ABSTRACT

REVERSIBLE DISSOCIATION AND CHARACTERIZATION
OF RABBIT MUSCLE α-GLYCEROPHOSPHATE DEHYDROGENASE

By William H. Holleman

Physical and chemical studies on native and dissociated rabbit muscle α-glycerophosphate dehydrogenase (α-GDH) have shown the native enzyme ($s^{o}_{20,w}$ = 4.86S, $D^{o}_{20,w}$ = 6.20 x 10^{-7} cm^2/sec, M^{o}_{w}(s/D) = 74,400) to consist of two noncovalently bound polypeptide chains ($s^{o}_{20,w}$ = 1.70S, $D^{o}_{20,w}$ = 4.1 x 10^{-7} cm^2/sec, M^{o}_{w}(s/D) = 40,000). Each mole of native enzyme was found to contain 2 moles of C-terminal methionine as determined by the carboxypeptidase technique, and 21 moles of free sulfhydryl groups as determined by both carboxymethylation of reduced protein and by performic acid oxidation. Fingerprinting of the tryptic peptides suggests the polypeptide chains are not grossly dissimiliar and may be identical. The partial specific volume of α-GDH in 0.1M KCl was determined, using density gradient techniques, to be 0.746 cc/g, in good agreement with the value calculated independently from the amino acid composition. The enzyme may be dissociated into stable subunits by either limited performic acid oxidation, followed by dialysis into 8.0M guanidine·HCl, or in the solvent system of 7.2M guanidine·HCl, 0.1M mercaptoethanol. Dilution of the guanidine·HCl dissociated enzyme ($s^{o}_{20,w}$ = 1.70S) at optimum conditions reversed the dissociation process with 90% recovery of enzyme activity. The optimum conditions for

reversal were 0.1M Tris·HCl, at pH 7.42, 0.001M EDTA, 0.2M mercaptoethanol and a final enzyme concentration of 0.025 mg/ml.

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INTRODUCTION

One of the ultimate problems of biochemistry is to determine the correlation between the structure and function of enzymes. It was hoped that by providing a detailed analysis of the structure of an enzyme additional knowledge concerning such a relationship would be gained. Preliminary studies (Deal et al., 1963) on α-glycerophosphate dehydrogenase from rabbit muscle had revealed a reversible denaturation. Because of this and the interesting properties described below this enzyme was chosen for further analysis. The molecular weight of native α-GDH seemed too large for a single polypeptide chain and therefore it was suspected that α-GDH was composed of two or more subunits. The original goal of this investigation was to find conditions for dissociation of the enzyme into stable subunits and to determine the size and number of the subunits of rabbit muscle α-glycerophosphate dehydrogenase by both chemical and physical methods. A study of reversal of the dissociation process was also planned pending successful dissociation of the enzyme into subunits. In the course of these investigations it became clear that the physical properties of native α-GDH reported in the literature were inconsistent. Since a reliable knowledge of the structure of the native enzyme was a prerequisite to a knowledge of the subunit structure, a detailed reinvestigation of the physical properties of native α-GDH was also conducted.

LITERATURE REVIEW

Occurence of α-glycerophosphate Dehydrogenase

α-glycerophosphate dehydrogenase (α-GDH) is an enzyme catalyzing the reduction of dihydroxyacetone phosphate (DHAP) to α-glycerophosphate (α-GP) accompanied by the oxidation of NADH to NAD^+. It was originally discovered by Meyerhof in 1919. The subject of this study is the water soluble cytoplasmic rabbit muscle α-glycerophosphate dehydrogenase. There is also a mitochondrial α-glycerophosphate dehydrogenase which differs from its cytoplasmic counterpart in that the coenzyme for the reaction is FAD rather than NAD^+ and the reaction greatly favors the oxidation of α-GP, whereas the cytoplasmic enzyme greatly favors the reduction of DHAP. For this reason the mitochondrial enzyme is known as α-glycerophosphate oxidase.

α-GDH activity has been demonstrated in insect muscles, different rat organs (Young and Pace, 1958b), components of the blood and is found to some extent in all the organs of both vertebrates and invertebrates (Delbruck et al., 1959; Zebe, 1960). With the notable exceptions of the Morris hepatoma 5123 and the ascites Ehrlich-Lettre tumor of the mouse (Morris et al., 1960), α-GDH activity is either lacking or very low in most malignant tissues. The observations of low levels of α-GDH activity are not due to the presence of an inhibitor, since the addition of tumor extracts to

extracts from normal tissues did not inhibit α-GDH activity in the normal tissue extracts.

Properties of Native α-glycerophosphate Dehydrogenase

Preparation and Purification of α-GDH

As in the original purification and crystallization (Baranowski, 1949) the standard method of preparation involves fractionation of the enzyme in the 42-60% saturated ammonium sulfate range. Of several new procedures and modifications (Disteche, 1948; Beisenherz et al., 1953) two have yielded significant increases in specific activity. Van Eys et al. (1959) obtained a three fold increase by adding a heat step, and of secondary importance, DEAE chromatography under conditions where all other proteins were absorbed. A recent procedure (Telegdi, 1964) claimed an activity three times that of any previous procedure. A rabbit muscle α-glycerophosphate dehydrogenase with a higher molecular weight and different crystalline form (Young and Pace, 1958a) is probably a dimer of the "standard" enzyme produced by either the isolation procedure or by the high concentration of ammonium sulfate (0.5M) in the solvent used for their molecular weight analysis.

Stability of α-GDH

The thermal stability of α-GDH has special interest because of the heat step sometimes employed for purification (Van Eys et al., 1959). Although the enzyme loses no activity upon standing at 25° for 30 minutes or for 2 weeks at 0° in 0.2M ammonium sulfate, the loss after 1 minute at

55° is 53% and the loss after 1 minute at 60° is 100% (Young and Pace, 1958a). The only information on stability in the pH range 5-9 has been obtained with the previously mentioned "unusual" enzyme (Young and Pace, 1958a). Using catalytic activity as the criterion for stability the enzyme is most stable at pH 5.7-6.2, is least stable at pH 7.0 and shows fair stability at pH 8.5. Beisenherz (1953) reported that, even in the absence of salts, the use of redistilled water prevented denaturation of α-GDH.

Physical Properties of α-GDH

The physical properties of native α-GDH in both the nucleotide-containing and nucleotide-free form have been studied in several laboratories. In either 0.32M ammonium sulfate or 0.1M malate, pH 6.28, both the native and the nucleotide-free protein yielded values[1] of $s^{\circ}_{20,w} = 4.9S$, and $D^{\circ}_{20,w} = 5.1 \times 10^{-7}$ cm^2/sec (Van Eys et al., 1959) which were only slightly dependent on protein concentration. Using the value[1] of 0.70 cc/g found for the partial specific volume, together with the sedimentation and diffusion data Van Eys et al. (1959) calculated a molecular weight for α-GDH of

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[1]Our data indicate a value of 0.746 cc/g for the partial specific volume of α-GDH in contrast to the results in the literature which are 0.70 cc/g (Van Eys et al., 1959) and 0.75 cc/g (Young and Pace, 1958a). Inspection of the data in Table 1 shows this value should be 0.70 cc/g. Our data also indicate a value of $D^{\circ}_{20,w} = 6.20 \times 10^{-7}$ cm^2/sec for the diffusion coefficient, in contrast to the result reported in the literature which is 5.1×10^{-7} cm^2/sec (Van Eys et al., 1959). Van Eys et al. (1959) calculated a value of 1.44 for the frictional ratio (f/f_o) while the value obtained by us is 1.23. Further discussion of these discrepancies and their effect on molecular weight calculations, may be found in the text.

78,000. From these combined data the frictional ratio (f_o/f) was calculated to be 1.44, which is larger than values usually obtained for globular proteins (Tanford, 1961). Ankel et al. (1960) using a solvent of 0.05M phosphate, 0.2M NaCl, pH 6.8, found an $s^{0.8\%}_{20,w}$ value of 4.94S for both the native and nucleotide free protein. Young and Pace (1958a), although using conditions (0.5M ammonium sulfate) which differed only slightly from the conditions of Ankel et al. (1960) and of Van Eys et al. (1959), found a considerably higher value of $s^{o}_{20,w}$ = 6.5S. As previously suggested these values may represent a dimer of the enzyme, or they may be due to aggregation. Although Young and Pace (1958a) reported a value[1] of 0.75 cc/g for the partial specific volume, reevaluation of their data suggests that 0.70 cc/g is a better value (see Table 1). Using their value of 7.2 cc/g for the intrinsic viscosity of α-GDH in ammonium sulfate, Young and Pace (1958a) calculated a molecular weight of 173,000, and a frictional ratio of 1.4, in good agreement with the frictional ratio of 1.44 found by Van Eys et al. (1959). These values for the frictional ration and the intrinsic viscosity are much higher than expected for globular proteins which have frictional ratios which range from 1.1 to 1.2 and 3.0 to 4.0 respectively (Tanford, 1961).

Table 1. Apparent[a] specific volume of α-GDH, taken from Young and Pace (1958a).

Determination	Specific Volume (cc/gm)
1	0.70
2	0.70
3	0.95
4	0.70
5	0.70
Mean	0.75
Standard deviation	0.11

[a]Enzyme (4 mg/ml) in 1M ammonium sulfate at 24°

Jirgensons (1965) has measured the optical rotary dispersion of α-GDH and calculated the Moffitt constant, b_o, of 208°, which corresponded to an α-helical content of 34%, measured at a λ_o of 216mu. For this calculation polyglutamic acid was used as a standard for 100% α-helix and it was assumed that the only kind of helical structure present was the right-handed α-helix.

Interaction of α-GDH with Aldolase

The first data suggesting interaction between α-GDH and aldolase was the observation (Baranowski, 1939; 1949b) that crystalline Myogen A contained α-GDH activity as well as aldolase activity. Several recrystallizations of the Myogen A did not separate the α-GDH activity from the aldolase activity. This interaction has been substantiated by Gulyi (1959), but they could not detect α-GDH activity in Myogen A, unless the

Myogen A was treated with 0.5M urea for two hours. Addition of purified aldolase or Myogen A to pure α-GDH resulted in a decrease of α-GDH activity and an increase in aldolase activity (Litvinenko, 1963). Sereda (1960) demonstrated that the incubation of α-GDH with aldolase resulted in a 15% increase in aldolase activity as well as protecting α-GDH from thermal denaturation. No physiological role has been assigned to these phenomena.

Prosthetic Groups of α-GDH

The most important prosthetic group of α-glycerophosphate dehydrogenase is NADH. As seen in Table II, various attempts to measure the amount of NADH bound to the enzyme have yielded

Table II. Binding of NADH by α-GDH.

Moles of NADH Bound	Method of Determination	Reference
1.0 per 70,000g	Fluorometric titration	Ankel et al., (1960)
1 per 65,000g	Sedimentation (400,000 x g)	Ankel et al., (1960)
1-3 specifically per 78,000g	Equilibrium dialysis	Van Eys et al., (1959)
31 non specifically per 78,000g	Equilibrium dialysis	Van Eys et al., (1959)
22 per 78,000g	Sephadex gel filtration	Pfleiderer and Aurricchio (1964)

different results ranging from 1 to 3. The fluorometric titration (Ankel et al., 1960) is based on the fact that when

free NADH binds to α-GDH, its absorbance maximum shifts from 463mu to 454mu and its fluorescence increases 7-10 fold. The dissociation constants for the two classes (Van Eys et al., 1959) of NADH binding found by equilibrium dialysis are: (1) 6.0×10^{-4} for the nonspecific class and (2) 1.8×10^{-6} for the specific class. While there is no explanation for the values of 1 mole of NADH bound per mole of enzyme obtained by Ankel et al. (1960) the Sephadex gel filtration technique (Pfleiderer and Auricchio, 1964) seems to have no experimental pitfalls or deficiencies and thus seems to us the best value. In connection with this data, it is of interest that Pfleiderer and Auricchio (1964) found one mole of NADH bound per 30,000-45,000 grams of protein for seven different dehydrogenases; this protein mass presumably represents the individual polypeptide chains, which therefore would be predicted to possess one active site per polypeptide chain.

In contrast to the majority of the other common dehydrogenases which catalyze reactions whose equilibrium favors oxidation, α-GDH catalyzes a reaction in the reductive direction. As a result of this, most of the other common dehydrogenases bind NAD^+ but not NADH. In contrast, α-GDH binds NADH and not NAD^+. Extensive attempts (Van Eys et al., 1958) to demonstrate NAD^+ binding have not been successful.

A second prosthetic group of α-GDH is ADP-ribose (Van Eys et al., 1964; Ankel et al., 1960; Celliers et al., 1963). It is possible that the ADP-ribose is a degradation product of bound NADH. The bound ADP-ribose is not an integral part

of the active site of α -GDH for its removal (Ankel et al., 1960) does not affect the specific activity, $s_{20,w}$, or turnover number of α-GDH; however its removal lowers the electrophoretic mobility and exposes an additional sulfhydryl group.

The ADP-ribose was first detected as a result of the observation by Baranowski (1949a) and Beisenherz et al. (1953) that α -GDH crystallized from rabbit muscle exhibited an abnormal ultraviolet spectrum in the 260-280mu region. Van Eys et al. (1959) was able to separate the bound ADP-ribose from α -GDH by treatment with charcoal, trichloroacetic acid, acid alcohol, heat, or by dialysis against cysteine at a pH above 8. Quantitative analysis of this nucleotide (Ankel et al., 1960) gave 2 moles of ribose, 2 moles of phosphate, and 0.9 moles of adenine per 70,000 grams of protein. The substance, demonstrated by enzymatic means to be neither NAD^+ or NADH, migrated identically in paper chromatography experiments in several different solvent systems with authentic ADP-5'-ribose.

The existence of a third prosthetic group has been reported (Van Eys, 1960) but its identity is uncertain. This compound, originally thought (Van Eys, 1960) to be 3-(4-methyl-5-(β-hydroxethyl)-thiazolyl)-succinic acid, was given the trivial name of thiamic acid. Celliers et al. (1963) have questioned this structure and Van Eys et al. (1964) indicated in a later report that the structure seemed less certain than it had previously. Thiamic acid (Van Eys, 1960) reactivated charcoal treated yeast or rabbit muscle α-GDH, suggesting that it might have some function in the enzyme. As additional

evidence for the presence of a thiazole derivative as part of the α-GDH molecule he showed that thiamine deficiency in rats resulted in a significant lowering of the α-GDH content.

Amino Acid Analysis of α-GDH

Van Eys et al. (1964) have calculated from the amino acid analysis a minimal molecular weight of 38,400 for the half molecule of α-GDH, based on 28 aspartic acid residues per half molecule of α-GDH. Since the molecular weight from physical measurements (Van Eys et al., 1959) was 78,000 a molecular weight of 76,800 was indicated. Approximately 4 moles of tryptophan were found per mole of α-GDH by both microbiological and spectrophotometric methods, after removal of the prosthetic groups with trichloroacetic acid.

Sulfhydryl Content of α-GDH

Ankel et al. (1960) reported the presence of 15 moles of cysteine per 70,000 grams of protein as determined by p-mercuribenzoate titration in neutral phosphate buffer. Van Eys et al. (1964) also using p-mercuribenzoate, as well as (di-3-carboxy-4-nitrophenyl) disulfide, found only 12 moles of cysteine in the presence of 8M urea and values as low as 8 in the absence of urea. When the free sulfhydryl groups were blocked by dinitrophenylation and then oxidized with performic acid, 1.7 moles of cysteic acid were found (Van Eys et al., 1964). This suggested that α-GDH had a maximum of 1 disulfide bond; these authors interpreted this as evidence that no disulfide bonds were present.

