Dehydrogenase. The *CH_3HgI modified GDH (1:1) was prepared by incubation of GDH with the mercurial for 5 minutes followed by dialysis. The native enzyme was treated in the same way except for the mercurial. The graph shows varying concentrations of CH_3CH_3HgI added to the CH_3HgI — modified GDH (1:1) and to native GDH (Control) versus ΔOD/min at 340 mμ. Enzyme concentration = 1.6 μg/ml. Symbols: (◐-◐) = *CH_3HgI treated enzyme; (●-●) = Control enzyme.

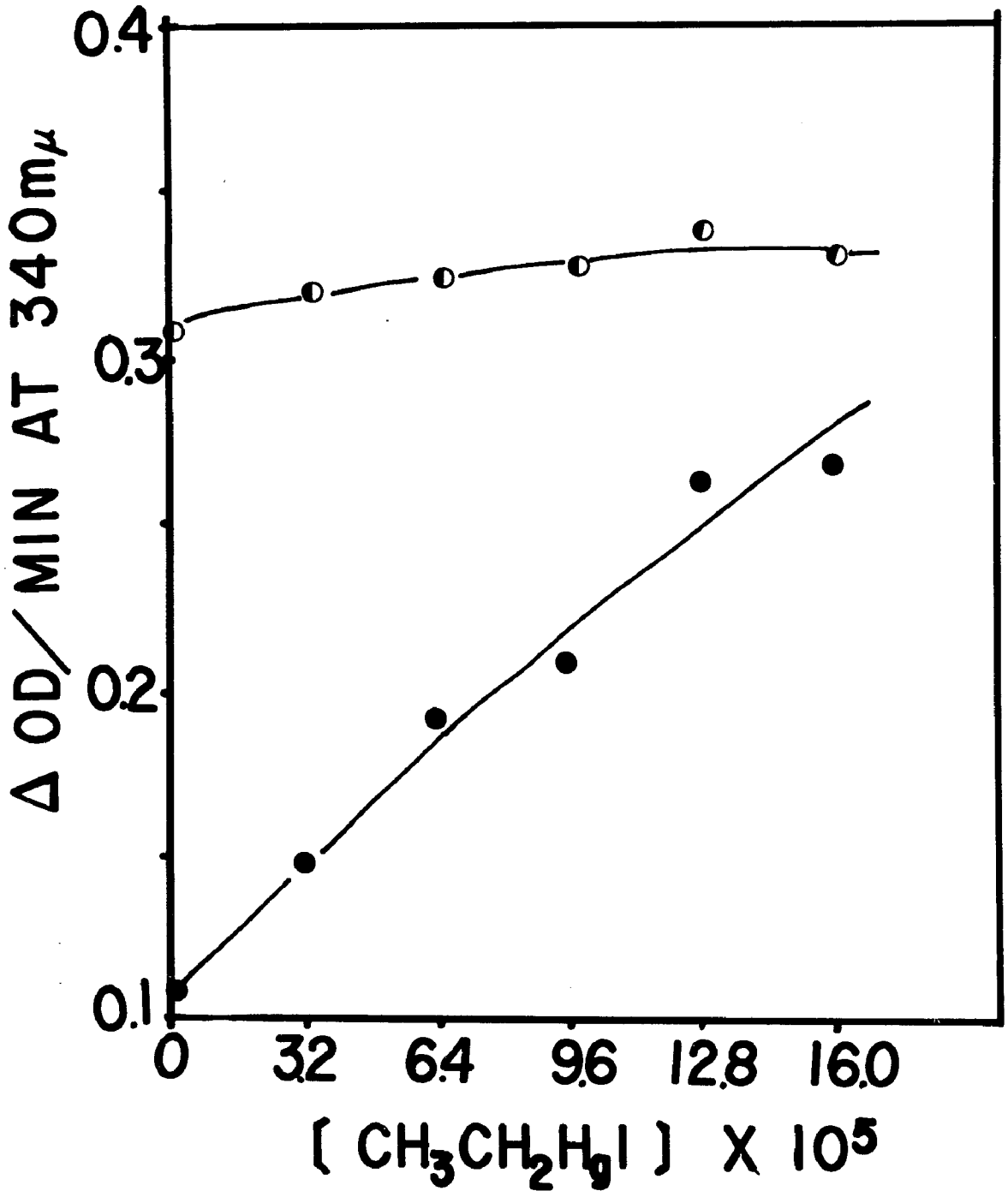

0.4
0.3
0.2
0.1
Δ OD/ MIN AT 340mμ
0
3.2
6.4
9.6
12.8
16.0
[CH3CH2HgI] X 10^5

FIGURE 13

Effect of Methylmercuric Iodide on the Catalytic Activity of Ethylmercuric Iodide Treated Glutamate Dehydrogenase. Various concentrations of $*CH_3HgI$ were added to the CH_3CH_2HgI modified GDH (1:1) and to native GDH (control).

The CH_3CH_2HgI modified GDH (1:1) was prepared in the same way as that with $*CH_3HgI$. It is assumed that free CH_3CH_2HgI was eliminated by dialysis. Enzyme concentration = 1.3 µg/ml; Symbols: CH_3CH_2HgI pretreated enzyme = (◐-◐); Native GDH = (●-●).

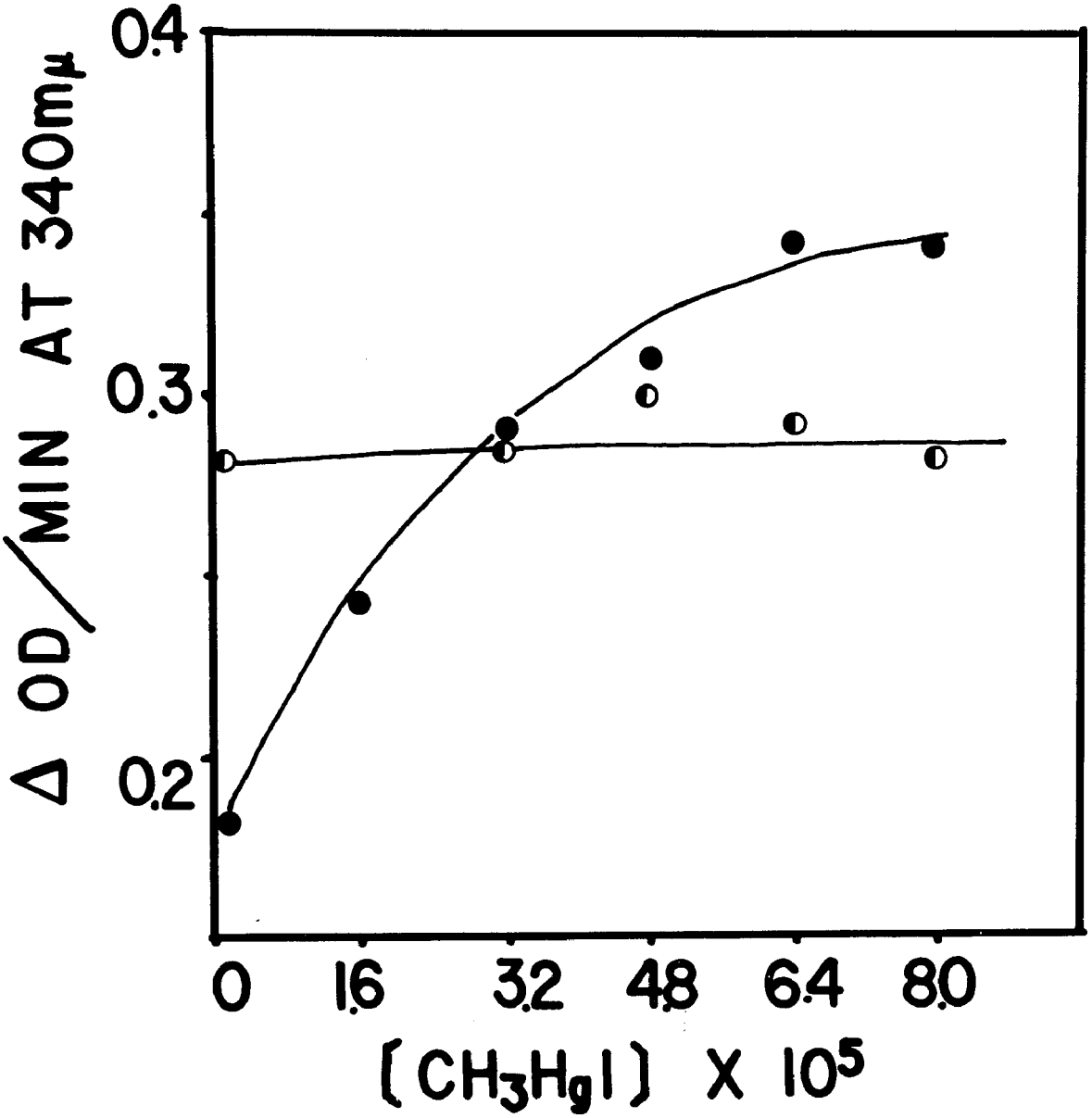
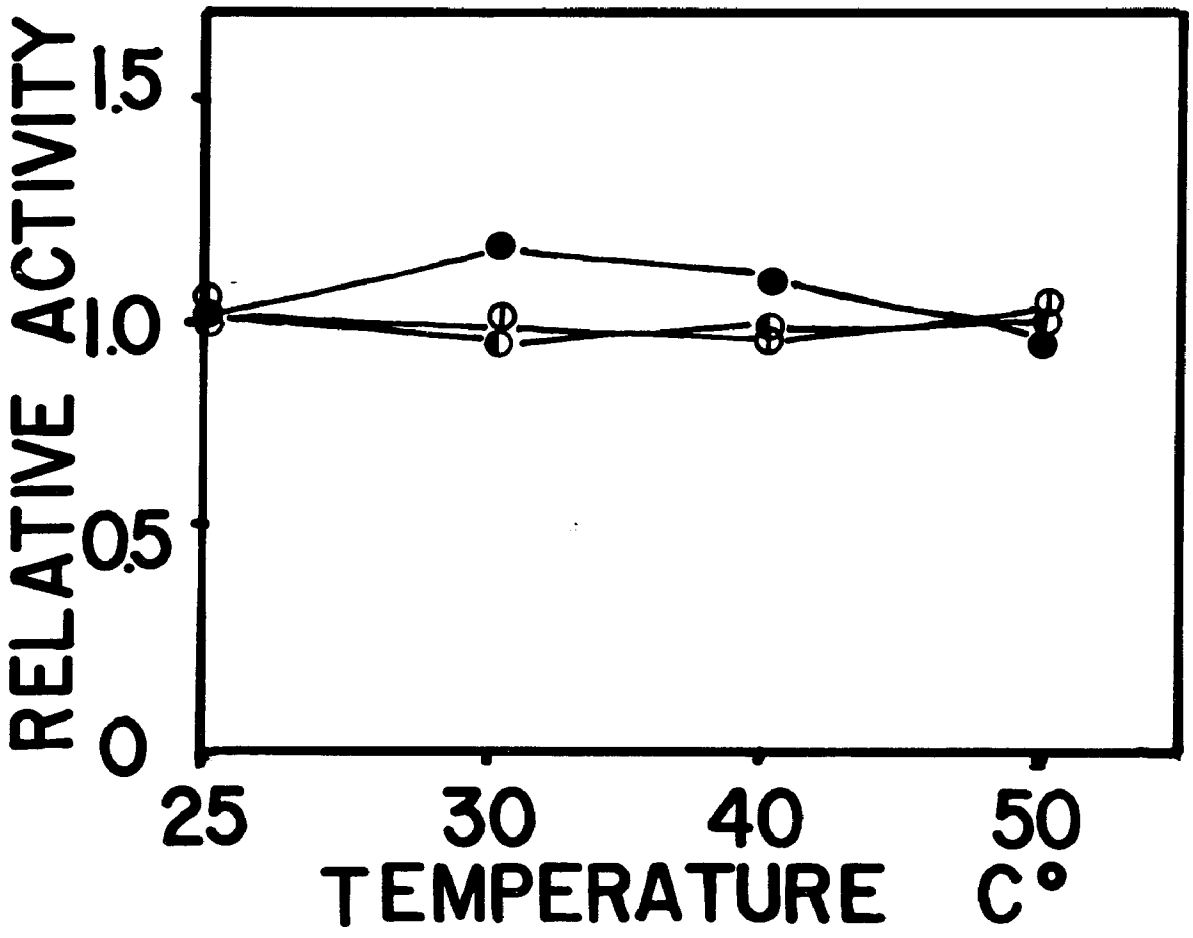

0.4
0.3
0.2
Δ OD/MIN AT 340mμ
0
1.6
3.2
4.8
6.4
8.0
[CH3HgI] X 10^5

FIGURE 14

The Effects of Heat on the Stability of Ethylmercuric Iodide Treated Glutamate Dehydrogenase. The experiment was performed as in figure 9 with untreated enzyme, added dimethylsulfoxide, and enzyme which had been treated with CH_3CH_2HgI in dimethylsulfoxide at a reagent/enzyme molar ratic of 1:1, and dialysed. Enzyme samples were exposed for 5 minutes to the temperatures shown and assayed for catalytic activity. Symbols: (●-●) = control enzyme; (◐-◐) = GDH + DMSO; (◑-◑) = GDH + CH_3CH_2HgI.


RELATIVE ACTIVITY
1.5
1.0
0.5
0
25
30
40
50
TEMPERATURE C°

its poor solubility.

These studies show, therefore, that one -SH group plays a critical role in the control of the catalytically active conformation of the enzyme.

RESULTS

PART II: PROPERTIES OF GLUTAMATE DEHYDROGENASE MODIFIED WITH VARIOUS SULFHYDRYL REAGENTS OR OTHER GROUP SPECIFIC AMINO ACID REAGENTS SINGLY AND IN COMBINATION WITH METHYLMERCURIC IODIDE

In the preceding chapter one specific sulfhydryl group has been described in GDH which can clearly be distinguished from all other -SH groups of the enzyme by its reactivity with an alkylmercuric iodide. As mentioned previously, other types of -SH reagents that have been described in the literature as enzyme modifiers (8,9,36,45,54,56,57), can also cause changes in catalytic activity, structure, and sensitivity of GDH towards allosteric reagents. The question now arises whether all such reagents are interacting with the same -SH group as the alkylmercuric iodide reagent, or whether their effects are independent. In order to answer this question, silver ion (Ag^+) and N-bromosuccinimide (NBS) were chosen as possible alternative -SH reagents. Their effects on GDH will be examined singly and in combination with the aliphatic mercurials.

It is also known that several other amino acid residues are important in the regulation of GDH by small molecules. For example, modification of GDH by trinitrobenzene sulfonate (TNBS) (for lysyl), tetranitromethane (TNM) and N-acetylimidazole (NaIm) (for tyrosyl), fluorodinitrobenzene (FDNB) (for lysyl,

tyrosyl and -SH), N-bromosuccinimide (NBS) (for tryptophanyl, tyrosyl, -SH), and acetic anhydride (for lysyl), in each instance results in changes in overall catalytic activity and/or sensitivity toward regulation. It is not known whether or not the amino acids modified by these reagents are in the same region of the protein and what relation they bear to the catalytic site or the critical -SH group.

If it were possible to modify more than one of these functional amino acids with a single reagent, more understanding could be gained regarding the spatial arrangement of the various active sites and the structure-function relationship in GDH. However, at the present time, few bifunctional enzyme modifiers are known which react simultaneously with two chemically different amino acid residues of the enzyme (30,59,76) and such modifiers for sulfhydryl, lysyl, and tyrosyl groups have not been found. Therefore, a different approach had to be taken. GDH was sequentially modified with two different reagents, each of them specific for one amino acid residue, and the "doubly modified" enzyme examined for its catalytic activity and sensitivity to allosteric reagents. In the present work the effect of CH_3HgI on GDH, in which either a lysyl group had been modified with TNBS, or a tyrosyl group with TNM, was studied with the hope of obtaining more knowledge about the unique relationship between various sites.