N-terminal Studies of α-GDH

Using the fluorodinitrobenzene technique Van Eys et al. (1964) found no free N-terminal amino acids, raising the possibility that the N-terminal amino acids might be N-acetyl derivatives, as in enolase and several other proteins(Winstead and Wold, 1964). Using the colorimetric technique of Ludoweig and Dorfman(1960) they calculated a value of 2 moles of acetyl per 78,000 grams of protein; unfortunately, the accuracy of this determination was limited by a large correction (50%) for the blank, because of the presence of adenosine diphosphate ribose (see Prosthetic Groups). The method involves deacetylation by hydrolysis of the N-acetyl bond in a 2N HCl methanol mixture. The methyl acetate product is spectrophotometrically determined as the hydroxylamine-ferric complex by its absorbance at 520 mu. This complex is formed in a reaction mixture containing hydroxylamine, ferric chloride, perchloric acid and HCl. Presumably ADP-ribose also reacts with this reagent to give a colored complex. Nevertheless, this analysis suggested the existence of 2 or 3 polypeptide chains in the native enzyme.

Fingerprinting of α-GDH

Further analysis of the number and identity of the polypeptide chains in α-GDH utilized the fingerprinting of tryptic peptides; this revealed no more than 16 peptides (Van Eys et al., 1964) despite repeated experiments. This is less than half the number of tryptic peptides which would be predicted if the native enzyme contained two identical

polypeptide chains.

Properties of Denatured α-GDH

Effect of Acid and Alkali

The reversible inactivation of rabbit muscle α-GDH in acid has been studied by several workers (Deal and Van Holde, 1962; Deal et al., 1963; Van Eys, 1963; Van Eys et al., 1964). Deal and co-workers reported an inactivation without dissociation into subunits in 0.01M citrate, pH 2.6; the Van Eys group reported dissociation into subunits in 0.01M glycine, pH 2.6, in the presence of 0.2M Na_2SO_4, as well as in 1.5M NH_4OH. Van Eys (1963) reported that alkali treated α-GDH moved with the salt boundary on Sephadex G-25 in high ionic strength solvent (0.35M ammonium sulfate); but in a low ionic strength solvent the protein was excluded from the Sephadex and came through the column with the solvent front. Both the alkali and acid treated α-GDH had sedimentation coefficients of $s_{20,w} = 2.6S$.

Effect of Other Dissociating Agents on α-GDH

Deal and Holleman (1964) reported that treatment of α-GDH with 8.5M guanidine·HCl in the presence of mercaptoethanol resulted in the splitting of the enzyme into subunits which have a molecular weight of 4×10^4 and a sedimentation coefficient of $s_{20,w} = 1.6S$. This dissociation was not observed if the mercaptoethanol was omitted. Chilson et al. (1965) have reported the reversible denaturation of α-GDH from guanidine·HCl. Sodium dodecyl sulfate, p-mercuribenzoate,

an inhibitor of α-GDH, urea or zinc ions did not dissociate the protein into subunits (Van Eys et al., 1964).

Properties of the Catalytic Reaction

Specificity of the α-GDH Reaction

Rabbit muscle α-glycerophosphate dehydrogenase is specific for L-glycerol-1-phosphate and dihydroxacetone phosphate. The reduction of 1,2-propanediol-1-phosphate attributed to α-GDH by Selinger and Miller (1949) has been shown by them to be due to a distinct 1,2-propanediol-1-phosphate dehydrogenase (Selinger and Miller, 1958). Borreback et al. (1965) have shown that α-GDH oxidizes NADPH at a rate approximately 10% the rate of NADH oxidation; values of the pH optimum shifts from pH 7.5 for NADH to pH 5.7 for NADPH. The reaction catalyzed by rabbit muscle α-GDH is stereospecific for β-NAD^+ (Levy and Vennesland, 1957); the oxidation of NADH, deuterated in the α-position proceeds with complete retention of the deuterium in the NAD^+.

pH for Optimum Activity

Young and Pace (1958a) using the higher molecular weight enzyme mentioned earlier, found pH optimums for dihydroxyacetone phosphate and α-glycerophosphate as substrates of 7.5 and 10.2 respectively. The rate of α-GP oxidation at pH 10.2 is about one-twelfth the rate of DHAP reduction at pH 7.5, using the same substrate concentrations.

Turnover Number and Substrate Km's

The equilibrium of the α-GDH reaction greatly favors the formation of α-glycerophosphate. Values of 4.6×10^{-12} M

(after extrapolation of the apparent equilibrium constants to zero ionic strength) and 5.7×10^{-12} M were found respectively by Burton and Wilson (1953) and Bücher and Klingenberg (1960). From equilibrium and free energy data (Burton and Wilson, 1953) for the oxidation of NADH a free energy change of -10.24 kcal. was calculated.

The turnover number for the reduction of DHAP (20°, pH 7, ionic strength 0.15) was calculated by Baranowski (1949b) to be 20,700 moles per minute per 78,000 grams of protein and by Beisenherz et al., (1953), 23,400 moles per minute per 78,000 grams of protein at 25°. Young and Pace (1958a) reported a much higher value of 35,000 moles per minute per 78,000 grams of protein at 22°. Other kinetic data are scarce. The approximate Michaelis constants at pH 7.0 have been reported as 1.1×10^{-4} M for α-GP, 3.8×10^{-4} M for NAD^+, 4.6×10^{-4} M (Bücher and Klingenberg, 1960) and 3.63×10^{-4} M (Blanchaer, 1965) for DHAP.

Inhibitors and Stimulators of the α-GDH Reaction

A large number of NAD analogs studied by Van Eys et al. (1959) were found to be potent inhibitors of α-GDH. The only exception was 4-(1-imidazoyl)NAD, which is neither a coenzyme nor an inhibitor. Ankel et al. (1960) showed that only 60% maximal activity is obtained if EDTA is not included in the reaction mixture. Emmelot and Bos (1962) reported a stimulatory effect of potassium cyanate on crude α-GDH preparations, which they attributed to a protective action of the cyanate anion against inactivation of the enzyme by heavy metals. Other

labortories using crystalline α-GDH (Boxer and Devlin, 1962; Borst, 1962; Pette and Ruge, 1963) could not reproduce this effect and Pette and Ruge showed that this stimulatory effect was due to the use of impure DHAP. KCN caused decomposition of this impurity to pyruvate which was a substrate for lactic dehydrogenase, present in the crude preparations of Emmelot and Bos (1962). Sacktor and Dick (1965) reported that many substituted cinnamic acids inhibited crystalline α-GDH, the most potent of these being p-nitrocinnamic acid which gave 50% inhibition at 3×10^{-4} M. This inhibition was not competitive with DHAP and could be reversed by dilution of the enzyme. Shonk (1962) reported that zinc ions at a concentration of 10^{-6} M strongly inhibited the enzyme in both directions.

Inhibition of α-GDH by α-glycerophosphate

Blanchaer (1965) reported that α-GP inhibits α-GDH and measured an inhibition constant of 5.6×10^{-5} M. Blanchaer (1965) also showed that there was a sharp decrease in DHAP reduction (in vivo) when the concentration of α-GP rose above 1 μmole per gram of muscle tissue. Consideration of the estimated concentrations of DHAP and α-GP present in muscle during contraction (680 and 60 mμ, respectively) suggests that α-GP may influence the pathway of triose phosphate utilization (Blanchaer, 1965).

Effect of Sulfhydryl Agents on α-GDH Activity

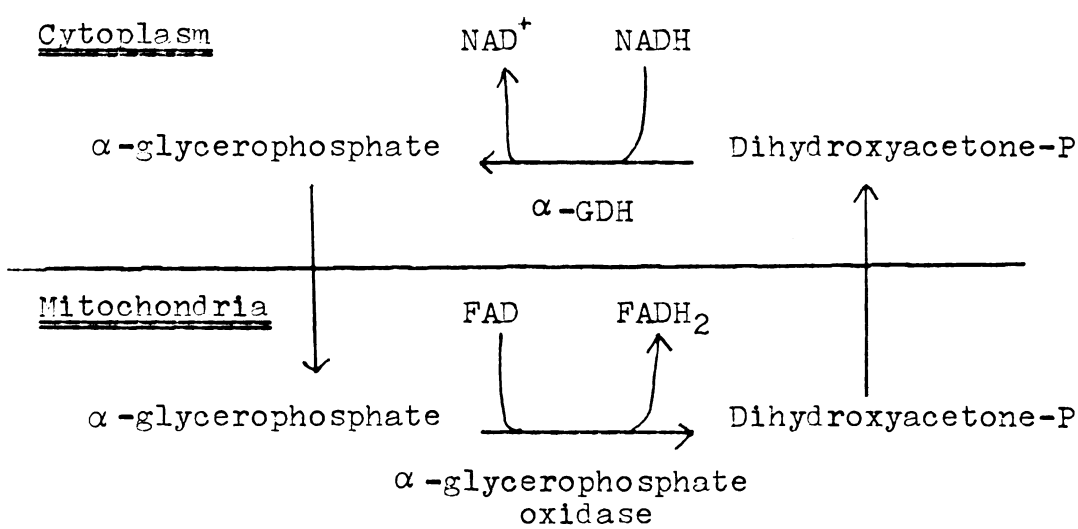
Van Eys et al. (1959) found that one mole of p-hydroxymercuribenzoate (PHMB) per 87,000 grams of protein completely inhibited α-GDH as did a concentration of 1×10^{-4} M

N-ethylmaleimide. Iodoacetic acid (1×10^{-4} M) gave only a 23% decrease in activity. In contrast to these results, Telegdi and Keleti (1964) reported that titration of 3 sulfhydryl groups per mole of α-GDH gave a 50% loss of activity and it was necessary to titrate 9-11 sulfhydryl groups in order to abolish all enzymatic activity.

Role of α-GDH in Cell Metabolism

α-glycerophosphate Shunt

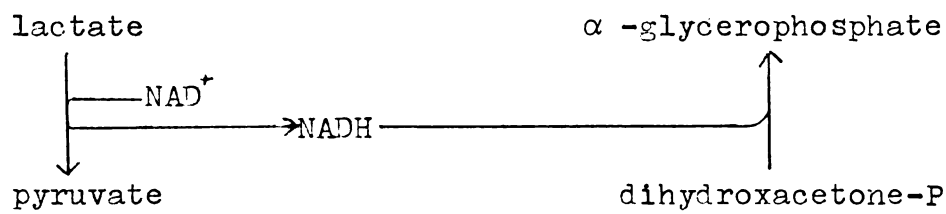
The role of α-GDH in cell metabolism, except for the production of α-GP for fat synthesis, remained largely unexplored until Lehninger (1951) noted that extramitochondrial NADH is not a substrate for the electron transport chain. A system for the transfer of hydrogen from the extramitrochondrial NADH to the respiratory chain was postulated by Zebe et al. (1956) utilizing the α-glycerophosphate cycle illustrated below.



Support for this model come from the demonstration that mitochondria from various sources contained a flavin linked enzyme that oxidized α-glycerophosphate by way of the phosphorylating electron transport chain (Sachtor et al., 1959; Klingenberg and Slenczka, 1959; Zebe et al., 1957). Recently an NAD-linked, mitochondrial α-glycerophosphate dehydrogenase has also been found (Tomita and Helling, 1965). The equilibrium of the α-glycerophosphate oxidase greatly favors the formation of dihydroxacetone phosphate as a result of the FAD potential. The alternate extramitochondrial reduction of DHAP and mitochondrial oxidation of α-GP thus form an effective system for shuttling reducing equivalents from extramitochondrial NADH to the intramitochondrial electron transport system. It should be noted that any dehydrogenase which is located in both the cytoplasm and mitochondria can provide a means of transferring electrons into the mitochondria, if the substrates are permeable to the mitochondria membrane. Two enzymes in this category are lactic dehydrogenase and β-hydroxybutyrate dehydrogenase (Boxer and Devlin, 1961).

α-glycerophosphate-pyruvate Dismutation

α-GDH may also play a role in the so-called "α-glycerophosphate-pyruvate dismutation" which is outlined in the diagram below (Klingenberg and Bücher, 1960). The result of



this cycle is the transfer of hydrogens from lactate to DHAP with the formation of an extra pyruvate. The end result is that lactate does not accumulate at the expense of pyruvate. An extra production of pyruvate from α-GP could allow the production of lactate without a corresponding lowering of the lactate-pyruvate redox potential because of a build up of lactate. This mechanism could provide a stabilization of the redox potential of the extramitrochondrial ATP system under inadequate oxygen supply. Such a system can also explain the lack of a change in the lactate-pyruvate ratio when there is a rise in the lactate level in skeletal muscle, heart, brain and liver of the rat (Hohorst et al., 1959; Bücher and Klingenberg, 1958) and during tetanic work of striated muscle (Sachs and Morton, 1956).

Absence of α-GDH Activity in Malignant Tissue

In most malignant tissues, α-GDH is either absent or present in very low levels (Boxer and Devlin, 1961). Holzer et al. (1958) first observed this characteristic in Ehrlich tissues and Yoshida hepatoma cells. In a variety of normal mammalian tissues, including regenerating liver and embryonic tissue the ratios of lactic dehydrogenase (LDH) to α-GDH was found (Boxer and Slonk, 1960) to vary between 0.5:1 and 7.0:1; in a large series of tumors of rodents and human beings this ratio ranged from 10:1 to several hundreds:1. The increase in the α-GDH; LDH ratio was due to a large decrease in the amount of α-GDH present in the tumors and not due to an increase in the amount of LDH (Boxer and Devlin, 1961).

MATERIALS AND METHODS

Materials

NADH, NAD^+, dihydroxyacetone phosphate-cyclohexylamine salt-dimethyl ketal•H_2O and α-glycerophosphate were obtained from the Sigma Chemical Co. NADH was prepared daily. A stock solution (2×10^{-2} M) of dihydroxyacetone phosphate was prepared for use by acidification with Dowex-50 (H^+) and heating for 4 hours at 40° in order to hydrolyze the ketal. This solution was stored frozen until used. Guanidine carbonate, iodoacetic acid and mercaptoethanol were products of the Eastman Kodak Chemical Co. All of the reagents used in the course of these investigations were reagent grade quality and solutions were made up in distilled deionized water.

Guanidine•HCl was prepared from guanidine carbonate by a slight modification of the method of Anson (1941). The carbonate salt was recrystallized twice from 95% alcohol, dried, and concentrated HCl added to the guanidine carbonate to a pH of 2. The guanidine•HCl was evaporated to near dryness, filtered, washed with cold (-20°) 95% alcohol and dried in a vacuum at 50° for several days. Urea was obtained from the J. T. Baker Chemical Co. and recrystallized from hot (40°) analytical 95% alcohol.

S-carboxymethyl cysteine was prepared by the method of Dickens (1933); it had a melting point of 185°, which compared well with the reported value of 184° (Dickens, 1933).

S-carboxymethyl cysteine sulfone was prepared by adapting the procedure of Moore (1963) for the preparation of cysteic acid. The carboxymethyl cysteine was oxidized with performic acid for 4 hours at 0°. Performic acid was prepared by mixing 1 ml 30% H_2O_2 with 9 ml 88% formic acid. The reaction was stopped by the addition of 0.3 ml of 48% HBr and the reactants removed with a rotary evaporator at 40°.

Rabbit muscle α-glycerophosphate dehydrogenase was obtained from the Calbiochemical Co. Lot numbers used were 54734, 63490, 63688, 63684, 45818, 52614, 54520, 52610. Trypsin (bovine pancreas) two times recrystallized was obtained from the Sigma Chemical Co. Carboxypeptidase-A treated with diisopropyl fluorophosphate was obtained from Worthington Corp.

Methods

Ultracentrifigal Analysis

Samples were prepared for sedimentation equilibrium and sedimentation velocity analysis by centrifigation of the protein from an ammonium sulfate suspension and dissolving the protein in the test solvent. This stock solution was then dialyzed against this solvent for 48 hours. For a series of experiments done at different protein concentrations, samples of the stock protein were diluted directly with the dialysate to give the desired concentrations.

Two Spinco analytical Model E ultracentrifuges equipped with phase-plate schlieren optics were used for these studies. The short column sedimentation techniques of Van Holde and

Baldwin (1958) were utilized. Techniques were developed for the use of multicell (2 or 3 cells) operation using the double sector cells equipped with sapphire windows and filled epon centerpieces. Equilibrium was judged complete in 36 hours for the guanidine•HCl-protein solutions and in 24 hours for the native protein. Average initial protein concentrations (C_o) were obtained from a series of synthetic boundary experiments performed at different protein concentrations immediately after completion of dialysis. The computations were performed on a Control Data Corporation 3600 digital computer using a program developed and extensively evaluated in this laboratory. The calculations include statistical analysis of the data and a plot of $1/r \cdot dc/dr$ versus ΔC.

Sedimentation velocity experiments were run at 59,780 rpm at low temperature; exact temperatures were obtained from an RTIC unit. The diffusion coefficients and synthetic boundary experiments were performed in a double sector synthetic boundary cell at approximately 5,000 rpm at a temperature of 5°. Diffusion coefficients were calculated using height to area analysis (Schachman, 1957). Densities of solvents were measured to three significant figures with hydrometers and viscosities were either obtained from the International Critical Tables or were measured using an Oswald free-fall viscometer.

Preparation of α-GDH Derivatives

Limited performic acid oxidation of α-GDH, in preparation for molecular weight analysis, was carried out by the method of Hirs (1956). The ammonium sulfate pellet was taken up in

88% formic acid and sufficient performic acid (1 ml 30% H_2O_2 + 9 ml 88% formic acid) added to give a 10 fold excess over the half-cysteine content of α-GDH. After oxidation the protein was dialyzed for 24 hours against 8.5M guanidine·HCl, 0.2M NaCl, 0.04M Tris·HCl, 0.001M EDTA, pH 8.3 to remove the oxidation reactants. Sedimentation equilibrium experiments were performed on the dialyzed sample.

Reduction and carboxymethylation of α-GDH was done according to the method of Crestfield et al. (1963) with the exception that the 8M urea was replaced by 8.5M guanidine·HCl to insure complete unfolding for reduction and alkylation with iodoacetic acid. Reduction of the protein (5-10 mg) was carried out with 0.1M mercaptoethanol in a 0.14M Tris·HCl, pH 8.6 buffer, under a nitrogen atmosphere. Iodoacetic acid (0.2 ml of 0.2M) was added to the reduced protein and allowed to react for 30 minutes. The reactants were removed from the protein by gel filtration on Sephadex G-75 which was equilibrated with 50% acetic acid. The acetic acid was removed from the protein by rotary evaporation at 40°.

The method of Crestfield et al. (1963) was also used to prepare carboxymethylated protein with the exception that the protein was not reduced prior to alkylation with iodoacetic acid.

Oxidation of α-GDH for amino acid analysis was accomplished by the method of Moore (1963). To 5-10 mg of protein, 2 ml of performic acid solution (1 ml of 30% H_2O_2 plus 9 ml of 88% formic acid) were added, reacted at 0° for 4 hours and the reaction stopped by adding 0.3 ml of 48% HBr.

Reactants were removed with a rotary evaporator at 40°.

Amino Acid Determinations

Samples for all of the amino acid determinations were desalted on Sephadex G-25 and lyophilized to dryness. For each analysis 5 to 10 mg portions of the dried protein or protein derivative were hydrolyzed in 6N HCl, in sealed tubes evacuated to 50 microns. Hydrolysis was carried out at 110° for 24 hours or more as desired. After hydrolysis the HCl was rapidly removed at 40° in a rotary evaporator, and the hydrolysate dissolved in 2 mls of a 0.2M citrate buffer, pH 2.2. Quantitative determination of the amino acids was performed on a Beckman Spinco Model 120 amino acid analyzer according to the method of Moore and Stein (1954).

Peptide Mapping

All protein used for peptide mapping was reduced and carboxymethylated in 8.5M guanidine·HCl according to the method of Crestfield et al. (1963). Trypsin (3 mg/ml) was added to 5 mg portions of the carboxymethylated α-GDH suspended in water, pH 8.6. The ratio of trypsin to carboxymethylated α-GDH was 1:50. A Radiometer automatic recording titrator, Model TTT-1/SBR2-SBU1/TTA31 was used to monitor the reaction by maintaining the pH at 8.6 by the addition of 0.01N NaOH. The reaction was 90% complete after 10 minutes of digestion and was stopped after 2 hours by adjusting the pH to 2.8 with 1N HCl. The reaction mixture was frozen, lyophilized and dissolved in a minimum amount of water. The mapping of the tryptic peptides was carried out by the technique

of Katz et al. (1959). Chromatography was performed with butanol :acetic acid:water (4:1:5) and electrophoresis was carried out at pH 3.7 in pyridine:acetic acid:water (1:10:289) for 90 minutes at 2,000 volts. The peptides were located with 0.2% ninhydrin or with one of several specific amino acid sprays (given in the text--see Table VI).

C-terminal Analysis

The carboxy-terminal amino acids of α-GDH were determined, using carboxypeptidase, by the method of Koorajian and Zabin (1965). This method involved the use of sodium dodecyl sulfate as a denaturing agent in order to make the carboxy terminal amino acids more susceptible to attack by carboxypeptidase. α-GDH (5 mg) was dialyzed overnight against 0.1M Tris·acetate, 0.01M $MgCl_2$, 0.001M EDTA, 0.5% sodium dodecyl sulfate, pH 7.7, then heated at 100° for 10 minutes. Carboxypeptidase-A was added to the protein (ratio of 1:20) and allowed to react at room temperature for the desired time. Protein concentrations were determined by measurement of O.D. at 280mμ using an extinction coefficient of 0.62 ml/mg cm^{-1} for α-GDH and 1.94 ml/mg cm^{-1} for carboxypeptidase-A (Neurath et al., 1947). After various intervals of time (5, 10 and 90 minutes) the reaction was stopped by the addition of 20% trichloroacetic acid (TCA) to a final concentration of 5% TCA. The precipitated protein was washed twice with water, and the supernatant and washings were combined and lyophilized. The dried sample was dissolved in 1 ml of 0.2M citrate buffer, pH 2.2 and the amino acids were analyzed on a Spino Model 120 amino acid analyzer.

Renaturation

α-GDH was centrifuged from an ammonium sulfate suspension and dissolved in the dissociation medium 7.5M guanidine·HCl, 0.1M Tris·HCl, 0.1M mercaptoethanol, 0.001M EDTA, pH 7.5, to give a final protein concentration of 1 mg/ml. After 2 hours at 0°, 25 µl of the denatured protein was diluted into 1.0 ml of the renaturing media at room temperature to give a final protein concentration of 0.025 mg/ml and a final guanidine·HCl concentration of 0.2M. The protein was allowed to renature for exactly 15 minutes at room temperature after which 10 µl of the protein was assayed for enzymatic activity as described below.

Enzyme Assay

α-GDH was assayed by following the decrease in absorbance at 340mµ using dihydroxyacetone phosphate (1×10^{-3}M) and NADH (2.5×10^{-4}M) as substrates with 0.1M Tris·HCl, 0.001M EDTA, pH 7.42 as the buffering agent. A Beckman DU Spectrophotometer with a Gilford Multiple Sample Absorbance Recorder attachment was used; the recorder speed was 1 in. per minute and the sensitivity of the detector was 1 O.D. full scale. Temperature was maintained at 25° by a constant temperature circulator. An enzyme unit is defined as 100 divided by the time in seconds required for a change of 0.1 O.D. per mg of protein per ml of assay mixture.

Partial Specific Volume

The partial specific volume ($\bar{v}$) of α-GDH was calculated from the amino acid composition by the method of Cohn et al., (1934). The partial specific volume was determined

experimentally by the falling drop method of Barbour and Hamilton (1926). The apparatus consists of a 50 x 1.5 cm jacketed column containing a mixture of bromobenzene and o-xylene, the density of which was adjusted to give a drop time of 30-60 seconds. The temperature of this column was maintained at $20^{\circ} \pm 0.005$. A 5 µl drop of the enzyme, prepared as described below, was applied to the top of the column and the time for the drop to fall between two marks approximately 25 cm apart timed to the hundredth of a second. The drop fell approximately 15 cm before reaching the first mark in order to allow for complete temperature equilibration of the drop. A standard curve of density versus reciprocal fall time, was prepared using KCl solutions of known density (0.998-1.004 gm/cc). The fall times of protein solutions of different concentrations were measured and their densities determined from the standard curve. The partial specific volumes were calculated using the following equation (Schachman, 1957).

$$\bar{v} = \frac{1}{d_o} - \frac{1}{x}\left(\frac{d - d_o}{d_o}\right)$$

where $\bar{v}$ = partial specific volume

d = density of the solution

d_o = density of solvent (0.1000M KCl)

x = grams of protein/ml

The protein samples were prepared by dialyzing against either distilled water or 0.1000M KCl for 48 hours at 4°. The protein concentration of the test sample was determined on a separate portion by evaporating a known volume of α-GDH to dryness in an evacuated P_2O_5 dessicator and subsequently drying

to constant weight in a 110° oven. The contribution due to the KCl solvent was accounted for and subtracted from the total weight.

Sulfhydryl Titration

Sulfhydryl groups were determined by the method of Boyer (1954) using parahydroxymercuribenzoate (PHMB) and following the increase in absorbance at 250 mμ. In order to insure complete access of the PHMB to the sulfhydryl groups, the titrations were carried out in the presence of 8M urea, pH 4.6. Guanidine·HCl was not used, although preferred because of its greater effectiveness as a denaturing agent, due to its absorbance at 250mμ. Glutathione (1×10^{-3}M) was used to standarize the PHMB (3×10^{-5}M) immediately before each titration. Experimental O.D. differences of 0.001 were routinely measured reproducibly with a Beckman DU monochromator equipped with an absorbance indicator. Proper controls were run to account for the absorbance of glutatione and α-GDH at 250mμ.

PHMB was purified (Boyer, 1954) for use by dissolving in a aqueous basic solution and precipitating by acidifying with concentrated HCl. This process was repeated, the crystals washed twice with distilled water and dried.

The concentration of α-GDH was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard. For the analysis α-GDH was dialyzed for 24 hours against 8M urea, pH 4.6.

RESULTS

Physical Properties of Native α-glycerophosphate Dehydrogenase

An accurate knowledge of the native enzyme is a prerequisite for determining the subunit structure of α-GDH. Due to the limited nature of the experiments reported in the literature and to an inability to reconcile our results with some of the values in the literature, a complete physical characterization of native α-GDH was undertaken. This included determination of $s^{o}_{20,w}$, $D^{o}_{20,w}$, molecular weight and partial specific volume. A major problem in the determination of the molecular weight of native α-GDH by sedimentation techniques was the presence of high molecular weight aggregates in native α-GDH. The first phase of this research was directed toward finding a solution to this problem.

Preparation of "aggregate free" α-GDH

The formation of aggregates was considered likely to be due to the interaction of reactive groups as a result of a slight unfolding, or to the formation of random interchain disulfide bonds. When high ionic strength (1.0M NaCl), or co-factor stabilization (0.1M NAD^{+}; 0.05M NADH) did not eliminate aggregates, α-GDH was chromatographed on Sephadex

G-100 or G-200 in the presence of a reducing agent (either 0.1M mercaptoethanol or 0.1M dithiothreitol (DTT)). Chromatography on Sephadex G-100 in the presence of 0.1M mercaptoethanol gave unsatisfactory results. Although chromatography on Sephadex G-200 initially appeared promising, aggregates formed upon standing, even in the presence of 0.1M mercaptoethanol. This suggested the need for a reducing agent sufficiently strong to maintain the thiol groups in the reduced form. α-GDH, chromatographed on Sephadex G-200 in the presence of 0.1M DTT, gave satisfactory results. Since the concentration of the protein eluted from the Sephadex columns was low (ca. 1 mg/ml), water was removed by layering Sephadex G-200 over dialysis casing which contained the deaggregated α-GDH. In approximately 10 hours, α-GDH was concentrated tenfold. This was kept in DTT and used for the following experiments.

Molecular Weight of α-GDH as Determined by Sedimentation Equilibrium

The results of a series of sedimentation equilibrium experiments conducted on α-GDH obtained in this manner are shown in Figure 1. Least square analysis of the extrapolation to zero protein concentration data yielded a weight average molecular weight of $M_w^{c=0} = 83{,}900$. The individual sedimentation equilibrium schlieren patterns clearly showed a small amount of aggregate at the bottom of the cell. Further evidence for the existence of significant amounts of aggregate is shown by inspection of Figure 2 where it is

Figure 1. Extrapolation of the apparent weight average (M_w) molecular weights of native α-GDH to zero protein concentration. Apparent molecular weights were determined by sedimentation equilibrium at a speed of 9,341 rpm and at 5°. Concentrations were evaluated as $(C_m+C_b)/2$. In order to remove high molecular weight aggregates, α-GDH was chromatographed on Sephadex G-200 using 0.1M dithiothereotol, 0.2M NaCl, 0.001M EDTA, 0.1M Tris·HCl, pH 7.45 as the elution solvent. All solvents used were thoroughly purged with nitrogen prior to use and precautions were made to keep the system oxygen free. The protein was then dialyzed against this solvent overnight prior to the sedimentation equilibrium experiments. The initial concentration (C_o) of the stock solution was obtained by averaging the values obtained from synthetic boundary experiments performed on the samples containing 15, 12, 9, 6, and 3 mg/ml.