Effect of Silver Ion on the Catalytic Activity of the *CH_3HgI Modified GDH

Silver ion (Ag^+) is a potent inhibitor of the catalytic activity of GDH and its reactivity with -SH groups in the enzyme has been reported (36,45,54). According to Hellerman et al (36), two -SH equivalents per enzyme protein of molecular weight of 25,000 (i.e. four per chain of M. W. 53,500) can be distinguished by their behavior towards Ag^+. The modification of both -SH groups is required for complete inactivation of the L-glutamate catalytic activity. From sedimentation studies (57) it was learned that the extent of GDH dissociation proceeds parallel to the stoichiometry of added Ag^+ to 10^6 grams of enzyme, (i.e. 1, 2 and 4 Ag^+ per chain of M.W. 53,500), respectively resulted in an increased number of components with smaller sedimentation coefficients. These studies raised the question whether the specific -SH group which is reactive towards CH_3HgI is identical with that modified with Ag^+ since the two reagents produce rather different changes in enzyme properties.

To answer this question, the effect of *CH_3HgI on GDH pretreated with silver ions was tested. As Figures 15 and 16 show, the effect of Ag^+ on the catalytic activity of GDH is overcome by the addition of *CH_3HgI. However, GDH which had been incubated with four equivalents of Ag^+ with almost complete loss of its catalytic activity could not be reactivated by the addition of *CH_3HgI (data not shown).

It is likely that methylmercuric iodide has a greater affinity for a critical residue in GDH than silver ion, and that

FIGURE 15

Effect of Ag^+ on the Catalytic Activity of Glutamate Dehydrogenase modified with Methylmercuric Iodide. The results are expressed as percent of activity remaining versus Ag^+ equivalents added to enzyme. The $*CH_3HgI$ modified GDH was prepared by incubation of GDH with $*CH_3HgI$ (1:1) followed by dialysis. Volumes of a 3.74×10^{-3} M $AgNO_3$ solution were added to 2.0 ml aliquots of native and modified enzyme (1 mg/ml in 0.05 M PO_4 buffer pH 7.5) to give the desired equivalent ratios. The volume changes due to addition of microliter quantities were neglected. After two minutes incubation, the enzyme solutions were diluted to twice volume and assayed for catalytic activity using 2 μg/ml of the enzyme in the assay mixture. EDTA was omitted in all the work with Ag^+. (Control GDH = (●-●); $*CH_3HgI$ treated GDH (1:1) = (◐-◐)).

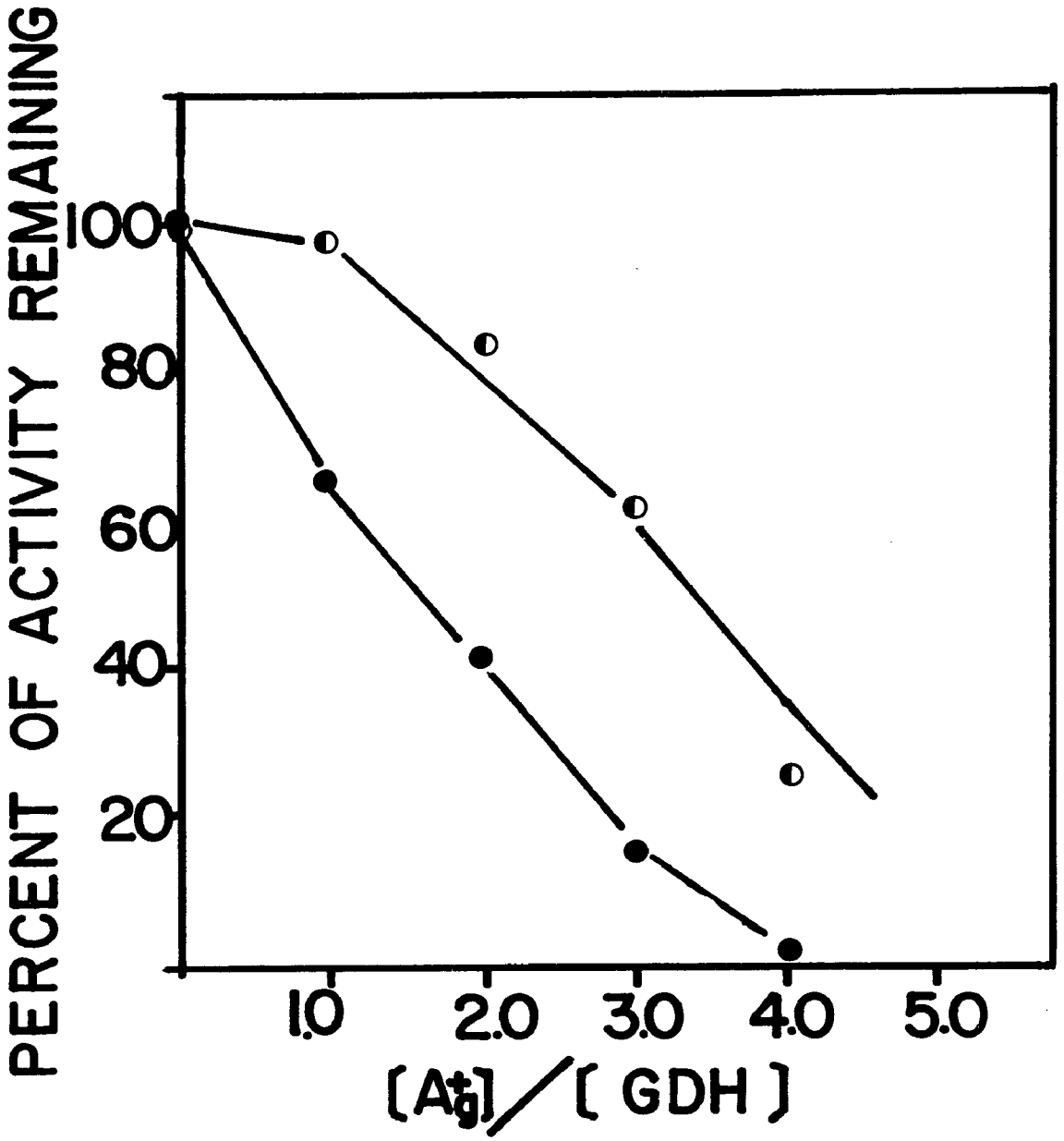
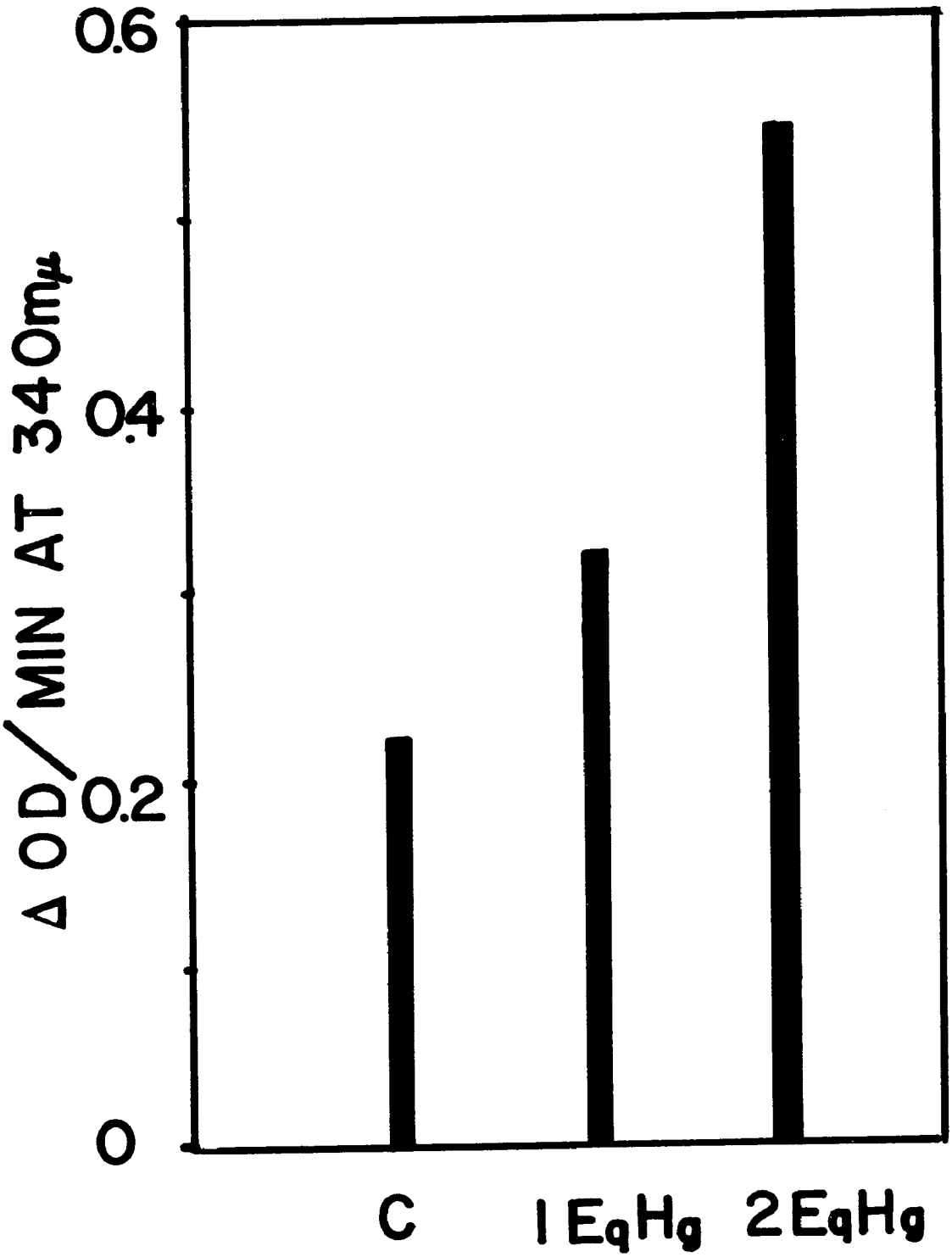

PERCENT OF ACTIVITY REMAINING
100
80
60
40
20
1.0
2.0
3.0
4.0
5.0
[ATP] / [GDH]

FIGURE 16

Effect of Ag^+ Treatment on the Response of Glutamate Dehydrogenase to Methylmercuric Iodide. In this experiment 2.0 ml of enzyme (2 mg/ml) in phosphate buffer pH (7.5) were incubated for two minutes with 2 equivalents of silver nitrate (3.7×10^{-3} M). To these mixtures, 1 and 2 equivalents of $*CH_3HgI$ were added sequentially at each interval. The volume changes due to addition of small quantity were neglected. An aliquot of enzyme was diluted ten times and assayed. EDTA was omitted from the standard assay medium. C stands for control which is in the absence of the mercurial. 1 EqHg and 2 EqHg refer to the addition of one or two equivalents of $*CH_3HgI$ to the pre-treated GDH.


0.6
0.4
0.2
0
Δ OD / MIN AT 340mμ
C
1 Eq Hg
2 Eq Hg

Ag^+ is less specific than the mercurial in its effects.

Effect of N-Bromosuccinimide on the Catalytic Activity of GDH

N-bromosuccinimide (NBS) modified tryptophanyl residues, and may possibly oxidize -SH groups in a protein (63,64). The preference of NBS for one amino acid over the other in GDH has not been established. The addition of NBS enhances the catalytic activity of GDH. The maximum stimulatory effect is achieved near a molar ratio of 5:1 of NBS/enzyme, and the activity is gradually reduced at higher ratios than that (66). The existence of a tryptophanyl residue has been postulated in the catalytic site of GDH. For the modification of tryptophanyl residues, NBS/GDH ratios of about 12:1 are required, a condition under which more than 80% of the catalytic activity has been eliminated (66).

The point of interest is now whether NBS, at the lower NBS/GDH ratios, oxidizes the specific -SH group which is reacting readily with CH_3HgI, and whether the stimulatory effect of NBS on GDH results from the modification of this particular -SH group. To find an answer to this question, the catalytic activity of the CH_3HgI treated enzyme (at a 1:1 mole ratio) was compared with that of control enzyme in the presence of varying amounts of NBS.

Figure 17 shows the increased catalytic activity of the control enzyme as a result of the addition of NBS between the molar ratios of NBS/GDH of 1:1 to 5:1. However, the mercurial treated enzyme did not show any stimulation of catalytic activity on addition of NBS. The activity remained unchanged up to a 3:1 ratio of NBS/GDH and inhibition set in as the ratio was

raised beyond this point.

In the next experiment, conditions were reversed. The results depicted in Figure 18 show that the control enzyme is stimulated as before by CH_3HgI, that the 1:1 NBS modified enzyme is stimulated less and that the NBS-treated GDH at a mole ratio of 5:1 has lost almost all its sensitivity to CH_3HgI. Moreover, all three curves show the same maximum extent of enzyme activity. This information seems to imply that NBS and CH_3HgI are reacting with the same site, although the affinity of NBS to the specific site must be less than that of the mercurial, since a NBS/GDH ratio of 5:1 eliminates CH_3HgI reactivity but a 1:1 ratio does not. An alternate explanation is that the two reagents are reacting at different sites but producing the same conformation of the enzyme.

The preceding experiments have shown that the effect of NBS on the catalytic activity of GDH is to some extent similar to that of the mercurial modifier. For additional comparison of the effects of the two reagents on the properties of GDH, the enzyme was modified with NBS and then tested for its sensitivity towards the allosteric modifiers ADP and GTP. Figure 19 shows that a mole ratio of NBS/GDH of 5:1 can completely block the ADP interaction with GDH just like CH_3HgI does at a 1:1 ratio, but a NBS ratio of 1:1 is not enough to control the regulatory activity of GDH. Figure 20 shows an analogous experiment with GTP, in which aliquots of 2.5×10^{-5} M GTP solution in 0.05 M phosphate buffer (pH 7.5) were added to NBS-modified enzymes. Here again the effect of GTP on the NBS modified enzyme was reduced. It

FIGURE 17

Effect of N-Bromosuccinimide on the Catalytic Activity of Glutamate Dehydrogenase Modified with Methylmercuric Iodide. Data are expressed as ΔOD/min. at 340 mμ as a function of the molar ratio of added NBS to enzyme. The $^{*}CH_3HgI$ modified GDH was prepared by incubation of GDH with $^{*}CH_3HgI$ (1:1), followed by dialysis. To 1 ml of a solution containing 1 mg/ml of $^{*}CH_3HgI$ modified enzyme, calculated quantities of 1.87×10^{-3} M NBS solution were added sequentially to give the desired mole ratios. Volume changes due to the addition of these minute amounts of NBS were neglected. Enzyme concentration = 2.5 μg/ml. Symbols: (● - ●) = control; (◐ - ◐) = $^{*}CH_3HgI$ modified enzyme.