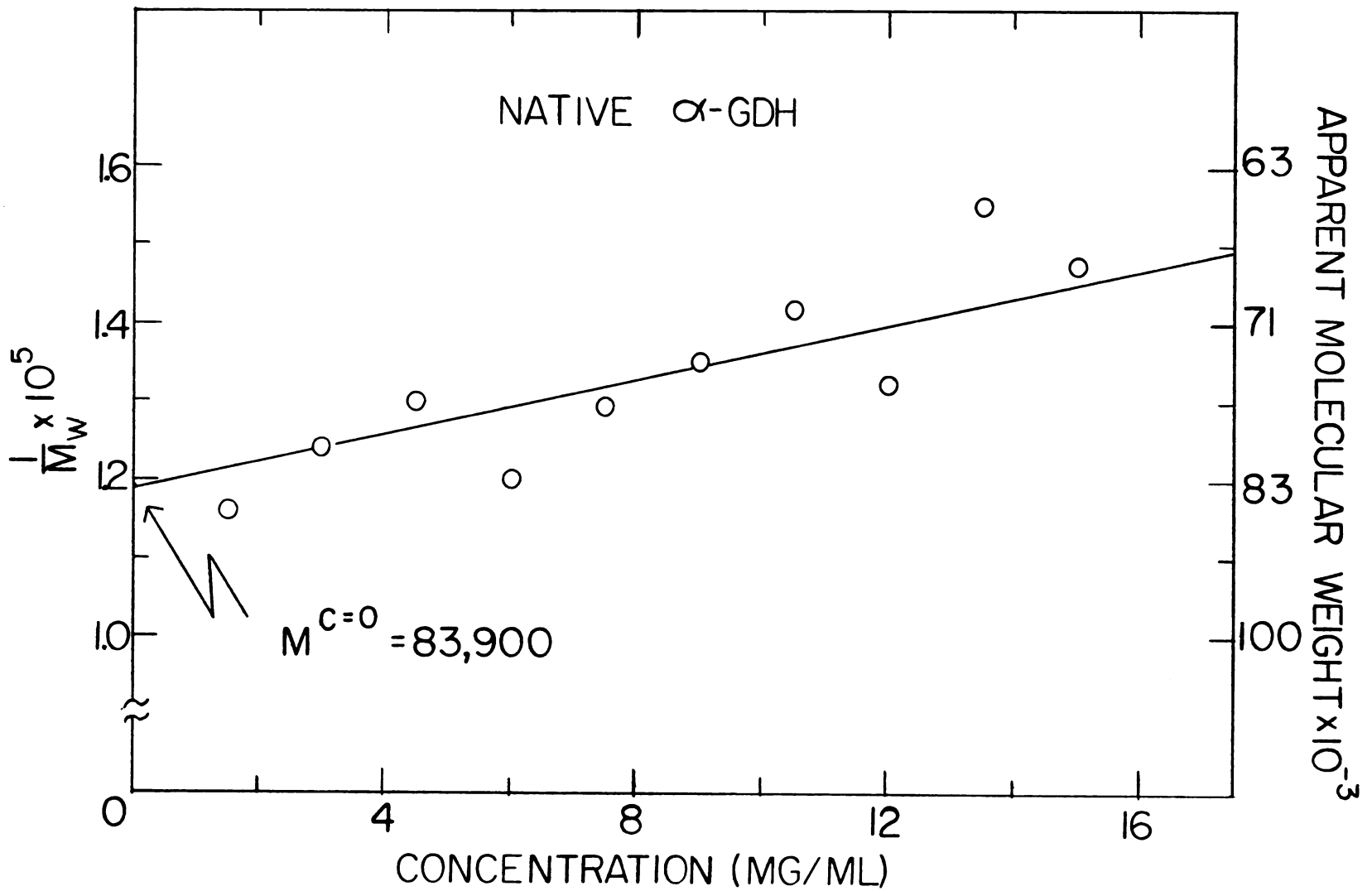
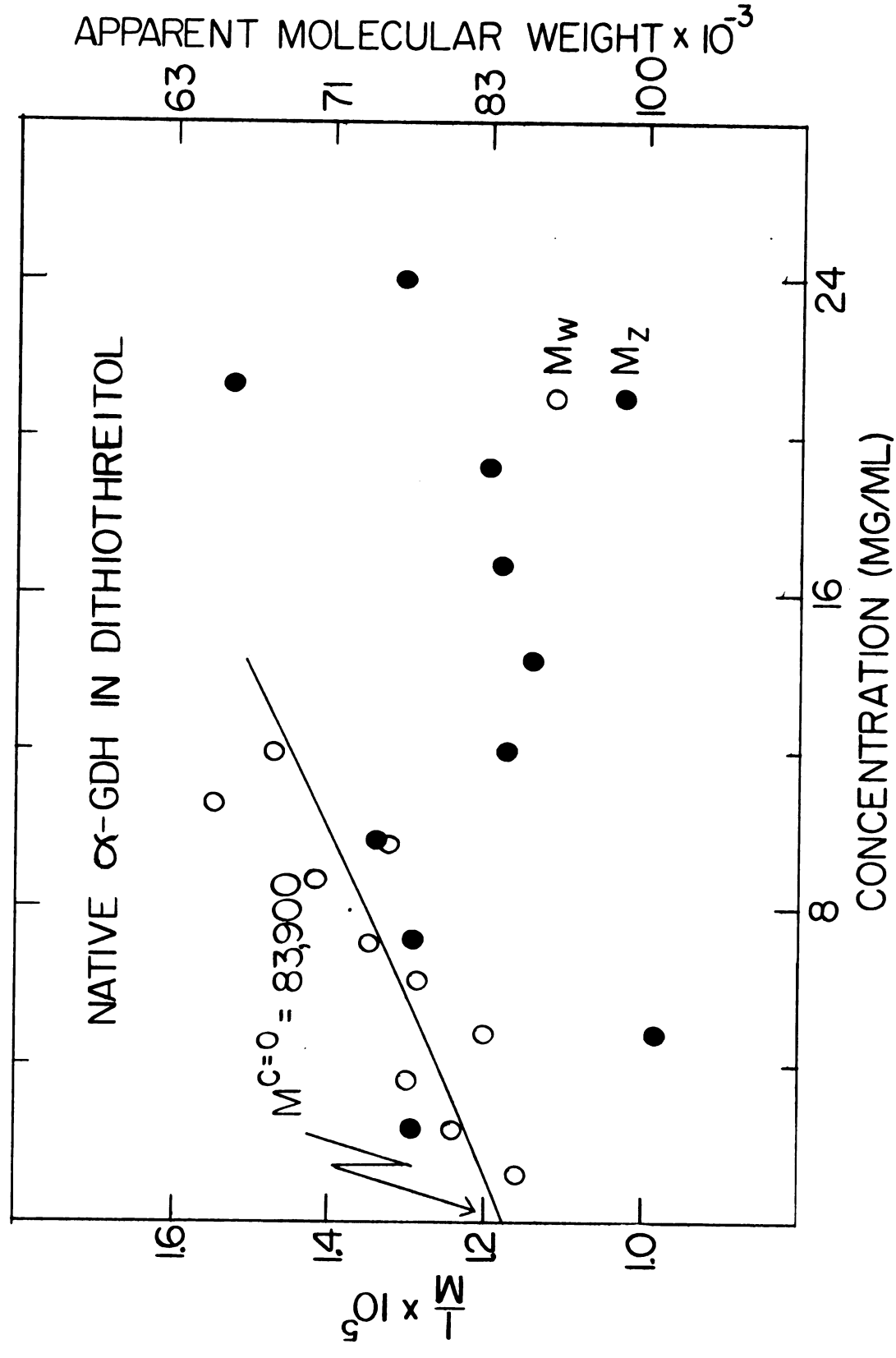

NATIVE α-GDH
1.6
1.4
1.2
1.0
0
4
8
12
16
63
71
83
100
$\frac{1}{M_w} \times 10^5$
$M^{c=0} = 83,900$
CONCENTRATION (MG/ML)
APPARENT MOLECULAR WEIGHT x 10^{-3}

Figure 2. Extrapolation of the apparent weight average (M_w) and z-average (M_z) molecular weights to zero protein concentration. This figure provides both M_w and M_z molecular weights for comparison purposes. The data are from the same experiments as those shown in Figure 1; see the legend in Figure 1 for experimental details. The high values of M_z (low values of $1/M_z$) at protein concentrations above 12 mg/ml indicate substantial aggregation of the enzyme.

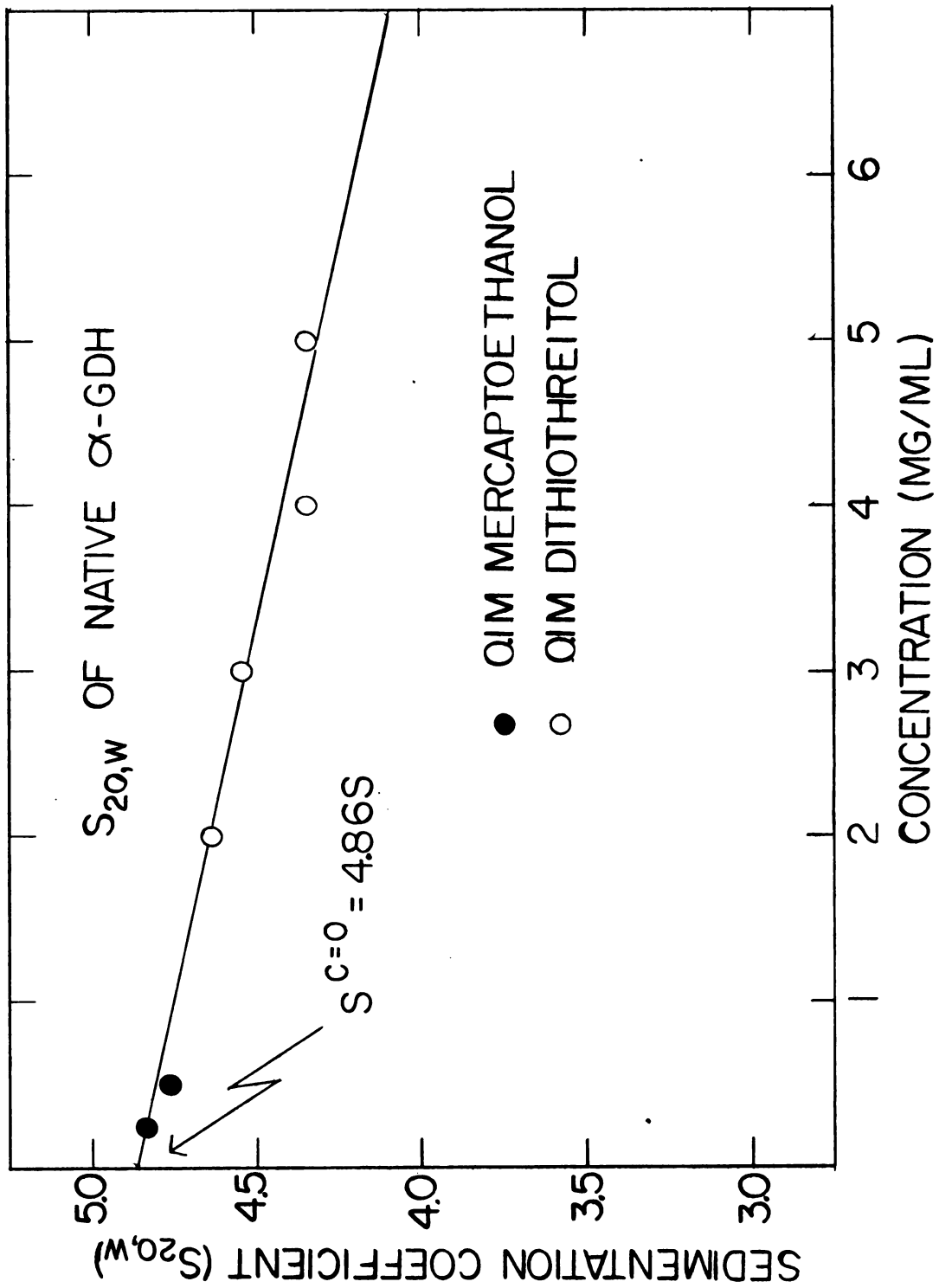

APPARENT MOLECULAR WEIGHT x 10^{-3}
63
71
83
100
NATIVE α-GDH IN DITHIOTHREITOL
$M^{c=0}$ = 83,900
M_w
M_z
1.6
1.4
1.2
1.0
$\frac{1}{M} \times 10^5$
8
16
24
CONCENTRATION (MG/ML)

seen that most of the z-average molecular weights at a given concentration are much higher than the corresponding weight-average molecular weights. As a result the weight-average molecular weight of 83,900 must be considered a maximum. Although there is a significant amount of aggregation in DTT, it does not approach the amount of aggregation observed in 0.1M mercaptoethanol. In further contrast to the results obtained in 0.1M mercaptoethanol, additional aggregation did not occur rapidly upon standing for several days. The evidence from the experiments above supports the theory that the aggregates in native α-GDH arise from the formation of random interchain disulfide bonds. Since the presence of aggregates prevented an unequivocal determination of the molecular weight of native α-GDH using sedimentation equilibrium techniques, we turned to an analysis using a combination of $s^{o}_{20,w}$ and $D^{o}_{20,w}$. This method is much less sensitive to aggregation since the sample is freed from aggregates in the sedimentation velocity experiments. This is because the aggregates sediment faster than the main protein boundary.

Determination of $s^{o}_{20,w}$, $D^{o}_{20,w}$, and $M_w(s/D)$ of native α-GDH

A series of sedimentation velocity experiments were carried out in parallel with the sedimentation equilibrium experiments, using the same stock enzyme preparation which had been deaggregated on Sephadex-G-200 in the presence of 0.1M dithiothreitol. For comparison, two values are included which were obtained using 0.1M mercaptoethanol rather than DTT as the reducing agent. As shown in Figure 3, there was

Figure 3. Extrapolation of sedimentation coefficients of native α-GDH to zero protein concentration. One sample was chromatographed on Sephadex G-200 in the presence of 0.1M dithiothreitol (open circles). A second sample was dissolved in a solvent containing 0.1M mercaptoethanol (filled circles). Both samples, containing 0.2M NaCl, 0.1M Tris·HCl, 0.001M EDTA, pH 7.45, in addition to the reducing agent, were dialyzed overnight. Sedimentation velocity experiments were conducted at 59,780 rpm at 5°.


SEDIMENTATION COEFFICIENT ($S_{20,w}$)
5.0
4.5
4.0
3.5
3.0
CONCENTRATION (MG/ML)
1
2
3
4
5
6
$S_{20,w}$ OF NATIVE α-GDH
$S^{c=0} = 4.86S$
● 0.1M MERCAPTOETHANOL
○ 0.1M DITHIOTHREITOL

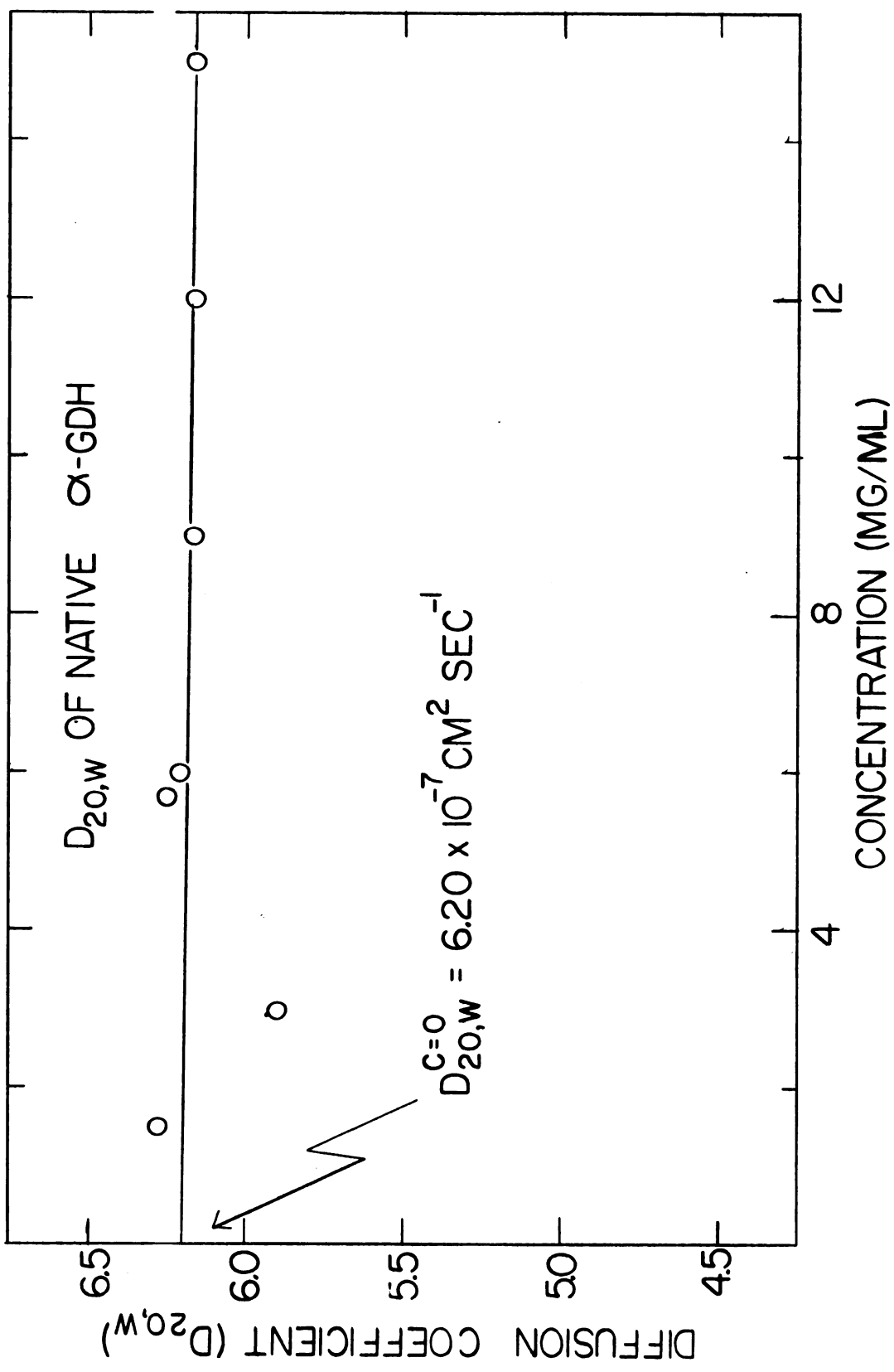
no significant difference between the two experiments. Extrapolation of the apparent sedimentation coefficients to zero protein concentration (see Figure 3) yields a sedimentation coefficient of $s^{o}_{20,w}$ = 4.86S which is identical to that reported by Van Eys *et al.* (1959).