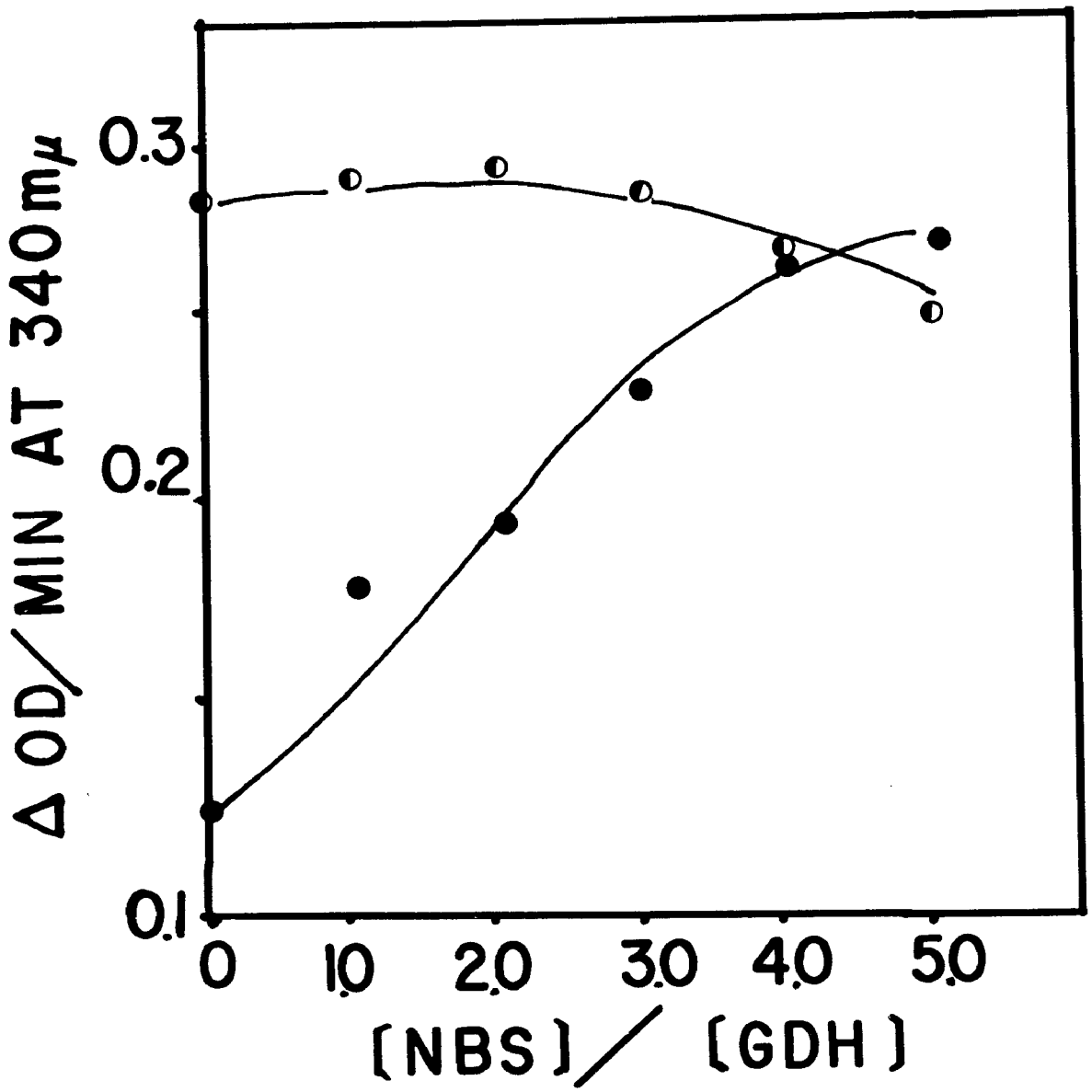
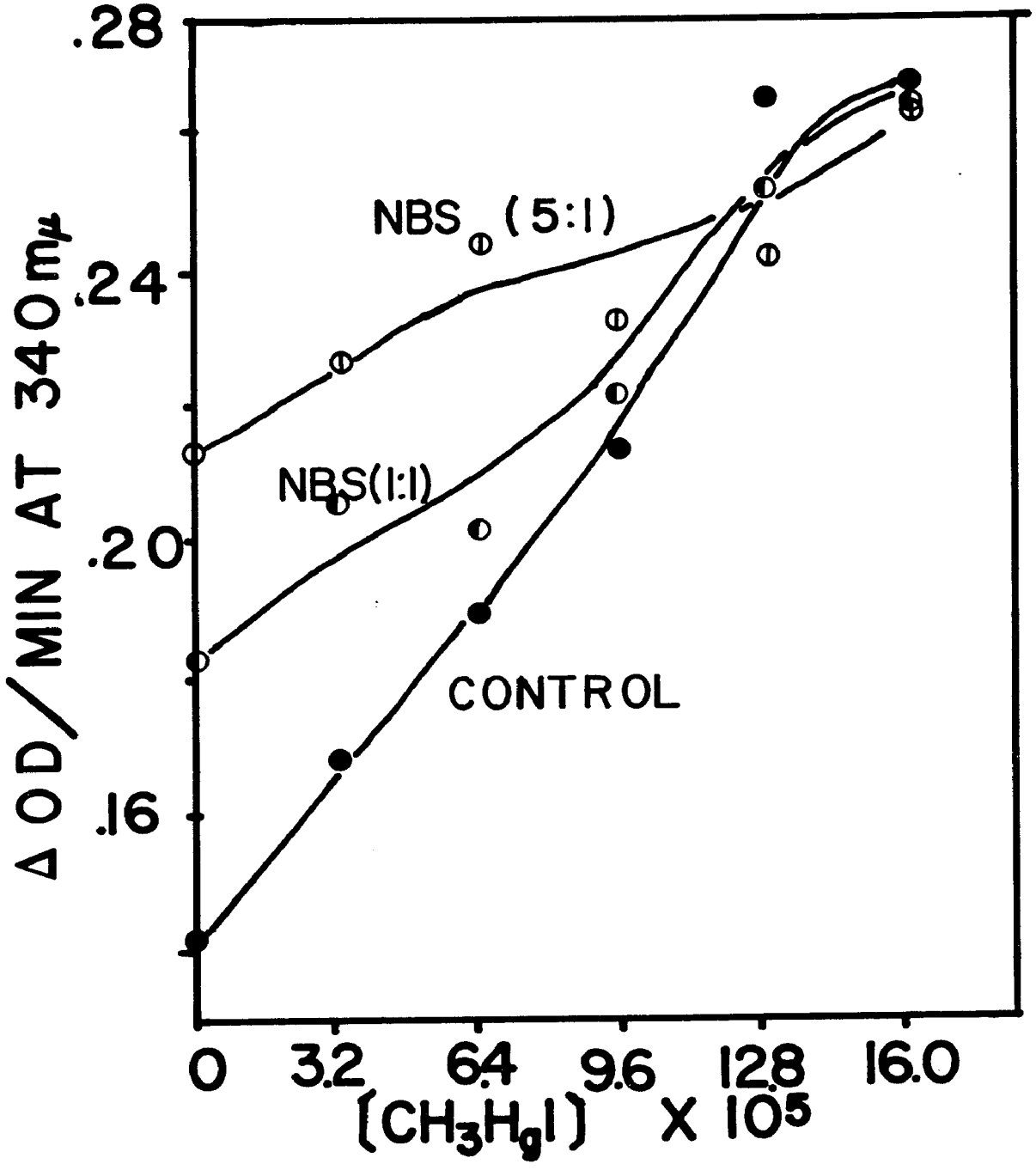

Δ OD/MIN AT 340mμ
0.3
0.2
0.1
0
1.0
2.0
3.0
4.0
5.0
[NBS] / [GDH]

FIGURE 18

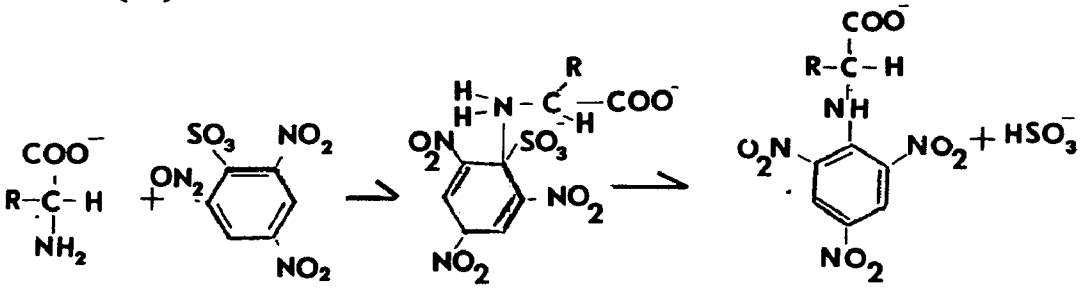
Effect of Methylmercuric Iodide on Glutamate Dehydrogenase Pretreated with N-Bromosuccinimide. GDH was pretreated with NBS at a molar of ratio of 1:1 and 5:1 for 5 minutes, respectively. Small amounts of 2×10^{-3} M CH_3HgI solution in dimethylsulfoxide were then added to the assay system to achieve the desired concentrations. Symbols: (● - ●) = control GDH; (◐ - ◐) = NBS/GDH pretreated 1:1; (Φ - Φ) = NBS/GDH pretreated 5:1. Enzyme concentration 2.0 µg/ml.


.28
.24
.20
.16
Δ OD / MIN AT 340 mμ
NBS (5:1)
NBS(1:1)
CONTROL
0
3.2
6.4
9.6
12.8
16.0
[CH3HgI] X 10^5

seems likely, therefore, that CH_3HgI and NBS attack the same site of GDH and have a similar mechanism of enzyme regulation, even though NBS has less specificity.

Effect of the Modification of Glutamate Dehydrogenase with Methylmercuric Iodide and Trinitrobenzenesulfonic Acid on the Catalytic Activity and on the Sensitivity Towards Allosteric Reagents

Changes in properties of GDH can also be elicited by reacting other amino acid residues with specific reagents. For instance, lysyl group (s) (ε-amino) of the enzyme react with acetic anhydride (15). This finding has created great interest in the role lysyl group (s) play in GDH. Trinitrobenzenesulfonic acid reacts with free alpha and epsilon amino groups of amino acids, peptides and proteins by the mechanism proposed by Palmer and Peters (46):



The specificity of the TNBS reaction has been studied by Kotaki et al (39), who reported that TNBS does not react with histidyl, guanidinyl or hydroxyl groups, but forms a liable complex with -SH groups which decomposes rapidly. In fact, amino groups are preferentially modified with TNBS even in the presence of free -SH groups, (44).

When TNBS is used for the modification of GDH, one particular lysyl group per enzyme chain of 53,500 is recognized as

FIGURE 19

Effect of Adenosine Diphosphate on the Catalytic Activity of Glutamate Dehydrogenase modified with N-Bromo-succinimide. NBS-modified GDH was prepared as before at a reagent/enzyme mole ratio of 1:1 and 5:1. Aliquots of a 7.5×10^{-3} M ADP solution in 0.05 M phosphate buffer (pH 7.5) were then added to the assay system to attain the desired concentrations. Symbols: (● - ●) = Control GDH; (◐ - ◐) = NBS modified GDH at 1:1 ratio; (◑ - ◑) = NBS modified GDH at 5:1 ratio. Enzyme concentration = 2 μg/ml.

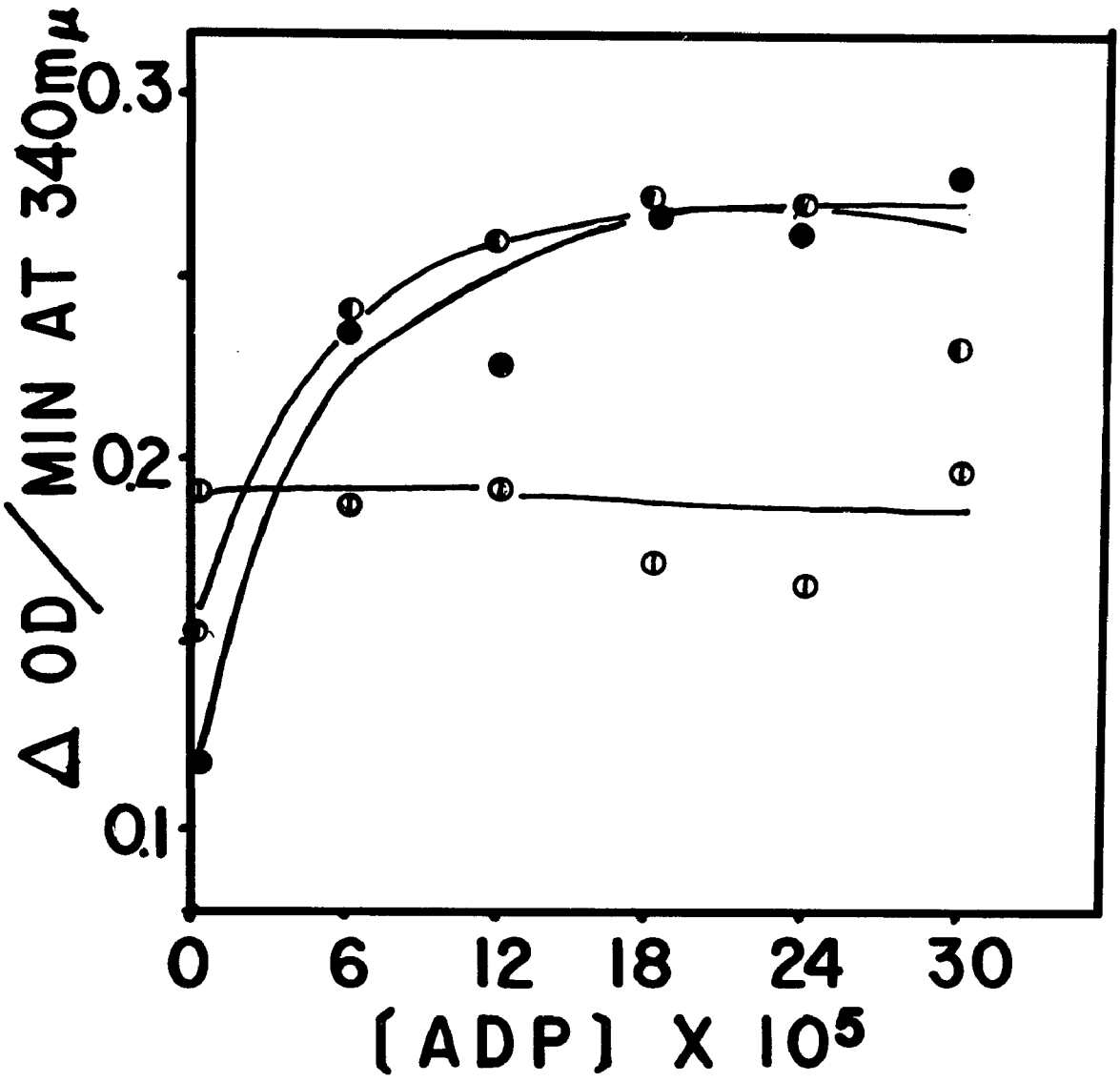
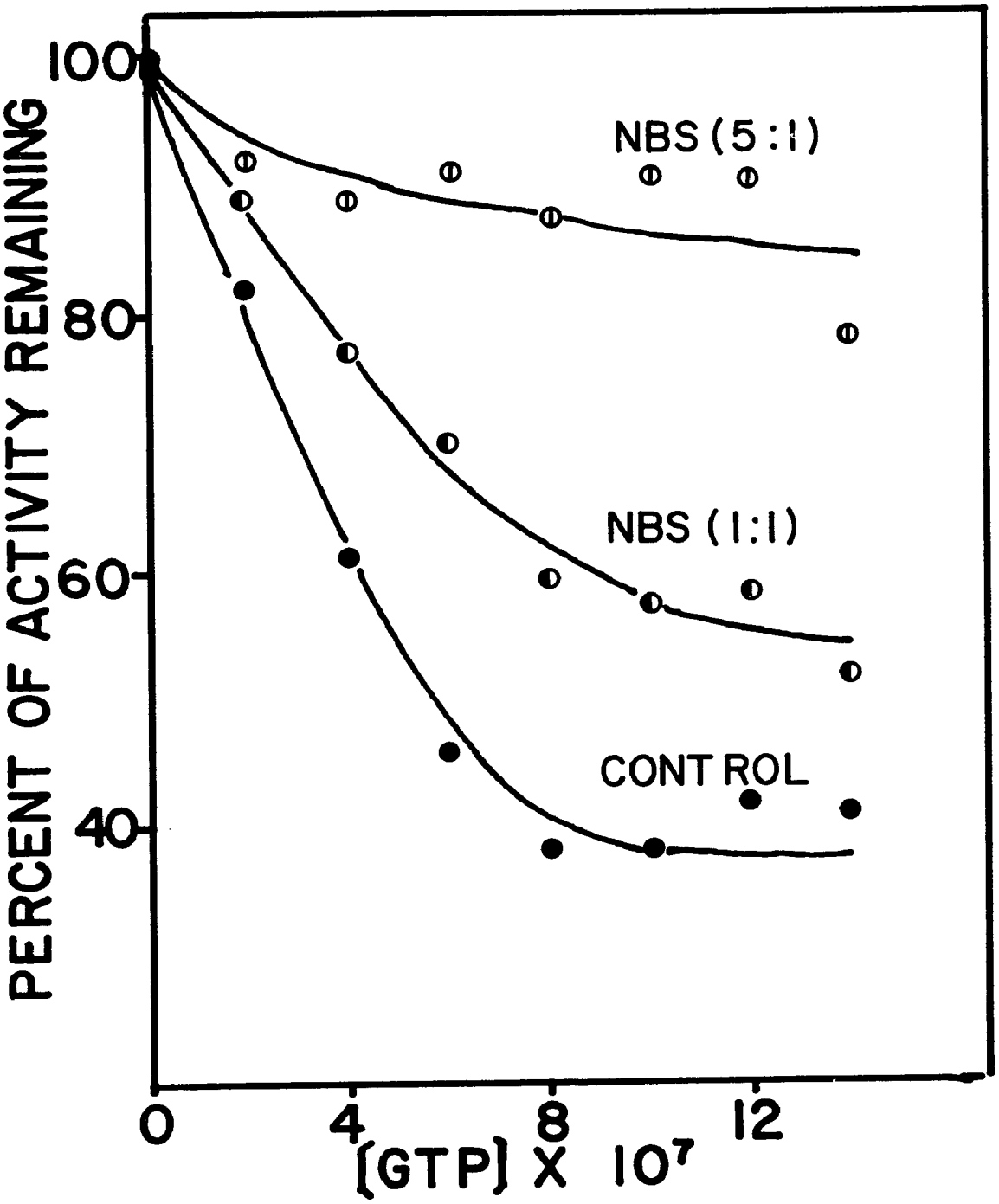