Since the determination of the molecular weight required both sedimentation and diffusion data it was necessary to determine the diffusion coefficient of native α-GDH. Extrapolation of the apparent[2] diffusion coefficients to zero protein concentration yielded a diffusion coefficient (see Figure 4) of $D^{o}_{20,w} = 6.20 \times 10^{-7} cm^2/sec$, which is independent of protein concentration. Using the value of $s^{o}_{20,w}$ = 4.86S and a value of $D^{o}_{20,w} = 6.20 \times 10^{-7} cm^2/sec$ for the diffusion coefficient a molecular weight of 74,400 is obtained. Using this molecular weight of 74,400 and a partial specific volume of o.746 cc/g, a frictional ratio of 1.23 is calculated. It is obvious that this data is internally consistent, in contrast to the data reported in the literature. This will be shown later in the discussion. Furthermore, assuming the molecular weight of 74,400 the sedimentation coefficient and diffusion

---

[2]Apparent simply denotes that the quantity is measured at a finite concentration and depends on concentration. This is in contrast to the value obtained by extrapolation to zero protein concentration, which is assumed to be an intrinsic property and thus not a function of protein concentration.

Figure 4. Extrapolation of the diffusion coefficients of native α-GDH to zero protein concentration. The protein was dialyzed 24 hours against 0.1M mercaptoethanol, 0.1M Tris·HCl, 0.2M NaCl, 0.001M EDTA, pH 7.42. The experiments were performed at approximately 3° using a speed of 5,200 rpm. $M_w(s/D)$ molecular weights were calculated from the Svedberg equation: $M = \frac{s\ R\ T}{D(1 - v\rho)}$


$D_{20,w}$ OF NATIVE α-GDH
$D^{C=0}_{20,w} = 6.20 \times 10^{-7}$ CM^2 SEC^{-1}
DIFFUSION COEFFICIENT ($D_{20,w}$)
6.5
6.0
5.5
5.0
4.5
4
8
12
CONCENTRATION (MG/ML)

coefficients both lie close to the curves expected for globular proteins, which is indirect evidence for their accuracy (see Figures 5 and 6).

Partial Specific Volume

As stated in the introduction, the literature value of 0.70 cc/g for the partial specific volume, gave ambiguous results for the subunit analysis of α-GDH. Using this value a molecular weight of 26,000 was obtained for the α-GDH subunit, thus indicating that α-GDH was composed of three polypeptide chains (see the subunit section). This result does not agree with the 2 moles of C-terminal (see Table I) or 2 moles of N-terminal (Van Eys et al., 1964) amino acids found per mole of α-GDH, which indicated 2 polypeptide chains per native enzyme unit. For this reason and because a precise value for partial specific volume was necessary for the calculation of reliable molecular weights, the partial specific volume of α-GDH was determined by two independent means: (1) a direct experimental measurement using the falling drop method of Barbour and Hamilton (1926) and (2) an indirect, empirical determination calculated from the amino acid composition (Cohn and Edsall, 1943).

The initial determination of the partial specific volume was conducted with α-GDH dialyzed in distilled water. It did not yield satisfactory results at the lower protein concentration (see Figure 7). Since protein-

Figure 5. Graphs of theoretical and empirical sedimentation coefficients as a function of molecular weights for globular proteins. Theoretical (——) sedimentation coefficients were calculated for spheres with a partial specific volume of 0.73 cc/g (see appendix for calculations). Empirical (---) values for sedimentation coefficients and molecular weights were obtained from the literature (See appendix for list of references).

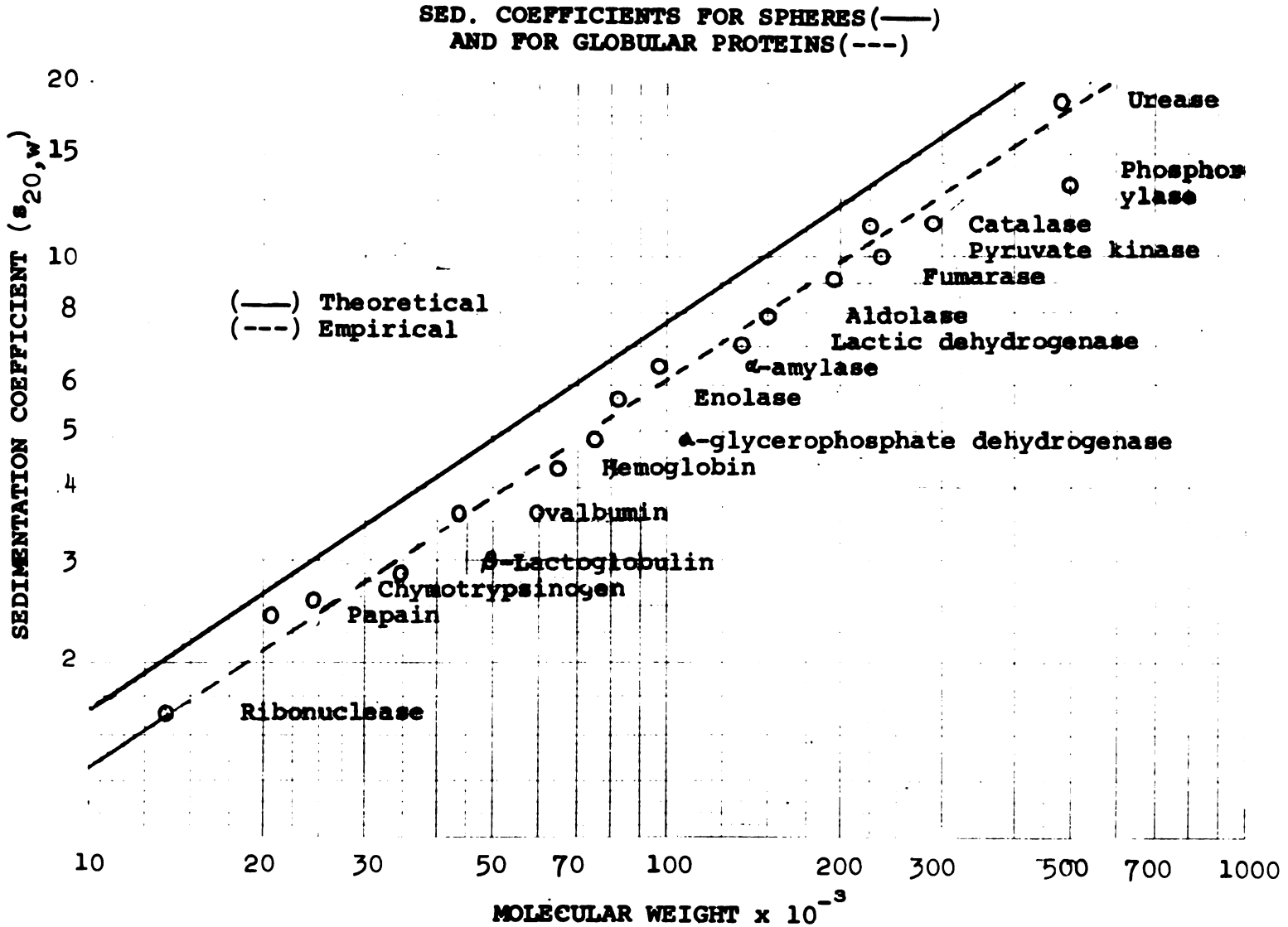

SED. COEFFICIENTS FOR SPHERES(——)
AND FOR GLOBULAR PROTEINS(---)
SEDIMENTATION COEFFICIENT ($s_{20,w}$)
20
15
10
8
6
5
4
3
2
(——) Theoretical
(---) Empirical
Urease
Phosphorylase
Catalase
Pyruvate kinase
Fumarase
Aldolase
Lactic dehydrogenase
α-amylase
Enolase
α-glycerophosphate dehydrogenase
Hemoglobin
Ovalbumin
β-Lactoglobulin
Chymotrypsinogen
Papain
Ribonuclease
10
20
30
50
70
100
200
300
500
700
1000
MOLECULAR WEIGHT x 10^{-3}

Figure 6. Graphs of theoretical and empirical diffusion coefficients as a function of molecular weights for globular proteins. Theoretical (——) diffusion coefficients were calculated for spheres with a partial specific volume of 0.73 cc/g (see appendix for calculations). Empirical (---) values for diffusion coefficients and molecular weights were obtained from the literature (see appendix for list of references).

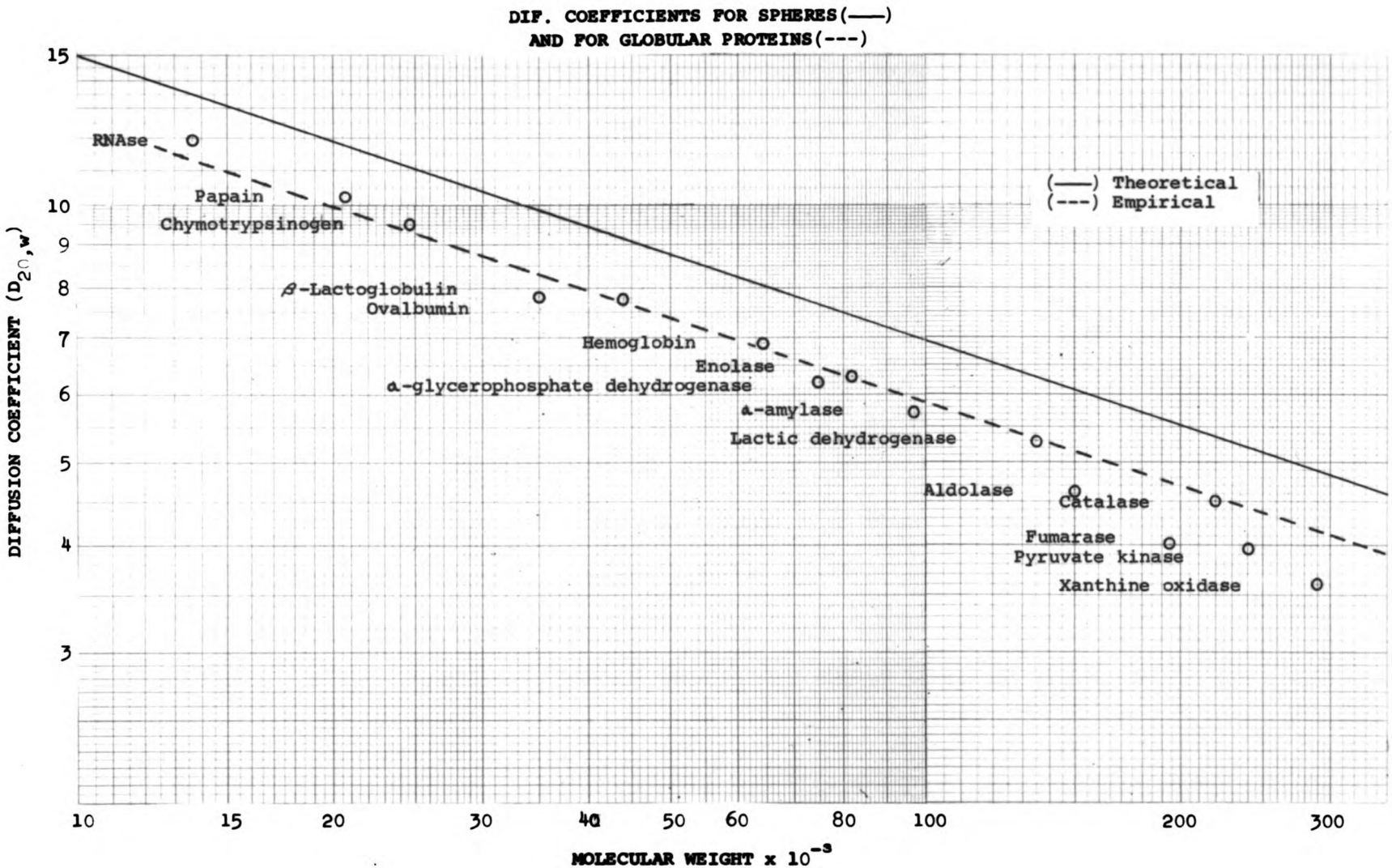
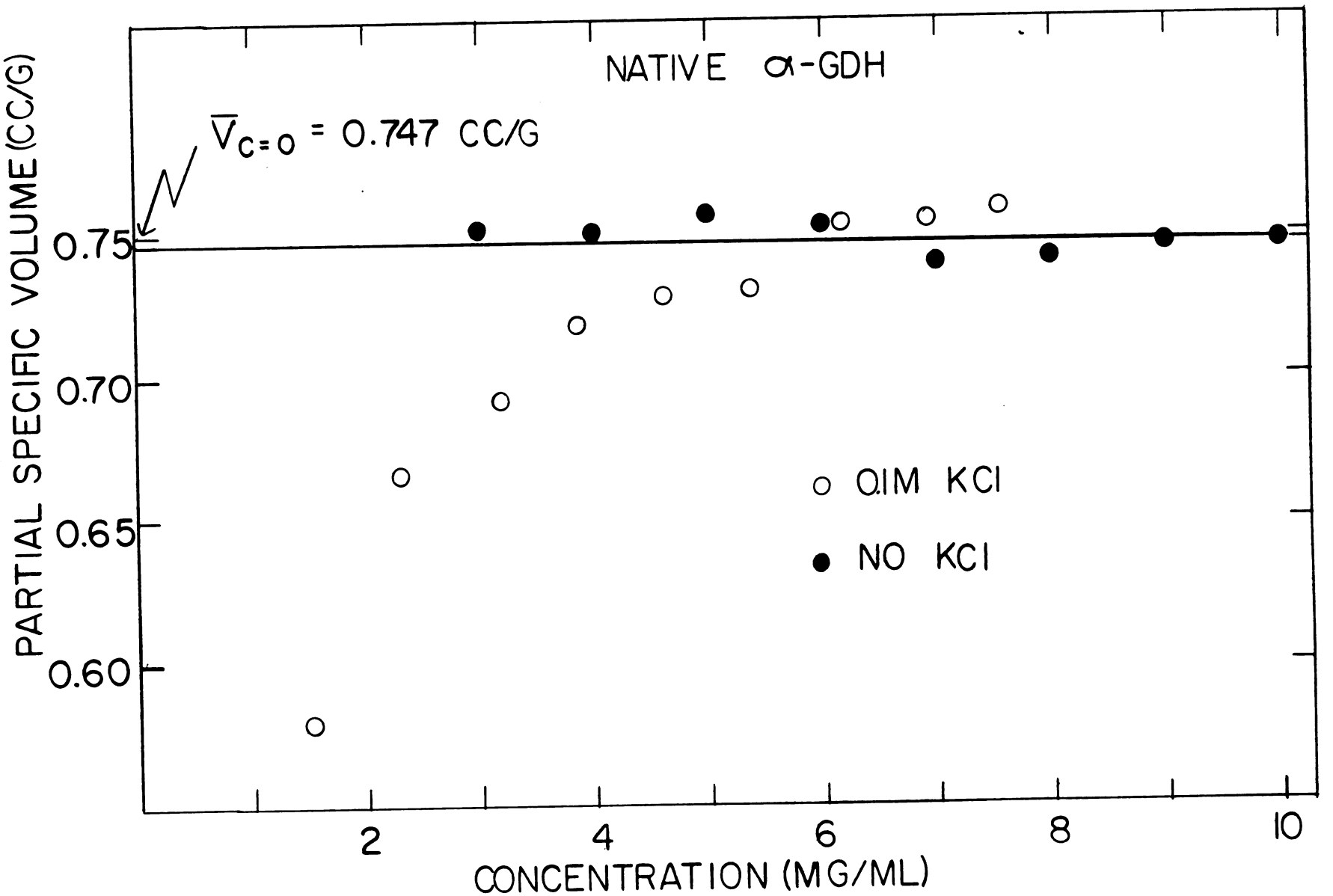

DIF. COEFFICIENTS FOR SPHERES(——)
AND FOR GLOBULAR PROTEINS(---)
(——) Theoretical
(---) Empirical
RNAse
Papain
Chymotrypsinogen
β-Lactoglobulin
Ovalbumin
Hemoglobin
Enolase
α-glycerophosphate dehydrogenase
α-amylase
Lactic dehydrogenase
Aldolase
Catalase
Fumarase
Pyruvate kinase
Xanthine oxidase
DIFFUSION COEFFICIENT ($D_{20,w}$)
MOLECULAR WEIGHT x 10^{-3}
15
10
9
8
7
6
5
4
3
10
15
20
30
40
50
60
80
100
200
300

Figure 7. Protein concentration versus apparent partial specific volume ($\bar{v}$) of α-GDH. Measurements were made on protein dissolved in two different solvents: water (closed circles) and 0.1000M KCl (open circles). Dilutions were made from a stock enzyme solution which was dialyzed against the appropriate solvent for 48 hours. Protein concentrations of the original stock solution in each series were determined by evaporation of a portion of the stock enzyme to dryness.


NATIVE α-GDH
$\bar{V}_{C=0}$ = 0.747 CC/G
PARTIAL SPECIFIC VOLUME (CC/G)
0.75
0.70
0.65
0.60
0.1M KCl
NO KCl
2
4
6
8
10
CONCENTRATION (MG/ML)

protein interactions are known to often be a significant factor in the absence of salt, the $\bar{v}$ measurement was repeated using 0.1000M KCl as solvent. Under these conditions precise measurements of $\bar{v}$ were possible and an average value of 0.746 cc/g was obtained for the partial specific volume of α-GDH (see Figure 7). It is also seen from Figure 7 that $\bar{v}$ is essentially independent of protein concentration.

McMeekin and Marshall (1952) have shown that the partial specific volume of proteins calculated from the amino acid composition agreed very closely with the values obtained by direct experimental measurements. Therefore the partial specific volume of α-GDH was calculated from the amino acid analysis listed in Table I. The partial specific volumes for the individual amino acids were obtained from Cohn and Edsall (1943) and the partial specific volume of cysteine was obtained from McMeekin *et al.* (1949). The value of 0.746 cc/g obtained by this method (see Table I) agreed very closely with the experimentally measured value of 0.747 cc/g and indicated that the value of 0.70 cc/g indicated by the data of Young and Pace (1958a) and the value reported by Van Eys *et al* (1959) was in error. A partial specific volume of 0.746 cc/g was also calculated from the amino acid composition of α-GDH reported by Van Eys *et al.* (1964). The excellent agreement between the values for $\bar{v}$ determined by these two widely different methods argues

Table I. Partial specific volume as calculated from amino acid analysis[a]

Amino acid residue	Number of residues/ molecule	(% by weight of residue)	Specific volume of residue	(% by volume of residue
Lysine	55	9.28	0.82	7.610
Histidine	18	3.25	0.67	2.178
Arginine	16	3.29	0.70	2.303
Aspartic acid	20	3.03	0.59	1.788
Asparagine[b]	36	5.40	0.60	3.240
Threonine	28	3.73	0.70	2.611
Serine	21	2.41	0.63	1.158
Glutamic acid	46	7.81	0.66	5.015
Glutamine[b]	36	6.06	0.67	4.060
Proline	31	3.96	0.76	3.096
Glycine	77	5.79	0.64	3.706
Alanine	67	6.27	0.74	4.640
Valine	62	8.09	0.86	6.596
Methionine	15	2.59	0.75	1.943
Isoleucine	55	8.19	0.90	7.371
Leucine	58	8.64	0.90	7.776
Tryosine	10	2.15	0.71	1.526
Phenylalanine	32	6.20	0.77	4.774
Half-cystine[c]	21	2.85	0.63	1.796
Tryptophane[d]	4	0.98	0.74	0.725
			Total	74.63

[a]See text for details.

[b]The 72 residues of ammonia were divided evenly between glutamine and asparagine.

[c]This value was determined separately, both as cysteic acid and as S-carboxymethyl cysteine (See Table III).

[d]Taken from the data of Van Eys et al.(1964).

strongly for the accuracy of the value of 0.746 cc/g.

Analysis for Disulfide Bonds and Sulfhydryl Groups of α-GDH

In order to evaluate the presence or absence of disulfide bonds in α-GDH, the two quantities which are needed are: (1) the total half-cystine content and (2) the number of free sulfhydryl groups; the number of disulfide bonds is then the difference between the two values. These values were obtained by measuring the sulfhydryl content of unreduced and reduced protein; the sulfhydryl content in the former case corresponds to the number of free sulfhydryl groups and in the later case to the number of free sulfhydryl groups plus twice the number of disulfide bonds. The sulfhydryl content values were obtained by two methods namely, (1) titration with para-hydroxymercuribenzoate and (2) by measurement of S-carboxymethylcysteine formed by reaction of α-GDH with iodoacetic acid.

PHMB titration.— The PHMB experiments were conducted in 3M urea, pH 4.6, to expose the sulfhydryl groups to the PHMB. The results are shown in Table II. If disulfide bonds were present in α-GDH the reduced protein should have a greater number of titratable sulfhydryl groups than the unreduced protein. Unfortunately, the results were neither consistent nor reasonable; a greater number of sulfhydryl groups were found in the unreduced samples (experiments 1 and 2) than in the reduced samples (experiments 3b and 4). However experi-

Table II. Titration of α-GDH with PHMB

Treatment	Exp. No.	No. of -SH groups per 76,000 g protein
None(ie., unreduced)	1	19.0
	2	20.4
	3a	15.2
Reduced and mercapto-ethanol dialyzed out	3b	15.1
	4	15.4
Carboxymethylation followed by reduction	5	4.8

ments 3a and 3b, which were done in parallel using the same stock solution, raised the possibility that there was no appreciable difference between unreduced and reduced protein. A direct measurement of the disulfide content of α-GDH was achieved by measuring with PHMB the sulfhydryl content of α-GDH carboxymethylated prior to reduction(Experiment 5). This suggested that α-GDH contained 1 or more disulfide bonds. The explanation for these results is not known; however one possibility is that when the enzyme is unfolded, as it is in urea, that very reactive sulfhydryl groups are exposed which then react to form disulfide bonds. Since the results obtained by PHMB titration were contradictory and did not allow unequivocal conclusions concerning the presence of disulfide bonds in native α-GDH, it was necessary to resort to more refined and more reliable chemical techniques. Carboxymethylation with iodoacetic acid was the technique chosen for additional experiments.

Carboxymethylation of α-GDH.—— The aim in this series of experiments was to determine values for the total number of half-cystines and free sulfhydryl groups in α-GDH by measurement of carboxymethyl cysteine, formed by reaction of reduced and unreduced α-GDH with iodoacetic acid respectively. Values of 20.8 and 15.9 (see Table III) moles of carboxymethyl cysteine per mole of protein were found for the reduced and unreduced protein respectively. This obviously suggested the presence of two disulfide bonds in the sample under study. But again the variability (12, 17, and 19 sulfhydryls per mole) in the experiments on unreduced, carboxymethylated protein cast doubt on the results. We thus turned to still another method, performic acid oxidation, to provide an analysis for total half-cystine, in an effort to firmly establish this value.

Performic acid oxidation of α-GDH.—— Analysis of the cysteic acid produced by the performic acid oxidation, yielded a value of 21.4 moles of half-cystine per mole of protein, in very good agreement with the value of 20.8 obtained from the reduced and carboxymethylated protein (see Table III). These data established firmly and reliably the half-cystine content of α-GDH at 21 moles per mole of protein, but the possible existence of disulfide bonds still remained to be answered.

Combination of carboxymethylation and performic acid oxidation.—— The following experiment was de-

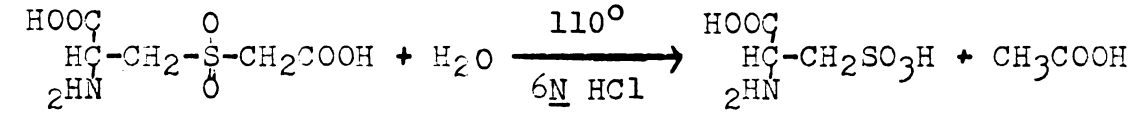
Table III. Analysis for sulfhydryl groups and disulfide bonds of α-GDH.

Treatment	Derivative measured	Number of residues found[a]			Ave.
performic acid oxidized	cysteic acid	20.6	21.4	22.1	21.4
reduced, carboxymethylated	carboxymethyl-cysteine	20.9	20.6		20.8
carboxymethylated	carboxymethyl-cysteine	11.7	16.7	19.4	15.9
carboxymethylated, performic acid oxidized	cysteic acid	7.2	8.1		7.7

[a]based on 56 aspartic acid residues per 76,000 grams of protein.

signed to provide a direct measurement of the disulfide bonds present in α-GDH. The first step was to carboxymethylate the protein in 8.5M guanidine·HCl and the second step was to oxidize the carboxymethylated protein with performic acid. All free sulfhydryl groups should have been carboxymethylated in the first step and all disulfide bonds oxidized to cysteic acid in the second step. Of the two products obtained by this procedure one, cysteic acid, should have provided a measurement of the disulfide bonds present in α-GDH, and the other, S-carboxymethyl cysteine sulfone, should have provided a measurement of the free sulfhydryl groups. The latter is produced by the oxidation of the S-carboxymethyl cysteine. Following such treatment (See Table III) α-GDH gave rise to a considerable amount of cysteic acid (8 moles per 76,000

grams of protein) suggesting the presence of 4 disulfide bonds. However examination of the structure of carboxymethyl cysteine sulfone suggested that some or all of the cysteic acid might have been formed from carboxymethyl cysteine sulfone during hydrolysis of the protein in 6N HCl, via the following reaction.



carboxymethyl cysteine sulfone — cysteic acid

To test this possibility, a mixture containing equimolar quantities of carboxymethyl cysteine and cysteine·HCl was oxidized with performic acid. A portion of the mixture was incubated in 6N HCl at 110° for 24 hours, while a second portion remained at room temperature. Both samples were than analyzed for cysteic acid. The sample which had been subjected to HCl hydrolysis had only 1/3 as much sulfone as the sample which had not been heated in 6N HCl, thus substantiating the theory that hydrolysis in 6N HCl at 110° for 24 hours results in a breakdown of carboxymethylcysteine sulfone with the possible formation of cysteic acid. As expected, some increase in cysteic acid was also observed. The experimental approach just described was thus unable to evaluate the existence of disulfide bonds. With this experiment this phase of the chemical analysis was terminated. Although the collective data (See Tables II and III) indica-

ted that disulfide bonds did exist in the particular samples analyzed, this did not prove that these disulfide bonds were an integral part of the original native α-GDH molecule. For example, the disulfide bonds could have been formed in the treating and handling of the enzyme. In conclusion the chemical analysis had provided a reliable value for the half-cystine content but was unable to unequivocally evaluate the possible presence of disulfide bonds. A final answer to this question was later obtained, however by molecular weight analysis of enzyme dissolved in a carboxymethylating dissociation media. This is described in the subunit section.