Δ OD / MIN AT 340mμ
0.3
0.2
0.1
0
6
12
18
24
30
[ADP] X 10⁵

FIGURE 20

Effect of Guanosine Triphosphate on the Catalytic Activity of Glutamate Dehydrogenase Modified with N-Bromosuccinimide. The date are expressed as the % of activity remaining as a function of GTP concentration. NBS-modified GDH was prepared as before at a reagent/enzyme molar ratio of 1:1 and 5:1 and aliquots of 2.5×10^{-5} M GTP solution in phosphate buffer (pH 7.5) were added to the assay system to attain the desired concentration. Symbols: (● - ●) = Control GDH; (◐ - ◐) = NBS modified GDH at 1:1 ratio; (◑ - ◑) = NBS modified GDH at 5:1 ratio. Enzyme concentration = 2 μg/ml.


100
80
60
40
PERCENT OF ACTIVITY REMAINING
NBS (5:1)
NBS (1:1)
CONTROL
0
4
8
12
[GTP] X 10⁷

the reactive group which regulates the catalytic activity, the sensitivity towards some of the allosteric regulators, and the physical properties of GDH; although there has been some discrepancy in interpretation regarding the relationship between the degree of TNBS modification and its inhibitory effect on catalytic activity (12). It is an interesting fact that the TNBS modified enzyme (at the mole ratio 1:1) has reduced sensitivity to the allosteric modifier GTP but not to ADP (12). In contrast, as has been shown before, CH_3HgI is an active modifier regulating the sensitivity to both ADP and GTP, and also the catalytic activity. The question arises whether there is any relationship between the mechanisms of which TNBS and of CH_3HgI modify the enzyme or whether TNBS and CH_3HgI react independently on GDH.

Therefore, GDH was first modified with TNBS at the mole ratio of 1:1 by the methods of Clark (12) and Goldbarb (32) with slight modification, and the effect of the mercurial on the activity of the TNBS-modified enzyme examined. Secondly, GDH was doubly modified with $^*CH_3HgI$ and with TNBS (each at a mole ratio of 1:1 to protein) and its sensitivity to the allosteric reagents tested.

Figure 21 shows the effect of CH_3HgI on the catalytic activity of the TNBS-modified GDH at a mole ratio of 1:1. No significant change in response of the TNBS modified enzyme to the mercurial was observed in the range of the mercurial concentrations tested. This suggests that the TNBS treated GDH at a mole ratio of 1:1 does not interfere with the function of the

mercurial on the GDH molecule. Moreover, the inhibition of the catalytic activity reported previously (12), with TNBS modification at a mole ratio of 1:1, was overcome by the mercurial (data not shown). This result was extended by comparing the sedimentation coefficients of TNBS treated enzyme and of the $*CH_3HgI$-TNBS treated enzyme (Figure 22). The TNBS treated GDH (lower picture) shows only one slow moving peak consistent with the report that the modified enzyme fails to aggregate (12). The doubly modified GDH associated partially, however, and forms one additional faster moving peak of higher molecular weight, showing that the mercurial can still cause aggregation of the enzyme after TNBS modification in much the same way as it affects the untreated enzyme.

Figure 23 shows the response of the control, the TNBS-modified, and the $*CH_3HgI$-TNBS modified enzymes to various concentrations of ADP. The catalytic activities of the control and the TNBS treated GDH were stimulated in the same manner by the addition of ADP; while the doubly treated GDH had completely lost its sensitivity to ADP. Apparently the mercurial affects the TNBS-modified GDH just like the native GDH as far as the response to ADP is concerned (See Figure 4). Therefore, it is reasonable to conclude that the modification of GDH with TNBS is independent of the mercurial modification.

As already reported (12,24) the modification with TNBS makes the enzyme less sensitive to GTP compared to the control enzyme. Therefore, the effects of double modification of the

FIGURE 21

Effect of Methylmercuric Iodide on the Catalytic Activity of Glutamate Dehydrogenase Modified with Trinitrobenzene sulfonate. GDH (10 mg/ml, in glycerol) was diluted 1:1 with 0.1 M phosphate buffer (pH 7.5). One ml of the GDH solution and 1 ml of 2×10^{-4} M TNBS solution in phosphate buffer were mixed at room temperature. The degree of modification was monitored at 340 mμ where the TNBS-GDH complex absorbs. When the absorbance at 340 mμ reached the calculated value corresponding to the modification of one amino acid residue per enzyme chain, the excess reagent was removed by passing the mixture through a Sephadex G-25 (Coarse, 15 x 16 mm) column, which was pre-equilibrated with buffer. The enzyme eluted from the column was detected with an ISCO spectrophotometer equipped with a flowthrough cell. The extent of the modification was re-examined spectrophotometrically at 340 mμ by using a molar extinction coefficient of 1.15×10^4 for the TNBS-amino acid complex at neutral pH. The amount of enzyme protein was determined by the Lowry method. Emzyme concentrations: 2.0 μg/ml of the control (●–●), and 3.1 μg/ml of the TNBS modified enzyme (Φ–Φ).

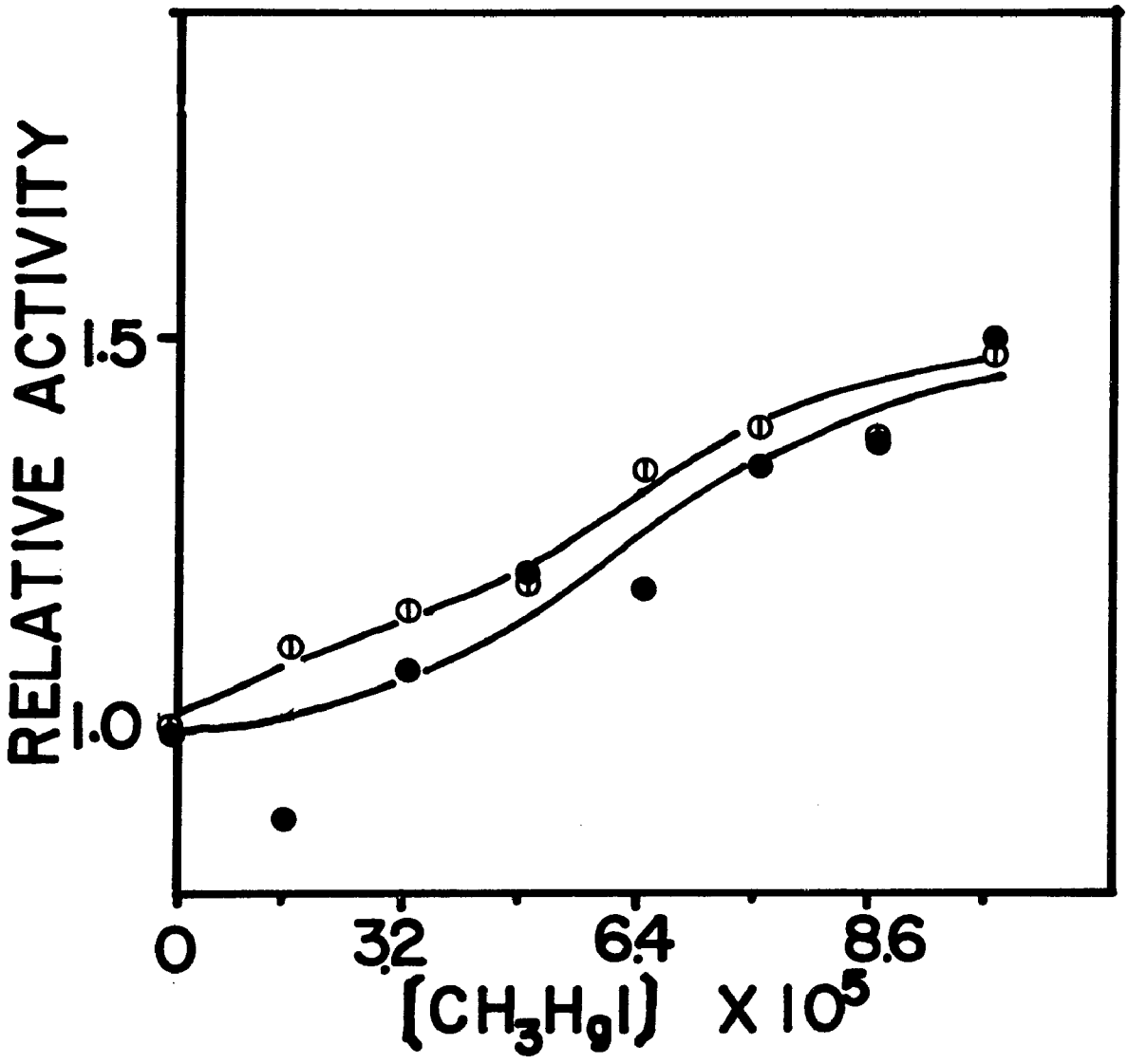
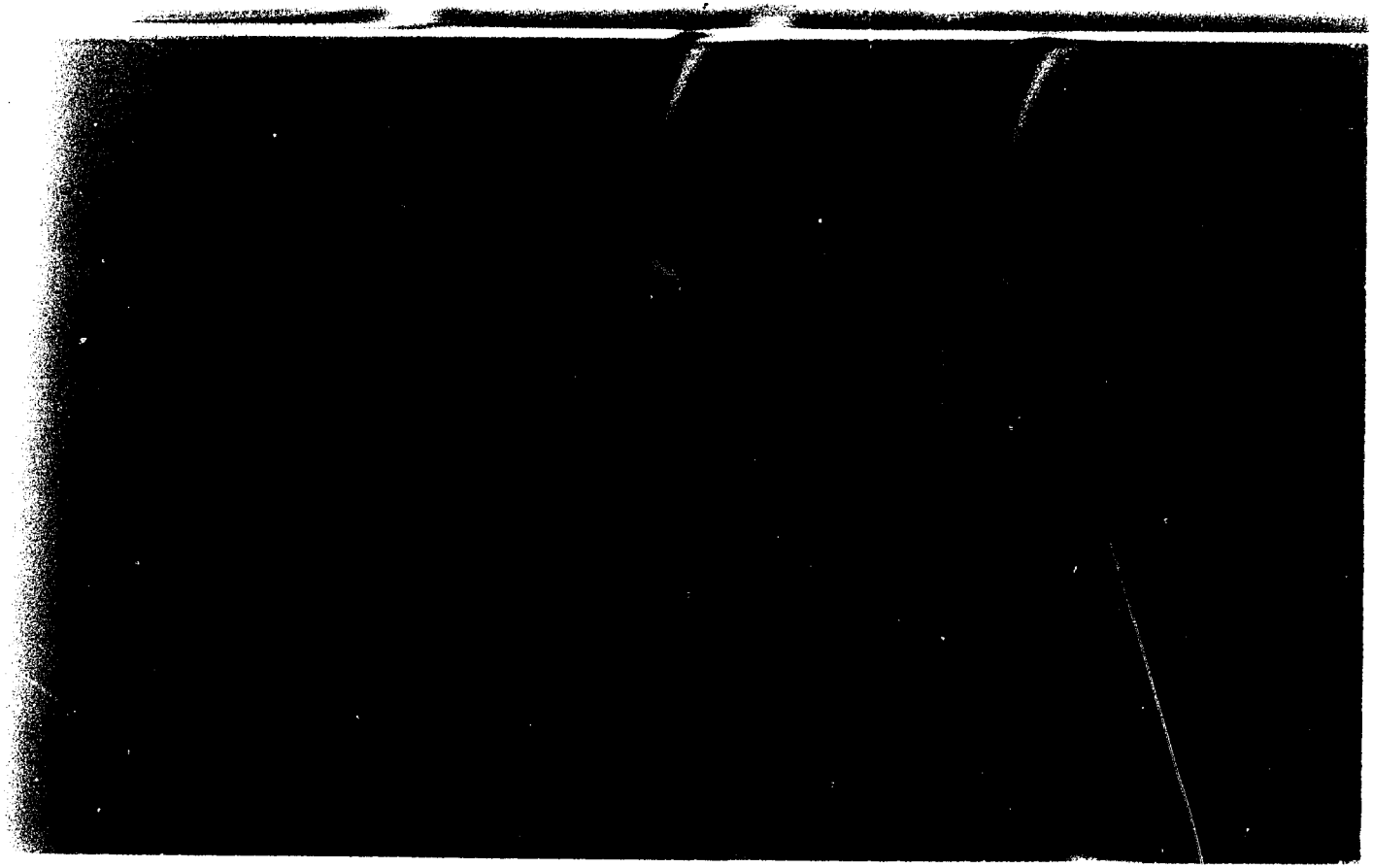

RELATIVE ACTIVITY
1.5
1.0
0
3.2
6.4
8.6
[CH3Hg1] X 10^5

FIGURE 22

Effect of the Modification with Trinitrobenzenesulfonic Acid, and with Trinitrobenzenesulfonic Acid plus Methylmercuric Iodide on the Sedimentation Velocity of Glutamate Dehydrogenase. A sample containing 1.45 mg/ml of the enzyme modified with $*CH_3HgI$ and TNBS, each at a mole ratio to protein of 1:1, (upper curve); and 1.55 mg/ml of enzyme modified with TNBS alone at a molar ratio of 1:1 were sedimented in 0.1 M phosphate buffer (pH 7.5). Sedimentation was from left to right in a Beckman Model E Analytical centrifuge with a rotor type AN-D at a speed of 59,780 at 20^{o} for 25 minutes.

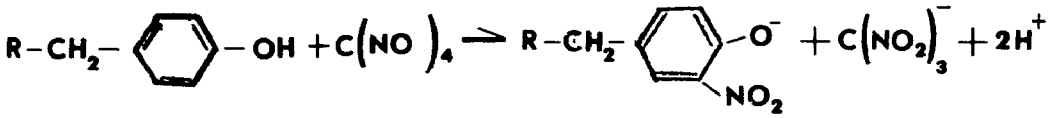


enzyme with mercurial and with TNBS on the GTP response were determined. Figure 24 shows the results. The TNBS modified enzyme was less sensitive to GTP than the control, and the *CH_3HgI-TNBS modified enzyme responded to GTP the least. This lack of response of the doubly modified GDH to GTP is probably due to the additive effect of both reagents. The present work suggests that although the binding sites for TNBS and *CH_3HgI and their effects on ADP sensitivity are different, they share a common effect on GTP sensitivity of the enzyme.

The Effects of Modifications of GDH with Tetranitromethane alone and with Tetranitromethane plus Methylmercuric Iodide

In GDH a tyrosyl residue has been proposed as the amino acid residue which binds to the aromatic moiety of GTP. When this specific tyrosyl residue is modified by various chemicals, the sensitivity of the GDH to GTP is reduced (49).

Sokolonsky et al (53,62) have proposed that tetranitromethane (TNM) is a favorable reagent for the modification of tyrosyl groups in various proteins because it reacts under mild conditions where the denaturation of proteins is minimized. The following reaction mechanism of TNM with the tyrosyl group has been proposed, and the specificity of the reaction has also been studied (62).



Price and Radda have reported (49) that when GDH is modified with TNM under controlled conditions, one tyrosyl group

FIGURE 23

Effect of Adenosine Diphosphate on the Catalytic Activities of Glutamate Dehydrogenase Modified with Trinitrogenzenesulfonate alone and in combination with Methylmercuric Iodide. The doubly modified enzyme was prepared by incubation of GDH with $^{*}CH_3HgI$ (1:1) and dialyzed. The mercurial modified GDH and the native enzyme were treated with TNBS in the same way as described in Figure 21. Reaction rates are given as (ΔOD/min. 340 mμ) as a function of ADP concentration, using enzymes concentration of 2.9 μg/ml for the control (●-●), 3.1 μg/ml for the TNBS modified (◐-◐), and 2.9 μg/ml for the doubly modified GDH (◑-◑). Substrate concentrations are given in the text.

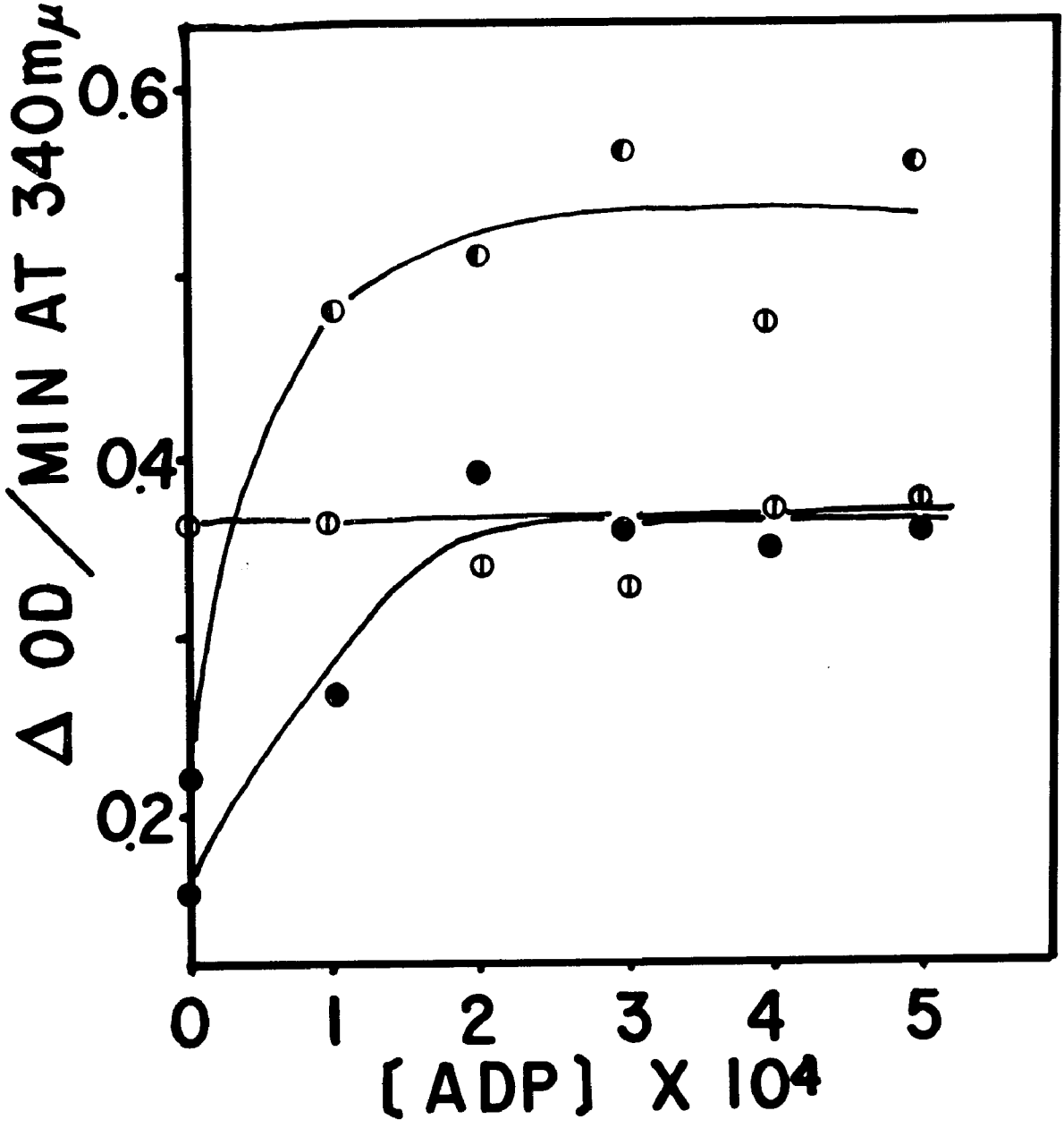
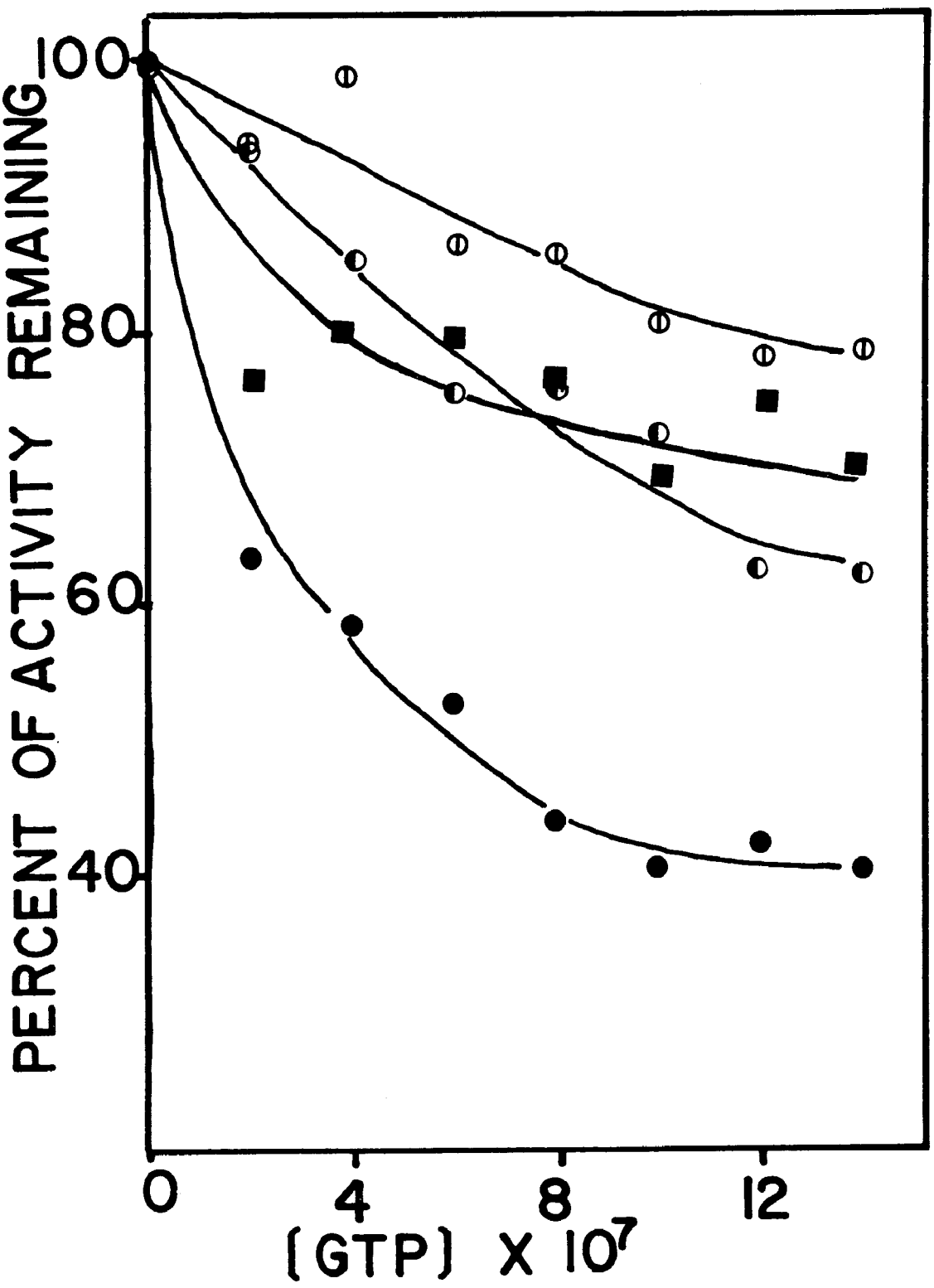

Δ OD / MIN AT 340mμ
0.6
0.4
0.2
0
1
2
3
4
5
[ADP] X 10⁴

FIGURE 24

Effect of Guanosine Triphosphate on the Catalytic Activity of Glutamate Dehydrogenase Modified with Trinitrobenzenesulfonate alone and in Combination with Methylmercuric Iodide. Data are expressed as % of activity remaining as a function of GTP concentration. The preparatory methods of the modification of GDH were the same as described previously. Enzyme concentrations: 2.9 μg/ml of the control (●-●); 3.1 μg/ml of the TNBS modified (◐-◐); 2.9 μg/ml of the doubly modified GDH (◑-◑) and 4.0 μg/ml of the *CH_3HgI modified GDH (■-■).


PERCENT OF ACTIVITY REMAINING
100
80
60
40
0
4
8
12
[GTP] X 10⁷

per subunit can be nitrated to form 3-nitrotyrosine, and the modified GDH exhibits the reduced sensitivity to GTP without any significant changes in catalytic activity, and sedimentation coefficient. This desensitization effect on GDH by TNM has been confirmed by Smith et al (61) who also pinpointed the location of the tryosyl residue modified by TNM in the tentative amino acid sequence of the GDH chain. The sensitivity of the TNM modified GDH to ADP, however has not been reported. CH_3HgI also reduces the sensitivites of GDH to both GTP and ADP effects, as shown in the first part of this dissertation. Therefore it should be interesting to determine if there is any interaction on the GDH molecule caused by the modification with mercurial and TNM, and whether it is possible to differentiate between the desensitizing effects of the mercurial and of TNM to GTP. The following experiments have been designed to answer those questions. The modification of GDH by TNM generally follows the method described by Price and Radda (49).

The precise determination of the extent of nitration, however, is difficult due to the rather small extinction coefficient of 3-nitrotyrosine ($\varepsilon 380 = 2,200$), and due to the fact that amino acid analysis is not applicable to measure the extent of the modification. The extent of modification achieved in the present work varied between 0.67 to 1.22 of 3-nitrotyrosyl group per GDH subunit. The amount of protein was re-examined by the Lowry method.

To prepare the $*CH_3HgI$-TNM doubly modified enzyme, the

*CH_3HgI bound enzyme at a mole ratio of 1:1 was made by the method described before, and then modified with TNM in the same way as the control enzyme.

First the catalytic activities of the TNM-modified enzyme, and the native enzyme were examined in the presence of CH_3HgI in the assay system, in order to see if the modification of GDH with TNM still allows the enzyme to respond to the mercurial effect. The result in Figure 25 shows that the response of the TNM-pretreated enzyme to the mercurial was decreased in the concentration range tested. The curves of the control enzyme and of the modified enzyme are plotted as average values of three independent experiments up to a concentration of 1.6×10^4 M mercurial. The TNM modified enzyme in the present work always resulted in a lower catalytic activity (about 30% less), compared to that of the native enzyme, in contrast to the report by Price and Radda (49).

Although the effect of ADP on the TNM-modified GDH had not been reported yet, it still provided an important point to be considered in studying the regulatory properties of GDH modified with both *CH_3HgI and TNM.

In Figure 26, it is shown that ADP stimulated the catalytic activities of the TNM modified and of the native enzymes in the same fashion.

The extent of the stimulatory effect of ADP on these enzyme activities however, varied from one enzyme preparation to another. Therefore, each spot in the native and the TNM-treated

FIGURE 25

Effect of Methylmercuric Iodide on the Catalytic Activity of Glutamate Dehydrogenase Modified with Trinitromethane. The native GDH was diluted to 5 mg/ml with 0.1 phosphate buffer (pH 7.6) and dialyzed against 200 ml of buffer overnight at 5^{o}. After dialysis, the enzyme was adjusted to 1.0 mg/ml. TNM was diluted 100 times with 95% ethyl alcohol, and added to the enzyme at a 4x molar excess. This small volume of alcohol did not affect the enzyme. The extent of nitration was monitored by the absorbance changes at 350 mμ, and at 380 mμ due to the formation of nitroform ion, and a 3-nitrotyrosine residue, respectively, on a DB-G spectrophotmeter. When the changes in absorbance corresponded to the calculated values for a molar ratio of 1:1, the reaction was stopped by gel filtration on Sephadex G-25 column (coarse 1.5 x 15 cm) equilibrated previously with phosphate buffer. The reagent alone did not show any significant changes in absorption during the incubation period. The purified enzyme was re-examined at 380 mμ to determine the extent of nitration in GDH. To prepare the $*CH_3HgI$-TNM doubly modified enzyme, the $*CH_3HgI$ bound enzyme (1:1) was made first and then modified with TNM in the same way as the control enzyme. (Continued on page 87)