Amino Acid Analysis of α-GDH

As mentioned previously an amino acid analysis was performed in order to calculate an empirical value for the partial specific volume of α-GDH and to obtain a detailed analysis for the number of disulfide bonds and sulfhydryl groups present in α-GDH. The results of a total amino acid analysis are shown in Table IV. Because the values obtained for aspartic acid remained constant during hydrolysis, the values for the other amino acids were based on aspartic acid, which was set equal to 1.000. The absolute values for the the individual amino acids were obtained by extrapolation of these ratios to zero time of hydrolysis for amino acids destroyed during hydrolysis and by appropriate extrapolation to the maximum value

Table IV. Total amino acid analysis of α-GDH at varying times of hydrolysis

Amino acid	Time of hydrolysis(hours) 24	48	72	96	Extrapolated value	Residue per 76,000 g protein	Values obtained by Van Eys et al. (1964)
Lys	0.984	0.965	0.958	0.994	0.975	55	60
His	0.330	0.320	0.315	0.326	0.325	18	18
Ammonia	1.336	1.273	1.218	1.394	1.291	72	82
Arg	0.290	0.291	0.297	0.298	0.288	16	16
Asp	1.000	1.000	1.000	1.000	1.000	56	56
Thr	0.472	0.468	0.451	0.427	0.495	28	26
Ser	0.347	0.312	0.291	0.267	0.357	21	24
Glu	1.456	1.455	1.467	1.465	1.461	82	82
Pro	0.552	0.551	0.510	0.578	0.556	31	32
Gly	1.374	1.349	1.437	1.355	1.374	77	80
Ala	1.148	1.154	1.129	1.108	1.198	67	68
Val	1.055	1.092	1.108	1.123	1.101	62	64
Met	0.308	0.255	0.271	0.267	0.264	15	16
Iso	0.973	0.936	0.970	0.973	0.984	55	54
Leu	1.041	1.046	1.056	1.070	1.035	58	60
Tyr	0.160	0.146	0.134	0.139	0.175	10	8
Phe	0.552	0.516	0.498	0.526	0.565	32	30
Cys[a]						21	20
Try[b]						4	4

[a]Results taken from Table III; based on analysis of carboxymethylated and performic acid oxidized protein

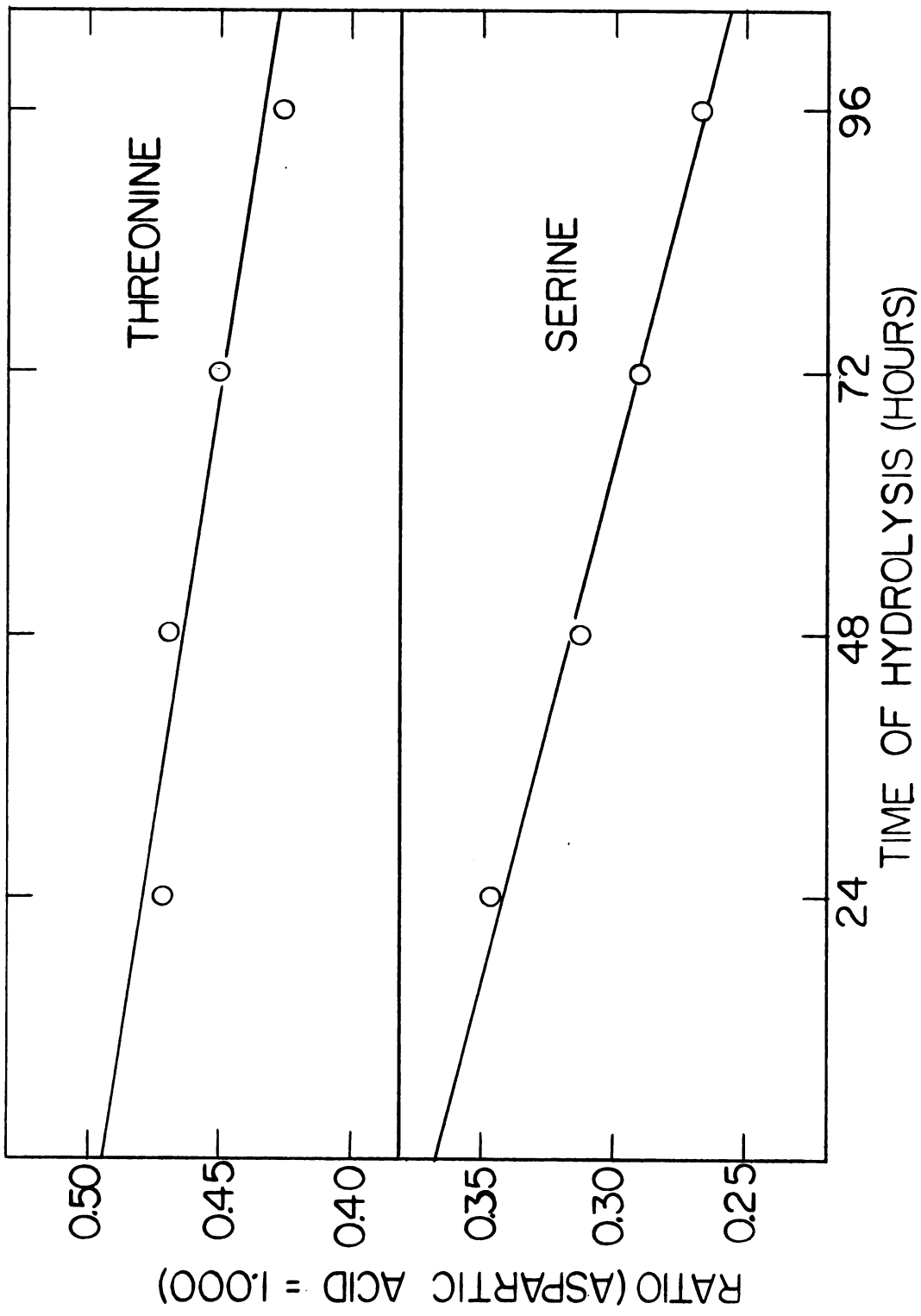
[b]Value obtained by Van Eys et al.(1964)

for those whichwere incompletely hydrolyzed after 24 hours. The use of leucine or arginine as standards gave essentially the same results as aspartic acid. Corrections for losses were made from observations of 24, 48, 72 and 96 hour hydrolysates of α-GDH. Losses during hydrolysis were significant in the cases of serine, threonine,(Figure 8) and tryosine. The molecular weight for α-GDH calculated on the basis of 56 residues of aspartic acid per mole of protein is 76,000. The agreement is good between our results and the results of Van Eys et al.(1964), which are shown in the extreme right column of Table IV.

Carboxy-terminal Analysis of α-GDH

As an independent means of determining the number of polypeptide chains in the native molecule of α-GDH a kinetic study of the appearance of C-terminal amino acids with time of digestion with carboxypeptidase was undertaken. Prior to digestion with DFP treated carboxypeptidase-A, α-GDH was thoroughly denatured by heating at 100° for 10 minutes in 0.5% sodium dodecyl sulfate. The digestion was stopped after the indicated time periods by precipatating the proteins with trichloroacetic acid; the amino acids in the supernatant were concentrated and analyzed on an amino acid analyzer. After 5 or 90 minutes of digestion (See Table V), methionine was the only amino acid released in significant amounts. These results are consistent with α-GDH being composed of two poly-

Figure 8. Destruction of serine and threonine during acid hydrolysis of α-GDH. Hydrolysis was performed in evacuated sealed tubes containing 6N HCl at 110°. The ratios are based on the value of 1.000 for aspartic acid.


THREONINE
SERINE
RATIO (ASPARTIC ACID = 1.000)
0.50
0.45
0.40
0.35
0.30
0.25
24
48
72
96
TIME OF HYDROLYSIS (HOURS)

peptide chains both of which contain a methionine residue on the carboxy-terminal end. The fact that no other amino acid is released after extended digestion with

Table V. Release of C-terminal amino acids with time.

Minutes of digestion[a] / Amino Acid	Moles amino acid per 76,000 grams of protein		
	5	10	90
Serine	trace	0.24	0.46
Glycine	trace	trace	trace
Methionine	1.86	2.11	2.03
Leucine	trace	0.23	trace

[a]ratio of carboxypeptidase-A to α-GDH was 1:20.

carboxypeptidase-A suggests that the amino acid adjacent to methionine is either lysine or arginine, for carboxypeptidase-A will not attack either carboxy-terminal lysine, arginine, or proline and may be blocked by glutamate or aspartate (Canfield and Anfinsen, 1963).

Fingerprinting of α-GDH

Amino acid analysis of α-GDH indicated a total of 71 lysines and arginine residues per 76,000 grams of protein. Assuming that trypsin is specific for lysyl and arginyl bonds, one would expect to find 72 peptides if α-GDH is composed of subunits which have no repeating amino

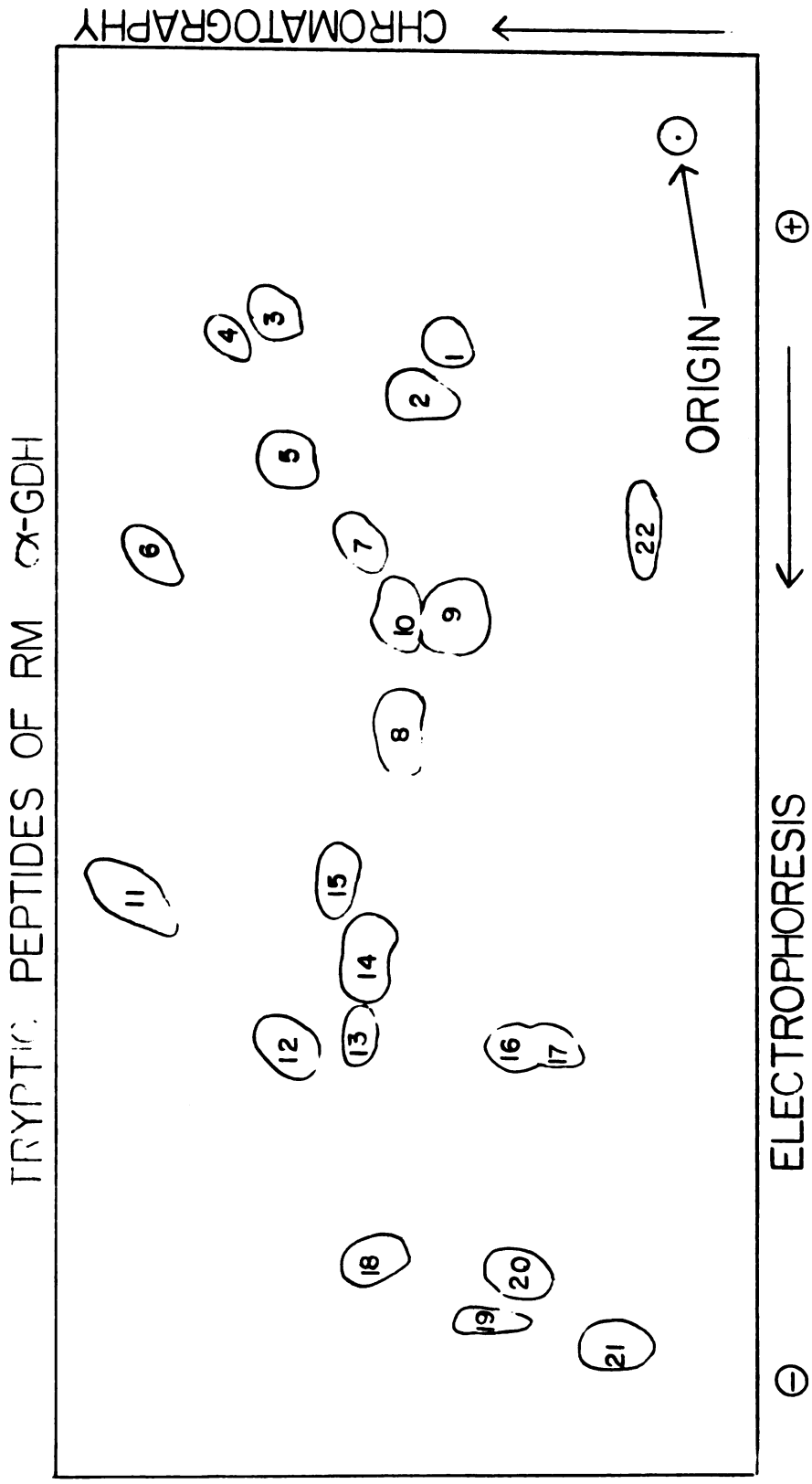
acid sequences, either in the same or in different polypeptide chains. On the other hand if the protein is composed of polypeptide chains whose amino acid sequences are wholly or partly unique, the number of peptides obtained will be some fraction of 71. The results of a typical fingerprint, obtained as described in materials and methods, is shown in Figure 9. A maximum number of 25 peptides was obtained with 22-23 being commonly found. Other peptide patterns obtained in a similiar manner were analyzed separately with specific spray reagents for peptides containing tyrosine, histidine, and arginine. As shown in Table VI, the number of peptides containing each of these amino acids was in all cases approximately

Table VI. Staining of peptides for specific amino acids

Spray reagent	No. of spots	No. of amino acid residues/ 76,000 g of α-GDH
Ninhydrin	20-25	71 lysine + arginine
Diazotized sulfanilic acid (Block, 1951)	5-6	18 histidine
α-Nitroso-β-naphthol Acher and Crocker (1952)	3	10 tyrosine
8-Hydroxyquinoline-sodium hypobromite (Jepson et al., 1953)	5-6	16 arginine

one third the number of residues present in the native molecule, which is consistent with the theory that α-GDH is composed of at least two and a maximum of 3 identical

Figure 9. A reconstructed peptide map of a trypsin digest of reduced carboxymethylated α-GDH. Chromatography was carried out in the vertical direction using butanol: acetic acid: water (4:1:5) as solvent. Electrophoresis was performed in the horizontal direction at 2000 volts for 100 minutes. Peptides 4, 8 and 13 contain tyrosine; 9, 13, 14, 19 and 22 histidine; and peptides 3, 8, 11, 15 and 16 contain arginine.


TRYPTIC PEPTIDES OF RM α-GDH
CHROMATOGRAPHY
ORIGIN
ELECTROPHORESIS
⊖
⊕
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22

polypeptide chains. It seems likely that extensive tests of other chromatographic solvent systems would have revealed conditions for better separation and thus yielded more peptides. Since it is likely that some peptides are very similiar and were not separated by the conditions used in the fingerprinting and due to the limited nature of the experiments, the number of peptides detected by this method will be a minimum.

Subunit Structure of α-glycerophosphate Dehydrogenase

As stated in the introduction, a principal objective of this research was to determine the number of polypeptide chains present in the native moleculae of α-GDH. This first phase of the research involved finding a dissociation system which produced stable subunits. When this was successfully accomplished, a complete physical characterization of the α-GDH subunits was undertaken. This included determination of the sedimentation coefficients, the diffusion coefficient and the molecular weight of the α-GDH subunits by both sedimentation equilibrium and by combination of the sedimentation and diffusion coefficients.

Attempted Dissociation of α-GDH in Urea

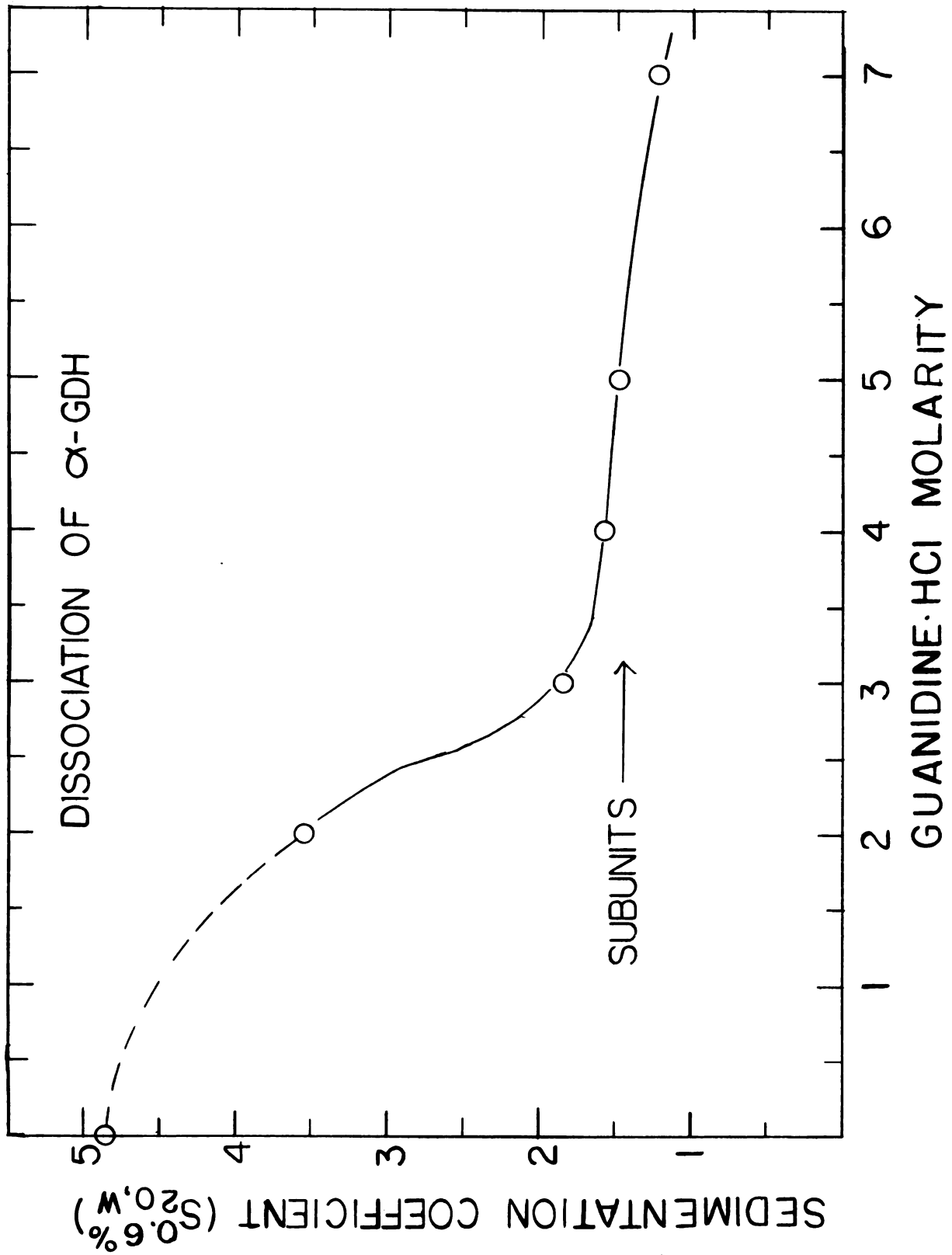
The results obtained in the carboxy terminal and in the fingerprinting analysis of α-GDH suggested that α-GDH was composed of two, or at most three, subunits. The next objective was to find conditions which would dissociate the enzyme into subunits. It had been shown previously by Deal *et al.* (1963) that the exposure of α-GDH to acid

(0.01M citrate, pH2.6) was ineffective as a means of dissociating the protein. Since urea is a convenient and inexpensive reagent which is often successful in dissociating proteins, its dissociating ability was tested on α-GDH. α-GDH in 8M urea aggregated even with the presence of the reducing agent mercaptoethanol(0.1M). This made accurate conclusions from the sedimentation equilibrium experiments impossible and necessitated consideration of other dissociating agents.