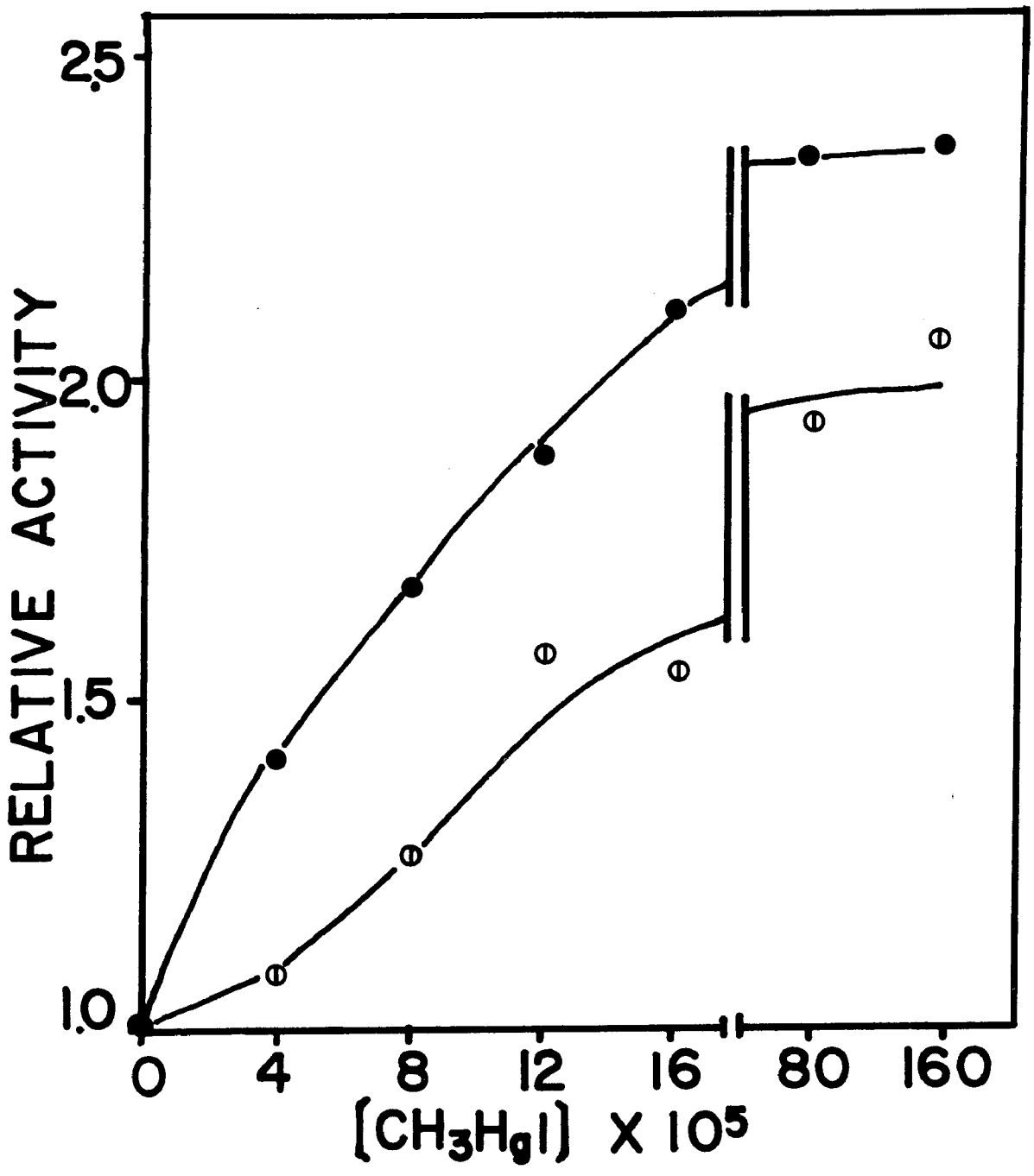

RELATIVE ACTIVITY
2.5
2.0
1.5
1.0
0
4
8
12
16
80
160
[CH3Hg1] X 10^5

FIGURE 25 (continued)

Each spot of the TNM-modified and the control curves up to the mercurial concentration of 16 x 10^{-5} M, is an average value of three individual experiments. The extent of the TNM-modification is varied from 0.94 to 1.22 tyrosyl/chain. The control enzyme (●-●) is 1.5 to 2.4 μg/ml, and the TNM modified enzyme (◐-◐) is 1.2 to 2.4 μg/ml.

enzyme curves is an average value of three independent experiments. The TNM modification did not prevent mercurial modification because the mercurial at a mole ratio of 1:1 was still able to antagonize the effect of ADP in the *CH_3HgI-TNM treated enzyme as well as that in the native enzyme. The desensitization of GDH to the GTP effect is therefore the major effect caused by TNM on the properties of GDH. The question is raised now whether the mercurial and the TNM work together or independently to antagonize the GTP effect. The *CH_3HgI-TNM modified GDH, the TNM modified GDH and the native GDH were studied for inhibition of catalytic activity by GTP.

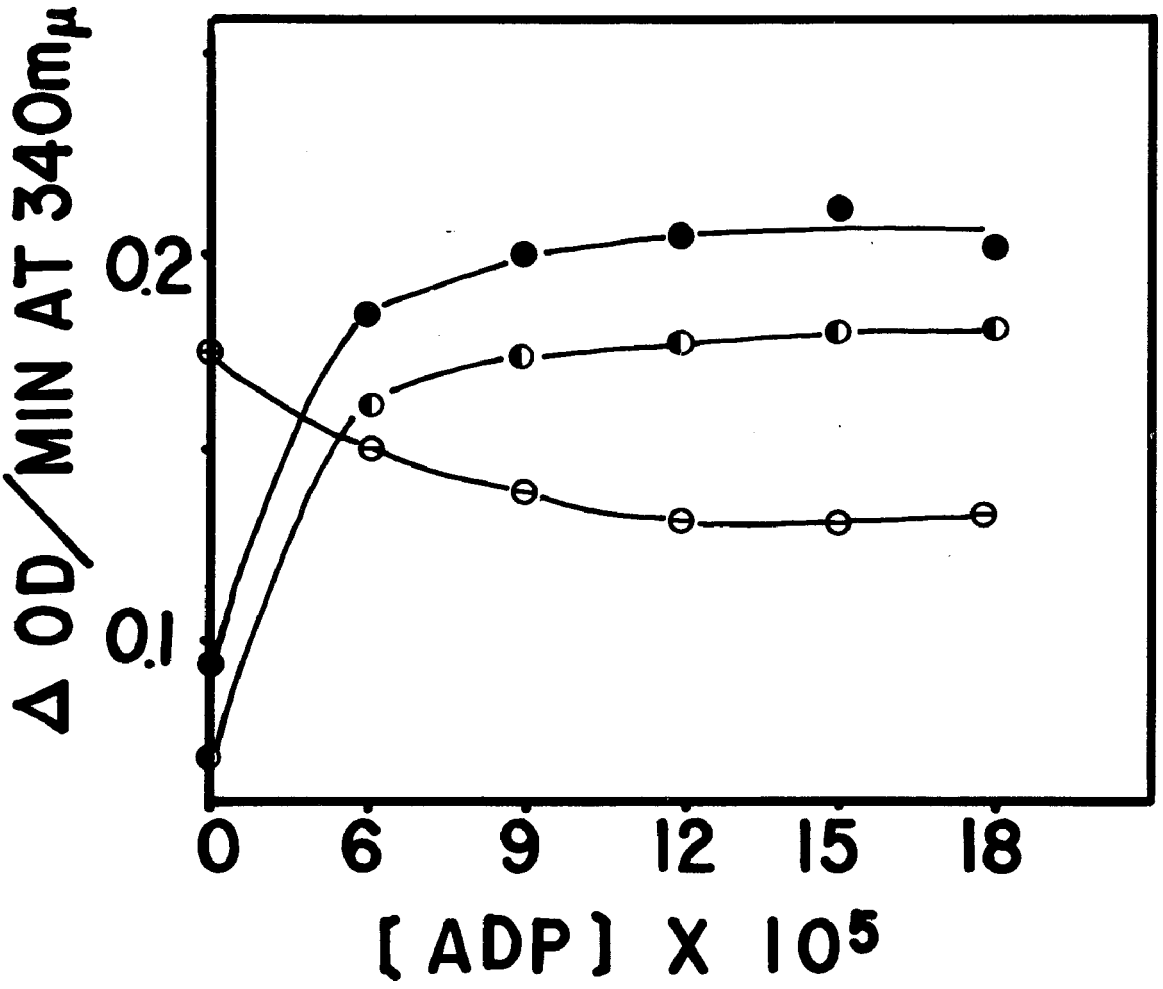
The results in Figure 27 show that the GTP sensitivity of the TNM modified GDH was less than of the native enzyme as expected, and the *CH_3HgI-TNM modified GDH was least sensitive of the three. From these results *CH_3HgI and TNM seem to work independently, and to have an additive effect against GTP. Unfortunately, in the present work the quantitative evaluation of the effect of both modifiers to GTP is not precise due to the uncertainty in the extent of modification with TNM in individual preparations.

One difference between the results by Price and Radda (49) and those in the present work can be seen; namely, the catalytic activity of the TNM-modified GDH was found to be reduced in the present work. This observation will be discussed later in the discussion.

It is likely that TNM and the mercurial may function

FIGURE 26

Effect of the Modification of Glutamate Dehydrogenase with Methylmercuric Iodide and Tetranitromethane on its Sensitivity to Adenosine Diphosphate. Each point for the TNM-modified, and the control curves is an average value of three identical experiments. The extent of the TNM-modification for the enzymes used varied from 0.94 to 1.22 tyrosyl per chain. Symbols: *CH_3HgI-TNM/GDH = (◑-◑); TNM-GDH = (◐-◐); Control = (●-●).


Δ OD/MIN AT 340mμ
0.2
0.1
0
6
9
12
15
18
[ADP] X 10⁵

independently against the GTP effect in the regulation of GDH, since CH_3HgI is able to regulate the sensitivity of the GDH to the ADP effect regardless of the presence of TNM, and the response of the enzyme to the effect of CH_3HgI is maintained after the enzyme is modified with TNM.

Summary: (Part II)

Two alternative chemicals expected to react with -SH groups were tested for their effects on the properties of GDH in connection with the effects by the organic mercurial.

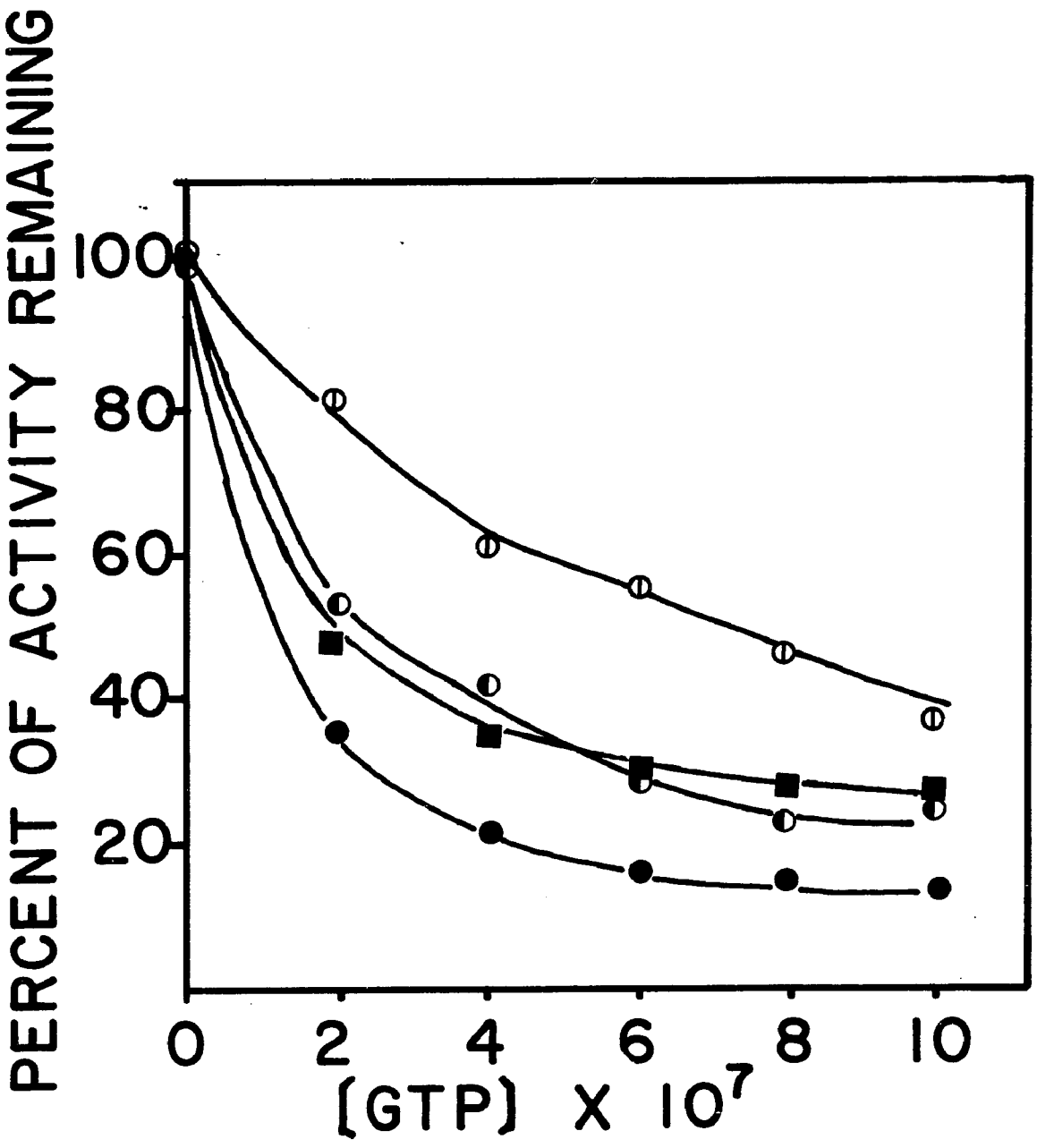
The effect of Ag^+, which is a potent inhibitor of GDH, when added to the enzyme stoichiometrically was overcome by the addition of $*CH_3HgI$.

N-bromosuccinimide, known as a modifier of tryptophan and possibly of -SH groups or tyrosine, was tested for its effect on the catalytic activity and the regulatory character of GDH. The addition of N-bromosuccinimide at molar ratios of NBS/GDH of 5:1 stimulated maximally the catalytic activity of the native enzyme, but GDH treated with mercurial at a molar ratio of 1:1 did not show stimulation of the catalytic activity on addition of N-bromosuccinimide.

A mole ratio of NBS/GDH of 5:1 could completely block the ADP effect on GDH, but a NBS ratio of 1:1 was not high enough to prevent inhibition of the ADP effect. With regard to the GTP effect, the enzyme modified with NBS at a ratio of 1:1 showed less sensitivity than the control, and the enzyme treated at a ratio of

FIGURE 27

Effect of the Modification of Glutamate Dehydrogenase with Methylmercuric Iodide and Tetranitromethane on its Sensitivity to Guanosine Triphosphate. The preparation of the enzymes was described in the legend of Figure 25. (*CH_3HgI-TNM modified GDH (1.18 tyrosyl/chain) and 1.5 µg/ml = (◐-◐), *CH_3HgI modified GDH 1.5 µg/ml = (■-■); TNM modified GDH (1.22 tyrosyl/chain) and 1.2 µg/ml = (◐-◐); and control GDH 1.5 µg/ml = (●-●)).


PERCENT OF ACTIVITY REMAINING
100
80
60
40
20
0
2
4
6
8
10
[GTP] X 10⁷

5:1 showed the least sensitivity.

It was concluded, therefore, that Ag^{+} and N-Bromo-succinimide display a different specificity from that for the organic mercurial.

The doubly modified GDH with either trinitrobenzene-sulfonate or tetranitromethane in connection with $*CH_3HgI$, showed complete loss of sensitivity to ADP, and the least sensitivity to GTP interaction. Whereas the enzyme modified singly with either the TNBS or TNM was fully sensitive to ADP, but less sensitive to GTP interaction. The mercurial and the other amino acid modifiers, therefore may react independently on the GDH molecule, yet work in an additive fastion in modifying its sensitivity to GTP.

DISCUSSION

The effects of CH_3HgI in stimulating the catalytic action and changing the regulatory responses of GDH to allosteric reagents have been interpreted in terms of enzyme conformational changes (8,9,72,82); but the details of such a mechanism have not been clarified. Further details of the effects of mercurial alone and in relation to other groups of specific reagents should, therefore, be of value.

The results obtained in the present work with C^{14} labeled organic mercurial have established that modification of a single -SH group per enzyme chain, results in maximum stimulation of the catalytic activity, and antagonizes the allosteric effects of various modifiers such as ADP and GTP. Consequently, one -SH group among the six in GDH plays a critical role in the regulatory process. It cannot be concluded whether this -SH group simply lies in a region of the protein which is important in conformational flexibility, or whether it comprises a part of the binding region for the various allosteric regulators. The facts that regulation is simply decreased rather than completely abolished and that the regulators are so diverse chemically makes a common binding region seem unlikely and favor a role in conformation rather than a direct role in binding. Other

types of -SH reagents have also been used to modify the enzyme and it has been reported that those -SH modifiers also show change in the catalytic activity and/or the sensitivity to the allosteric reagents (4,8,36,41,45,54-57). To find out whether the specific reactivity of the single -SH group is only directed to the mercurials of type R-Hg-X, experiments were conducted to differentiate between the effects caused by modifying this specific -SH group with the mercurial, and the effect caused by other modifiers namely Ag^+ and NBS.

The results have shown that the aliphatic mercurials are specific for one -SH group, in contrast to some of the more general -SH reagents tested.

As reported previously, several other amino acid residues in addition to -SH groups have been identified in the enzyme as being important in the conformational transitions which accompany its regulation. Although de Prisco (19) has recently reported an interaction between tyrosyl and lysyl residues related to regulation of GDH, correlations between the roles of these different amino acids in the protein have never been made before. Therefore, GDH was sequentially modified with two different reagents, each specific for one amino acid residue, and subjected to tests of its properties. The effect of CH_3HgI was also examined on GDH modified either with TNBS (for lysine) or with TNM (for tyrosine). Using the "doubly modified" enzyme, the mercurial was just as effective as it was in the native enzyme.

Although it is recognized that the reaction of one specific -SH group with CH_3HgI is a significant process in the regulation of GDH function, the precise action of the mercurial bound to GDH at a mole ratio of 1:1 has not been determined. Therefore, a hypothetical explanation will be attempted, which also emphasizes the importance of hydrophobic regions for enzyme properties.

Since cysteinyl, methionyl and alanyl groups are considered to be hydrophobic (43,69), it may be reasonable that the -S-Hg-CH_3 group, an expected reaction product between the -SH group and the mercurial, may create more of a hydrophobic site in GDH than a simple -SH group. Thus, the modification of the specific -SH group with the mercurial will give a more regional hydrophobic character to the enzyme and this will cause some conformational changes favorable to the catalytic action and less susceptible to regulation by allosteric modifiers (8,9,82). Heitmann has also demonstrated (35) that an increment of one methylene group in a fatty acid chain increases the stability of its micelle structure which is due to the hydrophobic character of the additional carbon moiety, and that one cysteinyl residue has the same stabilizing effect on the micelle structure as one methylene moiety does. This supports the idea that an -SH group modified with the mercurial would increase the hydrophobicity of the protein structure. In fact, CH_3CH_2HgI, a compound having a longer aliphatic chain, is likely to bind the same site as the CH_3HgI (Figures 12 and 13), and was able to stimulate

the catalytic activity, although the maximum stimulation by the longer chain compound could not be obtained in this study due to its poorer solubility. Charman et al (10) have studied the exchange reaction of the aliphatic moiety of organic mercurial compounds with one another from the viewpoint of kinetics. It is not known whether the methyl group of CH_3HgI is subjected to exchange with the ethyl group of CH_3CH_2HgI in the enzyme system. This point needs to be investigated at a later date.

The stimulatory effects of the aromatic mercurials phenylmercuric acetate (36,41,52) and parahydroxymercuribenzene sulfonate (26,71) on GDH have also been reported. The former compound unlike CH_3HgI, produces a decrease of about 25% in the sedimentation coefficient of GDH (41,57), even at concentrations when the aromatic mercurial increases the catalytic activity of the enzyme. This evidence suggests a different effect on structure for the aromatic than that for the aliphatic mercurial compound (57). Thus, the aromatic mercurial seems to exert a somewhat different effect on protein structure than the aliphatic compound.

It is important to examine whether CH_3HgI is really specific for the -SH group. As has been shown in Table 3, the specificity of $*CH_3HgI$ towards a -SH group containing compound is definitely high. Other amino acids tested with $*CH_3HgI$ in this work are commonly found as active amino acid residues in various enzymes (30,59), but do not show any binding affinity for $*CH_3HgI$ under the condition tested.

In a great number of other enzymes only a relatively small number of amino acid residues have been recognized as contributors of important functional groups (30). This fact must also be true in GDH. Hellerman et al showed (36) that two equivalents of essential -SH's were present in 25,000 g of GDH (or 4 equivalents per chain of 53,500). Bitensky et al speculated (8) that one out of eight -SH groups in GDH must be substituted to produce the organic mercurial effects described.

Pfleiderer et al reported (47) that 2.23 mole residues of bound N-acetyl-4-sulphamoylphenyl maleimide (ASPM) per 1000,000 equivalent of GDH (or 1 per chain) resulted in complete inhibition, although they have reported in their subsequent paper (37) that ASPM reacts more specifically with lysyl groups, at pH 7.3 than with -SH groups, whereas the latter groups react with ASPM at pH 6.9. The results of the present research have shown by direct labeling that one -SH residue per chain of GDH has a high specificity for the mercurial.

The present studies have also shown that Ag^+ is non-specific and NBS must be less specific toward the one -SH group than the mercurial. Silver ion acts as a strong inhibitor for the enzyme with complete suppression of the catalytic activity of native GDH at a mole ratio (Ag^+/GDH) of 4:1, in agreement with the reports by Hellerman et al (36), and by Rogers (54).

It may be, however, that the 4 equivalents of Ag^+/GDH subunit which are required to inhibit the catalytic activity, as Hellerman et al stated, may involve a substantial proportion

of all -SH groups of the enzyme, and the Ag^+ ion is non-specific in its action. It would appear that the progressive effects on catalytic activity resulting from this non-specific binding of Ag^+ also correlates with the finding of Rogers et al that Ag^+ results in progressive dissociation. (57).

The total number of -SH groups reported per unit chain varies with the researchers; 6 -SH groups per chain were reported by Smith et al in their magnificent work on the amino acid sequence of one GDH subunit chain (61). Other reported numbers are 8 by Appella et al (3), and 5 (8.95 per 10^5 g protein) by Sund et al (67).

NBS used in proteins as a chemical modifier of tryptophan may possibly react to oxidize -SH groups (64). The modification with NBS results in striking inhibition of the catalytic activities of various enzymes (13,30,59,63,64). Dihydrofolate reductase, however at low NBS/enzyme ratios shows an increase in catalytic activity (25), and Summers has found (66) that NBS stimulates the catalytic activity of GDH at low reagent ratios and inhibits only at higher levels. He speculated that the stimulatory effect by NBS on the GDH activity might be due to the modification of -SH groups up to the mole ratios NBS/GDH of 5 to 7:1, followed by the modification of tryptophanyl residues with loss of the catalytic activity at mole ratios higher than 7:1. The present studies have confirmed and extended the effect of NBS on GDH.

The enzyme which had been treated with NBS at mole

ratios of 1:1 and 5:1 respectively, was subjected to additional treatment with CH_3HgI. The enzyme modified with NBS at a 1:1 ratio was less sensitive to mercurial by about 50%, while NBS treatment at a 5:1 ratio almost completely abolished the mercurial response. NBS modification had similar effects on the effectiveness of the allosteric reagents ADP and GTP; namely enzyme treated with NBS at a 1:1 ratio responded to ADP while that treated at a 5:1 ratio was unresponsive to either ADP or GTP. The results indicate that NBS and the mercurial are producing the same effect on the enzyme, but that NBS is less specific. Although NBS is currently considered to oxidize -SH groups and/or tryptophanyl residues the real mechanism of its action on GDH is not clear. Most recently it was observed by Yielding (78) that bromine water could stimulate the catalytic activity of L-glutamate activity of GDH in the same manner and the possibility of tyrosine bromination must be considered. This question remains to be clarified.

In the preparation of "doubly modified" enzymes, it is important to consider whether the modification of the protein with the first reagent effects the reactivity with the second reagent. There are two ways to examine this point: 1) a determination of the effects of modification on binding of the second reagent; and a test of the reversal of the order of addition of the two reagents; 2) a determination of the sensitivity of the catalytic activity of the modified enzyme to the effects of the second compound, and a test of the reverse order. It

was confirmed that the enzyme was still responsive to the second reagent. The properties of the TNBS modified GDH have been studied extensively by Freedman et al (24) and Clark (12). They have established that the TNBS modified enzyme is desensitized against the GTP, but not the ADP effect. Similar results were obtained in the present studies. When the $*CH_3HgI$-TNBS modified GDH and the TNBS modified GDH were studied with respect to the effects of allosteric modifiers, the effects of $*CH_3HgI$ and TNBS on GDH appeared to be independent, and this suggests a critical role for lysyl and -SH groups in the regulation of GDH.

The modification of tyrosyl residues in proteins with TNM has been established by Sokolovsky et al (62). Price and Radda (49) have studied the effects of modifying tyrosyl residues with N-acetyl imidazole (NAIm) and with TNM on the properties of GDH. However, as described before, the control of the extent of modification of GDH with TNM meets considerable difficulty, due to the rather small extinction coefficient of the reaction product (3-nitrotyrosine E = 2,200 at 380 mμ). Although the nitroform ion ($C(NO_2)_3^-$) has a large extinction coefficient of 14,400 at 350 mμ, the extent of changes at 350 mμ and at 380 mμ do not always correspond in the results obtained in the present study.

Therefore, when amino acid analysis is not used for determining the extent of modification, absorption at 380 mμ, due to the formation of 3-nitrotyrosine may be the best indicator.

In the present work it was observed that the TNM

modification resulted in partial loss of catalytic activity (about 30% less), which was not observed by Price and Radda. Several explanations can be offered for this phenomenon: (1) Alterration of the enzyme protein by treatment with TNM may make GDH unstable; (2) The enzyme is less stable at the higher pH of the assay (8.6 in this work; 7.6 in Price and Radda's); and, (3) A non-specific inactivation accompanying the modifying procedures may occur.

According to Price and Radda the modified enzyme is not unstable so that reasons (2) and/or (3) seem most likely.

The effects of the various allosteric reagents on the modified enzymes, are summarized in Table V.

Based on this information, an attempt has been made to build a hypothetical scheme for the regulation of GDH.

First of all, the data obtained here are related to the well known scheme of the concentration dependent polymer-monomer equilibrium of GDH (page 3) and the possible relation between the active -SH group and the tyrosyl or the lysyl group are speculated.

Here there are two different kinds of regulatory mechanisms to consider. First, a number of allosteric modifiers affect reversibly the catalytic activity and conformation of native GDH. The native enzyme is sensitive to L-leucine and ADP as stimulatory allosteric effectors toward L-glutamate activity,

TABLE V

Relationship between the Specificity of Chemical Modification of Amino Acids and the Resulting Change in the Sensitivity of Glutamate Dehydrogenase to Allosteric Reagents. The chemicals used for the modification are shown in the left column and the corresponding amino acid residues modified shown to the right. A single arrow (↓) stands for reduction of sensitivity to the corresponding allosteric reagent, whereas double arrows (↓↓) mean additive effect to GTP action.

TABLE V

Modification	Amino Acid Group Modified	Response to modifier ADP	GTP
*CH_3HgI	Cysteinyl	↓	↓
NBS (1:1)	Cysteinyl (tyrosyl?)	↓	↓
NBS (5:1)	Cysteinyl (tyrosyl?)	↓	
TNBS	Lysyl	unchanged	↓
TNM	tyrosyl	unchanged	↓
*CH_3HgI-TNBS	cysteinyl, lysyl	↓	↓↓
*CH_3HgI-TNM	cysteinyl, tyrosyl	↓	↓↓

and to GTP, Zn^{++}, and steroids as inhibitory allosteric effectors, because of the shifts in conformation accompanying binding of these reagents. These changes are also reflected in the polymer-monomer equilibrium. In the second mechanism, there is regulation through specific (probably covalent) modification of functional amino acid side chains with resulting conformational changes. For example, when the specific -SH group is modified with the mercurial, the enzyme is stabilized in a conformation with increased catalytic activity and a concomitant resistance to allosteric reagents evoked changes in conformation. Thus ADP is no longer effective and the effectiveness of GTP is partially reduced. This remaining sensitivity to GTP can be reduced further by the covalent-type modification of the lysyl residue and the tyrosyl residue, which seems to result in additional conformational changes. Thus it is most likely that the specific -SH group to which the mercurial binds plays a critical role in the regulation of the enzyme. Moreover, the lysyl and tyrosyl residues identified by additional covalent modification also play a similar important regulatory role. This illustrates that such covalent changes serve an important controlling function in allosteric changes. Such interdependence of covalent and allosteric control may also be seen in biological systems. For example, with glutamine synthetase the gamma-glutamyltransfer activity is modulated by adenylation of a tyrosyl residue, and after such modification the enzyme becomes sensitive to such allosteric modifiers as histidine, tryptophan, AMP or CTP (65). An additional example is phosphorylase which in the

"b" form (inactive) can be stimulated by the allosteric modifier AMP, but when phosphorylated by ATP to the "a" form no longer shows sensitivity to AMP (33,34,40). In both cases, the covalent binding of the ligand alters both the nature of the enzymes and their response to the regulatory reagents.

It seems, therefore, that this "multi type" regulatory mechanism is of general importance.

GDH has been studied by a number of workers as an in vitro model for understanding the catalytic and regulatory mechanisms. Such information from the in vitro study may lead to understanding not only general mechanisms for enzyme regulation but also to specific control of this important enzyme in vivo.

In considering the in vivo regulation of GDH both mechanisms proposed in the present work seem attractive. In spite of the many allosteric modifiers identified for GDH in vitro, it is not yet possible to discuss in vivo mechanisms. Likewise it is too early to compare the mercurial, TNBS or TNM to any materials existing in vivo systems which would produce covalent changes. The present studies however have advanced our understanding of the possible structural basis for enzyme regulation.

BIBLIOGRAPHY

1. Anderson, B. M., Anderson, C. D. and Churchich, J. E.: Inhibition of Glutamate Dehydrogenase by Pyridoxal-5-phosphate. Biochemistry, 5:2893-2900, (1966).

2. Anderson, B. M. and Reynolds, M. L.: Inhibition of Glutamate Dehydrogenase by N-Alkylnicotinamide Chlorides. J. Biol. Chem., 241:1688-1693, (1966).

3. Appella, E. and Tomkins, G. M.: The Subunits of Bovine Liver Glutamate Dehydrogenase: Demonstration of a Single Peptide Chain. J. Mol. Biol., 18:77-89, (1966).

4. Baker, B. R.: Design of Active-Site-Directed Irreversible Enzyme Inhibitors. Wiley, New York (1967), p. 166.

5. Baldoni, A. A. and Miyashiro, J. J.: Preparation of Organic-mercuric Compounds. United States Patent, 2914, 451, (1959).

6. Beilstein Organische Chemie, Band III-IV, System Nummer 195-449.

7. Bitensky, M. W., Yielding, K. L. and Tomkins, G. M.: The Effect of Allosteric Modifiers on the Rate of Denaturation of Glutamate Dehydrogenase. J. Biol. Chem., 240:1007-1082, (1965).

8. Bitensky, M. W., Yielding, K. L. and Tomkins, G. M.: The Reversal by Organic Mercurials of "Allosteric" Changes in Glutamate Dehydrogenase. J. Biol. Chem., 240:668-673, (1965).

9. Bitensky, M. W., Yielding, K. L. and Tomkins, G. M.: Reciprocal Changes in Alanine and Glutamate Dehydrogenase Activities after Exposure of Crystalline Bovine L-glutamate Dehydrogenase to Organic Mercury. J. Biol. Chem., 240:663-667, (1968)

10. Charman, H. B., Hughes, E. D., Ingold, Sir C. K., and Thorpe, F. G.: Mechanism of Electrophilic Substitution at a Saturated Carbon Atom. Part III. Kinetics, Stereochemistry, and Mechanism of the Three-alkyl Mercury-Exchange Reaction. J. Chem. Soc., 1121-1133, (1961).

11. Churchich, J. E.: The Binding of NADH by Glutamate Dehydrogenase as Measured by Polarization of Fluorescence. Biochem. Biophys. Acta, 147:32-38, (1967).

12. Clark, C. E.: Dissertation, 1970. University of Alabama in Birmingham, Birmingham, Alabama.

13. Cohen, L. A.: Group Specific Reagents in Protein Chemistry. Ann. Rev. Biochem., 37:683-726, (1968).

14. Colman, R. F. and Frieden, C.: On the Role of Amino Groups in the Structure and Function of Glutamate Dehydrogenase. 1. Effect of Acetylation on Catalytic and Regulatory Properties. J. Biol. Chem., 241:3652-3660, (1966).

15. Colman, R. F. and Frieden, C.: Cooperative Interaction Between the GTP Binding Sites of Glutamate Dehydrogenase. Biochem. Biophys. Res. Comm., 22:100-105, (1966).

16. Cross, D. G. and Fisher, H. G.: The Mechanism of Glutamate Dehydrogenase Reaction. III. The Binding of Ligands at Multiple Subsites and Resulting Kinetic Effects. J. Biol. Chem., 245:2612-2621, (1970).

17. diPrisco, G.: Desensitization of the Allosteric Sites of Glutamate Dehydrogenase by Fluorodinitrobenzene. Biochem. Biophys. Res. Comm., 26:148-152, (1967).

18. diPrisco, G.: Tyrosyl and Lysyl Residues Involved in the Reactivity of Catalytic and Regulatory Site of Crystalline Beef Liver Glutamate Dehydrogenase. Biochemistry, 10:585-589, (1971).

19. diPrisco, G. and Strecker, H. J.: Effects of Phosphate and Other Ionic Compounds on the Activity of Crystalline Beef Liver Glutamate Dehydrogenase. European J. Biochem., 9:507-511, (1969).

20. Dodd, G. H. and Radda, G. K.: 1-Anilinonaphthalene-8-Sulphonate, a Fluorescent Conformational Probe for Glutamate dehydrogenase. Biochem. J., 114:407-417, (1969).

21. Eisenberg, H. and Tomkins, G. M.: Molecular Weight of the Subunits, Oligomeric, and Associated Forms of Bovine Liver Glutamate Dehydrogenase. J. Mol. Biol., 31,37-49, (1968).

22. Engel, D. C. and Dalziel, K.: Kinetic Studies of Glutamate Dehydrogenase. Biochem. J., 118,409-419,(1970).

23. Fisher, H. F., McGregor, L. L. and Power, U.: The Nature of the Alkaline Dissociation of the Glutamic Dehydrogenase Molecule. Biochem. Biophys. Res. Comm., 8:402-406, (1962).

24. Freedman, R. B. and Radda, G. K.: Chemical Modification of Glutamate Dehydrogenase by 2,4,6-Trinitrobenzene Sulphonic Acid. Biochem. J., 114:611-619, (1969).

25. Freishein, J. H. and Huennekin F. M.: Effect of N-Bromo-succinimide on Dihydrofolate Reductase. Biochemistry, 8:2271-2276, (1969).

26. Frieden, C.: Glutamic Dehydrogenase. 1. The Effect of Coenzyme on the Sedimentation Velocity and Kinetic Behavior. J. Biol. Chem., 234:809-814, (1959).

27. Frieden, C.: Glutamic Dehydrogenase. II. The Effect of Various Nucleotides on the Association-Dissociation and Kinetic Properties. J. Biol. Chem., 234:815-820, (1959).

28. Frieden, C.: Glutamic Dehydrogenase. V. The Relation of Enzyme Structure to the Catalytic Function. J. Biol. Chem., 238:3286-3299, (1963).

29. Frieden, C.: Unusual Inhibition of Glutamate Dehydrogenase by Guanosine Di- and Triphosphates. Biochem. Biophys. Acta, 59:484-486, (1962).

30. Glazer, A. N.: Specific Chemical Modification of Proteins. Ann. Rev. Biochem., 39:101-124, (1970).

31. Glover, D. J. and Landsman, S. C.: Spectrophotometric Method for Determination of Tetranitromethane in Solution and in Air. Anal. Chem., 36:1690-1691, (1964).

32. Goldfarb, A. R.: A Kinetic Study of the Reactions of Amino Acids and Peptides with Trinitrobenzenesulfonic Acid. Biochem., 5:2570-2574, (1966).

33. Haschke, R. H., Heilmeyer, L. M. G., Meyer, F. and Fisher, E. H.: Control of Phosphorylase Activity in a Muscle Glycogen Particle. III. Regulation of Phosphorylase Phosphatase J. Biol. Chem., 245:657-663, (1970).

34. Heilmeyer, L. M. G., Jr., Meyer, F., Haschke, R. H. and Fisher, E. H.: Control of Phosphorylase Activation by Calcium J. Biol. Chem. 245:6649-6656, (1970).

35. Heitmann, P.: A Model for Sulfhydryl Groups in Proteins. Hydrophobic Interactions of the Cysteine Side Chain in Miceles. European J. Biochem., 3:346-350, (1968).

36. Hellerman, L., Schellenberg, K. A. and Reiss, O. K.: L-Glutamic Acid Dehydrogenase II. Role of Enzyme Sulfhydryl Groups. J. Biol. Chem., 233: 1468-1478, (1958).

37. Holbrook, J. J., and Jeckel, R.: A Peptide Containing a Reactive Lysyl Group from Ox Liver Glutamate Dehydrogenase. Biochem. J., III: 689-694, (1969).

38. Kirtley, M. E., and Koshland, D. E., Jr.; Models for Cooperative Effects in Protein Carrying Subunits, Effects of Two Interacting Ligands. J. Biol. Chem., 242:4192-4205, (1967).

39. Kotaki, A., Harada, M. and Yagi, K.: Reaction Between Sulfhydryl Compounds and 2,4,6-Trinitrobenzene-1-Sulfonic Acid. J. Biochem., 55:553-561, (1964).

40. Mayer, F., Heilmeyer, L. M. G., Haschke, R. H. and Fisher, E. H.: Control of Phospholylase Activity in a Muscle Glycogen Particle. I. Isolation and Characterization of the Protein-Glycogen Complex. J. Biol. Chem., 245:6642-6648, (1970).

41. Mildvan, A. S., Greville, G. D.: Activation of Ox-liver L-Glutamate Dehydrogenase by Phenylmercuric Acetate and Adenosine 5'-Diphosphate. Biochem. J., 82:22-23, (1962).

42. Monod, J., Wyman, J. and Changeux, J. P.: On the Nature of Allosteric Transition. A Plausible Mode. J. Mol. Biol., 12:88-118, (1965).

43. Nemethy, G. and Scheraga, H. A.: The Structure of Water and Hydrophobic Binding in Proteins. III. The Thermodynamic Properties of Hydrophobic Bonds in Proteins. J. Phys. Chem., 66:1773-1789, (1962).

44. Okuyama, T. and Satake, K.: The Preparation and Properties of 2,4,6-Trinitropheylamino Acids and Peptide. J. Biochem. (Tokyo), 47:454-466, (1960).

45. Olson, J. A. and Anfinsen, C. B.: Kinetic and Equilibrium Studies on Crystalline L-Glutamic Acid Dehydrogenase J. Biol. Chem., 202:841-845, (1953).

46. Palmer, D. W. and Peters, T., Jr.: Automated Determination of Free Amino Groups in Serum and Plasma Using 2,4,6-Trinitrobenzenesulfonate. Clin. Chem., 15:891-901, (1969).

47. Pfleiderer, G., Holbrook, J. J., Nowicki, L. and Jeckel, S. R.: The Importance of -SH groups for Enzymic Activity. VI. A Method for Labeling the Essential Cysteine Residues in Native Beef Liver L-Glutamate Dehydrogenase. Biochem. Z., 346:297-300, (1966).

48. Piszkiewicz, D., London, M., and Smith, E. L.: Bovine Liver Glutamate Dehydrogenase Sequence of a Hexadecapeptide Containing a Lysyl Residue Reactive with Pyridoxal-5-Phosphate. J. Biol. Chem., 245:2622-2626, (1970).

49. Price, N. C. and Radda, G. K.: Desensitization of Glutamate Dehydrogenase by Reaction of Tyrosine Residues. Biochem. J., 114:419-427, (1969).

50. Reisler, E. and Eisenberg, H.: Study on the Viscosity of Solution of Govine Liver Glutamate Dehydrogenase and on Related Hydrodynamic Models: Effect of Toluene on Enzyme Association. Biopolymers, 9:877-889, (1970).

51. Reisler, E., Pouyet, J. and Eisenberg, H.: Molecular Weights, Association and Frictional Resistance of Bovine Liver Glutamate Dehydrogenase at Low Concentrations. Equilibrium and Velocity Sedimentation, Light Scattering Studies and Settling. Experiments with Microscopic Models of the Enzyme Oligomer. Biochemistry, 9:3095-3102, (1970).

52. Reiss, O. K., Geiger, P. J., and Hellerman, L.: Facilitation of Catalysis by L-Glutamate Acid Dehydrogenase in the Presence of Certain Mercury Compound. Fed. Proc., 18:1219, (1959), Abstract.

53. Riordan, J. F., Sokolovsky, M. and Vallee, B. L.: Enviromentally Sensitive Tyrosyl Residues. Nitration with Tetranitromethane. Biochemistry, 6:358-160, (1967).

54. Rogers, K. L.: Protein Fluorescence and Inactivation of Glutamate Dehydrogenases During Treatment with Silver. Enzymologia, 36:358-361, (1967).

55. Rogers, K. S., Geiger, P. J., Thompson, T. E., and Hellerman, L.: Molecular Weight of Chicken Liver Glutamic Acid Dehydrogenase in Relation to the Activation Effect of Methylmercuric Bromide. J. Biol. Chem., 238:481-482, (1963).

56. Rogers, K. S., Hellerman, L. and Thompson, T. E.: L-Glutamate Dehydrogenase III. Molecular Size of Bovine Glutamate Dehydrogenase and the Methylmercuric Bromide-Activated Enzyme in the Concentration Range of Enzymatic Assay. J. Biol. Chem., 240: 198-200, (1965).

57. Rogers, K. S., Thompson, T. E., and Hellerman, L.: The Effects of Silver Ions, Organic Mercurials and pH on the Dissociation of Bovine Liver Glutamate Dehydrogenase. Biochim. Biophys. Acta, 64: 202-204, (1962).

58. Scheraga, H. A., Nemethy, G., and Steinberg, I. Z.: The Contribution of Hydrophobic Bonds to the Thermal Stability of Protein Conformations. J. Biol. Chem., 237: 2506-2508, (1962).

59. Shaw, E.: Selective Chemical Modification of Proteins, Physiological Reviews, 50: 244-296, (1970).

60. Simpson, R. B.: Association Constants of Methylmercury with Sulfhydryl and Other Bases. J. Am. Chem. Sci., 83: 4711-4717, (1961).

61. Smith, E. L., Landon, M., Piszkiewicz, D., Brattin, W. J., Langley, T. J., and Melamed, M. D.: Bovine Liver Glutamate Dehydrogenase: Tentative Amino Acid Sequence: Identification of a Reactive Lysine: Nitration of a Specific Tyrosine and Loss of Allosteric Inhibition by Guanosine Triphosphate. Proc. Natl. Acad. Sci., 67: 724-730, (1970).

62. Sokolovsky, M., Riordan, J. F. and Vollee, B. L.: Tetranitromethane. A Reagent for the Nitration of Tyrosyl Residues in Proteins. Biochem., 5: 3582-3589, (1966).

63. Spande, T. F. and Witkop, B.: Determination of the Tryptophan Content of Proteins with N-Bromosuccinimide. Methods in Enzymology, 11: 498-506, (1967). Academic Press, N. Y.

64. Spande, T. F. and Witkop, B.: Reactivity Toward N-Bromosuccinimide as a Criterion for Buried and Exposed Tryptophan Residues in Proteins. Methods in Enzymology, 11: 528-532 (1967), Academic Press, N. Y.

65. Stadtman, E. R., Shapiro, B. M., Ginsburg, A., Kingdon, H. S., and Denton, M. D.: Regulation of Glutamine Synthetase Activity in Escherichia coli. Brookhaven Symposia in Biology, Vol. 2 (No. 21), 378-396, (1968).

66. Summers, M. R.: Dissertation, 1970. University of Alabama in Birmingham, Birmingham, Alabama.

67. Sund, H., and Åkeson, Åke.: Die Aminosäurezusammensetzung der Glutaminsäuredehydrogenase aus Rinderleber. Biochem. Z., 340:421-435, (1964).

68. Sund, H., Burchard, W.: Sedimentation Coefficient and Molecular Weight of Beef Liver Glutamate Dehydrogenase at the Microgram and the Milligram Level. European J. Biochem., 6:202-206, (1968).

69. Tanford, C.: Isothermal Unfolding of Globular Proteins in Aqueous Urea Solution. J. Am. Chem. Soc., 86:2050-2059, (1964).

70. Thompson, W. and Yielding, K. L: 8-Anilino Naphthalene Sulfonate Binding as a Probe for Conformational Changes Induced in Glutamate Dehydrogenase by Regulatory Reagents. Arch. Biochem. Biophys., 126:399-406, (1968).

71. Tomkins, G. M. and Yielding, K. L.: Regulation of the Enzymatic Activity of Glutamate Dehydrogenase Mediated by Changes in its Structure. Cold Spring. Harbor Symposia, XXVI, 331-341, (1961).

72. Tomkins, G. M., Yielding, K. L., Bitensky, M. W. and Talal, N.: Studies on the Regulation of Glutamate Dehydrogenase Structure and Activity. Symposium on Regulation of Enzyme Activity. Sixth International Congress of Biochemistry, 9:694-695, (1964).

73. Tomkins, G. M., Yielding, K. L. and Curran, J.: Steroid Hormone Activation of L-Alanine Oxidation Catalyzed by a Subunit of Crystalline Glutamic Dehydrogenase. Proc. Natl. Acad. Sci., 47:270-278, (1961).

74. Tomkins, G. M., Yielding, K. L., Talal, N. and Curran, F.: Protein Structure and Biological Regulation. Cold Spring Harbor Symposia on Quantitative Biology, XXVIII, 461-471, (1963).

75. Tomkins, G. M., Yielding, K. L., and Curran, J.: The Influence of Diethylstilbestrol and Adenosine Diphosphate on Pyridine Nucleotide Coenzyme Binding by Glutamate Dehydrogenase. J. Biol. Chem., 237:1704-1708, (1962).

76. Wold, F.: Bifunctional Reagents. Methods of Enzymology, 11:617-640, (1967). Academic Press, New York.

77. Yielding, K. L.: Persistance of the Effects of Regulatory Reagents on Conformation of Catalytically Inactive Glutamate Dehydrogenase. Biochem. Biophys. Res. Comm., 29:424-429, (1967).

78. Yielding, K. L.: In preparation, (1971).

79. Yielding, K. L., and Tomkins, G. M.: Inhibition of Glutamic Dehydrogenase by o-Phenanthroline and its Analogs. Biochem. Biophys. Acta, 62:327-331, (1962).

80. Yielding, K. L. and Tomkins, G. M.: Structural Alterations in Crystalline Glutamic Dehydrogenase Induced by Steroid Hormone. Proc. Natl. Acad. Sci., 46:1483-1488, (1960).

81. Yielding, K. L. and Tomkins, G. M.: An Effect of L-Leucine and Other Essential Amino Acids on the Structure and Activity of Glutamate Dehydrogenase, Proc. Natl. Acad. Sci., 47:983-989, (1961).

82. Yielding, K. L., Tomkins, G. M., Bitensky, M. W. and Talal, N.: Reagent Induced Changes in the Structure and Catalytic Activity of Glutamic Dehydrogenase. Canadian J. Biochem., 42:727-743, (1963).

83. Yielding, K. L., Tomkins, G. M., Holt, B. B., Summers, M. R. and Gaudin, D.: Alterations in the Structure and Activity of Glutamate Dehydrogenase as a Model for Enzyme Regulation. Excerpta Medica International Congress Series, 132:503-507, (1966).

84. Yielding, K. L., Tomkins, G. M., Munday, J. S., and Curran, J.: The Effects of Steroid Hormones on the Clutamic Dehydrogenase Reaction. Biochem. Biophys. Res. Comm., 2:303-306, (1960).

85. Yielding, K. L., Tomkins, G. M., Trundle, D. S.: Metal-Ion Requirement for Pyridine Nucleotide-Induced Disaggregation of Glutamate Dehydrogenase. Biochem. Biophys. Acta. 77:703-705, (1963).