Effect of Guanidine·HCl Concentration on the $s_{20,w}$ of α-GDH

From the previous section the need for a denaturing agent stronger than 8M urea was obvious. Since guanidine·HCl was the strongest protein denaturing agent available, it was tested for dissociation ability. Preliminary experiments indicated that 7M guanidine·HCl dissociated α-GDH into stable subunits, if 0.1M mercaptoethanol was present. The experiments which follow were designed to determine the minimum guanidine·HCl concentration at which dissociation occured. For this experiment the sedimentation coefficients of α-GDH in various concentrations of guanidine·HCl were determined (see Figure 10); the solutions also contained 0.1M mercaptoethanol. The protein concentration was 6 mg/ml for these experiments; further experimental details are given in the legend for Figure 10. Evaluation of the experiments at guanidine·HCl concentrations of 0.5M and 1M was impossible because the enzyme pre-

Figure 10. Effect of guanidine·HCl concentration on the sedimentation coefficient of α-GDH. The solvent used in all experiments was 0.1M Tris·HCl, 0.2M NaCl, 0.001M EDTA, 0.1M mercaptoethanol, pH 7.45, plus the indicated concentration of guanidine·HCl. All experiments were done at 5-7° and the protein concentration was 6 mg/ml. The samples were dialyzed for a minimum of 12 hours. The rotor speed setting was 59,780 rpm. Viscosities of the guanidine·HCl solutions were determined from the data of Kawahara and Tanford (1966) taking into account the additional viscosity due to the salts and the mercaptoethanol.


DISSOCIATION OF α-GDH
SUBUNITS
SEDIMENTATION COEFFICIENT ($S^{0.6\%}_{20,w}$)
GUANIDINE·HCl MOLARITY
1
2
3
4
5
6
7

cipitated from solution under these conditions. The sedimentation coefficient of α-GDH in 2M guanidine·HCl was $s^{0.6\%}_{20,w} = 3.6S$, which was a significant decrease from the corresponding value of $s^{0.6\%}_{20,w} = 4.2S$ for the native enzyme. The nature of the 3.6S species is not known but it may be due to a slightly unfolded native molecule or may represent a rapid equilibrium mixture consisting of the native enzyme and the subunit. Dissociation of α-GDH occured at concentrations of guanidine·HCl 3M and above. The slow decrease in sedimentation coefficients in the guanidine·HCl range of 3 to 7M suggested that some unfolding of the enzyme continued to occur as the guanidine·HCl concentration was increased.

$s^{o}_{20,w}$, $D^{o}_{20,w}$, and $M^{o}_{w}(s/D)$ of the α-GDH Subunit

The next phase of the research involved determination of the molecular weight of α-GDH by two independent methods, namely (1) sedimentation equilibrium and (2) combination of sedimentation and diffusion coefficient data, using the Svedberg equation. The two series of experiments were carried out simultaneously and used the same stock subunit solution; the series of equilibrium experiments is described in the next section. Although α-GDH dissociated in 3M guanidine·HCl the earlier experience with the pronounced tendency of the enzyme to aggregate provided a convincing argument for the use of higher concentrations of guanidine·HCl than 3M to avoid aggregation. Therefore these two series of experiments

were performed in 7.2M guanidine·HCl with 0.1M mercaptoethanol also present. A series of sedimentation velocity experiments at different protein concentrations was conducted in the dissociating system described previously. Extrapolation of the apparent sedimentation coefficients to zero protein concentration yielded a value of $s^{o}_{20,w}$ = 1.70S(see Figure 11).

From a similiar series of experiments at different protein concentrations the diffusion coefficient of dissociated α-GDH was also measured so its molecular weight could be determined by combination of sedimentation and diffusion data. A value for the diffusion coefficient of $D^{o}_{20,w} = 4.1(\pm 0.2) \times 10^{-7} cm^2/sec$ was obtained upon extrapolation of the apparent diffusion coefficients to zero protein concentration(see Figure 12). The reasons for the scatter of the diffusion coefficients were not obvious; but as a result of the scatter, the value for the diffusion coefficient was not very accurate. Using the value of $s^{o}_{20,w}$ = 1.70S for the sedimentation coefficient and the value of $D^{o}_{20,w} = 4.1 \times 10^{-7} cm^2/sec$ for the diffusion coefficient a subunit molecular weight of $M^{o}_{w}(s/D)$ = 40,000 $\pm$ 2,000 was calculated. This value for the molecular weight is approximately one-half the value of $M^{o}_{w}(s/D)$ = 74,400 for native α-GDH. These data suggested that α-GDH consisted of two polypeptide chains. The sedimentation equilibrium molecular weight analysis of the subunits described in the next section complements

Figure 11. Extrapolation to zero protein concentration of the sedimentation coefficients of α-GDH subunits in guanidine mercaptoethanol. The protein was dialyzed for 24 hours against 7.2M guanidine·HCl, 0.2M NaCl, 0.1M mercaptoethanol, 0.001M EDTA, and 0.1M Tris·HCl, pH 7.45. The experiments were performed at 5° with a rotor speed setting of 59,780 rpm.

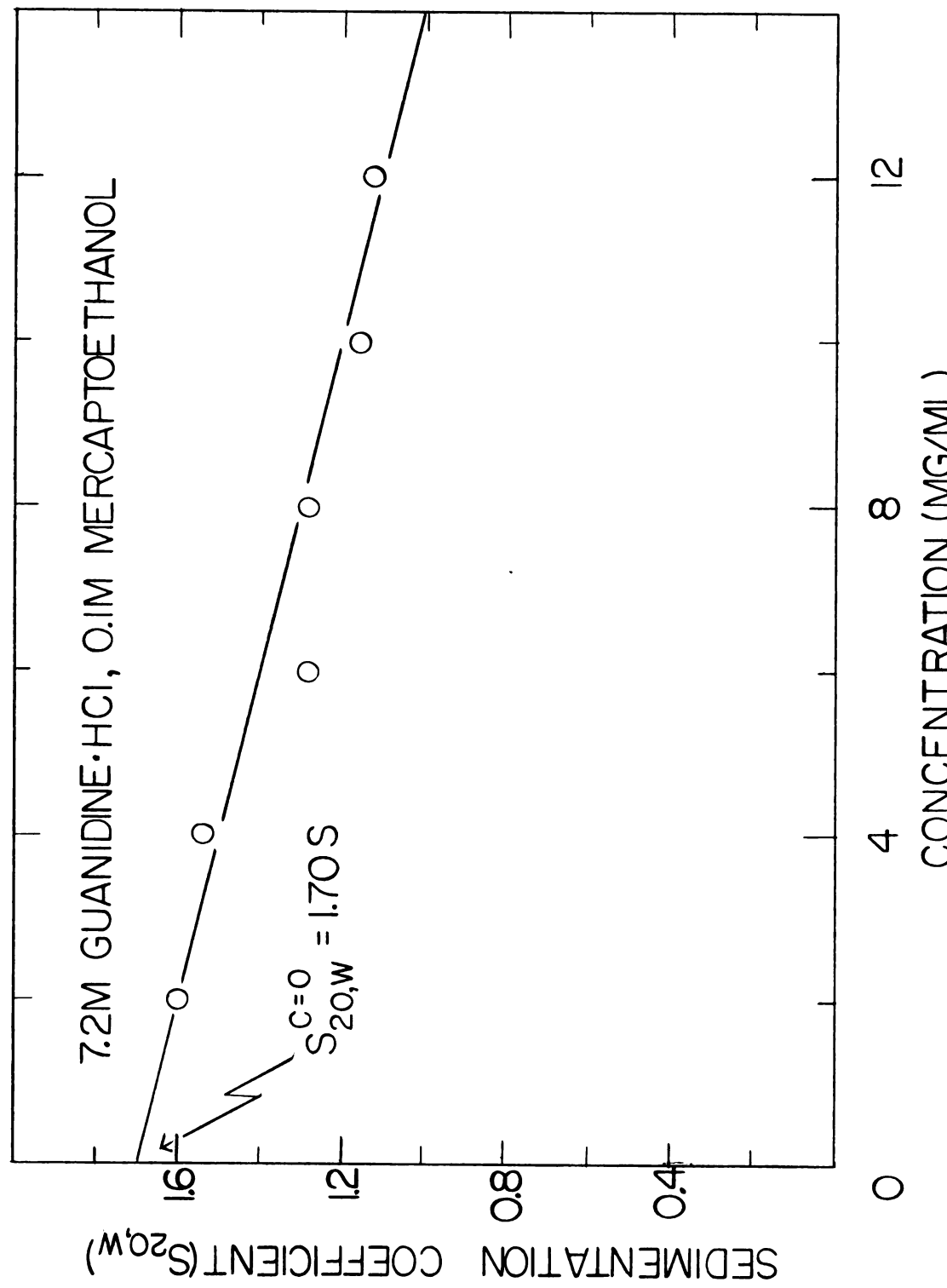
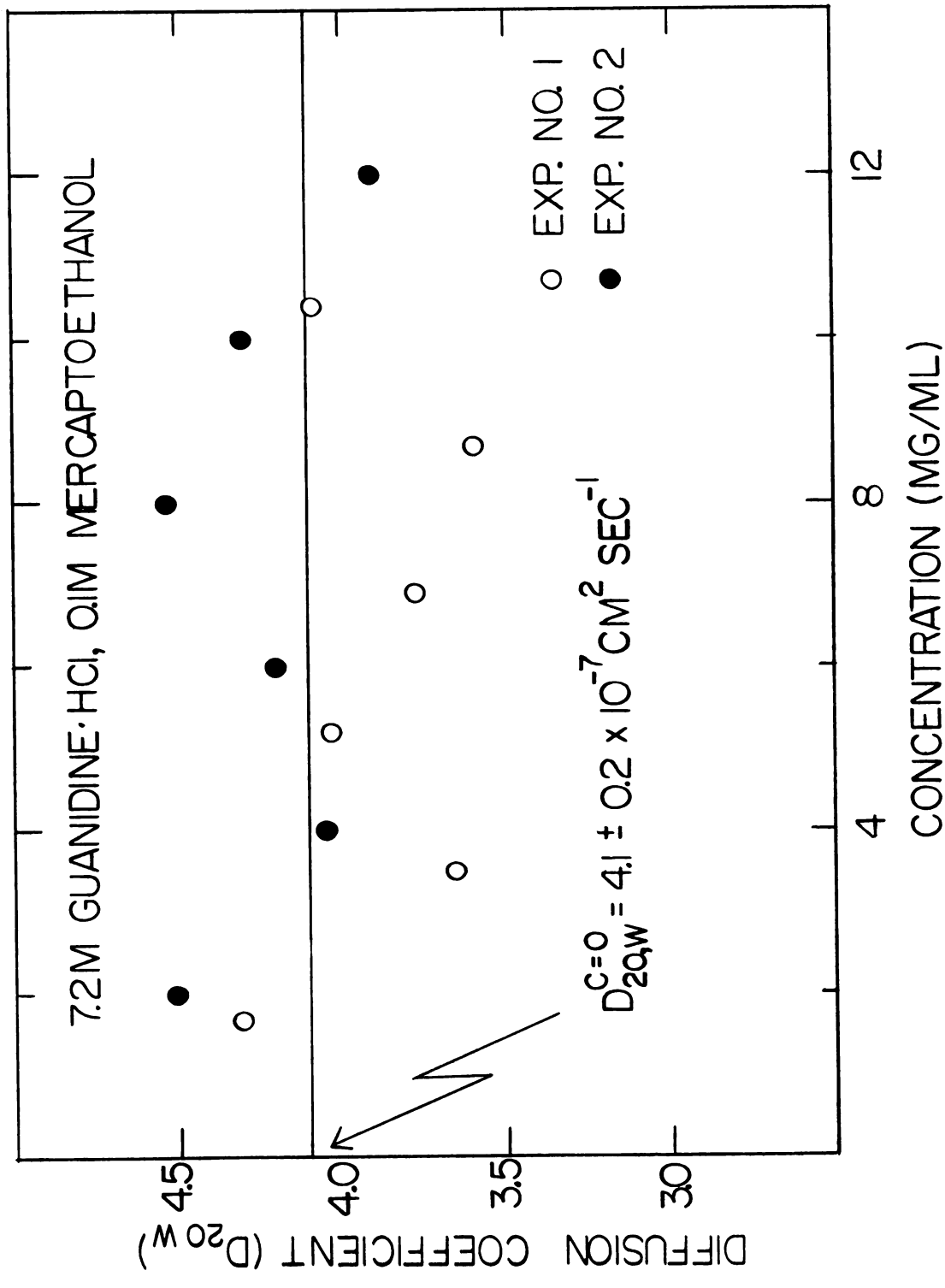

7.2M GUANIDINE·HCl, 0.1M MERCAPTOETHANOL
$S^{c=0}_{20,w}$ = 1.70 S
SEDIMENTATION COEFFICIENT ($S_{20,w}$)
1.6
1.2
0.8
0.4
0
4
8
12
CONCENTRATION (MG/ML)

Figure 12. Extrapolation to zero protein concentration of the diffusion coefficients of α-GDH subunits in guanidine·HCl, mercaptoethanol. The diffusion experiments were performed in 7.2M guanidine·HCl, 0.2M NaCl, 0.1M Tris·HCl, 0.001M EDTA, 0.1M mercaptoethanol, pH 7.45 at a speed of 5,200 rpm and a temperature of 5°. The closed and open circles refer to two separate series of experiments.


7.2M GUANIDINE·HCl, 0IM MERCAPTOETHANOL
○ EXP. NO. 1
● EXP. NO. 2
$D_{20,w}^{C=0} = 4.1 \pm 0.2 \times 10^{-7} CM^2 SEC^{-1}$
DIFFUSION COEFFICIENT ($D_{20,w}$)
4.5
4.0
3.5
3.0
4
8
12
CONCENTRATION (MG/ML)

the results described above by providing another independent measure of the subunit molecular weight.

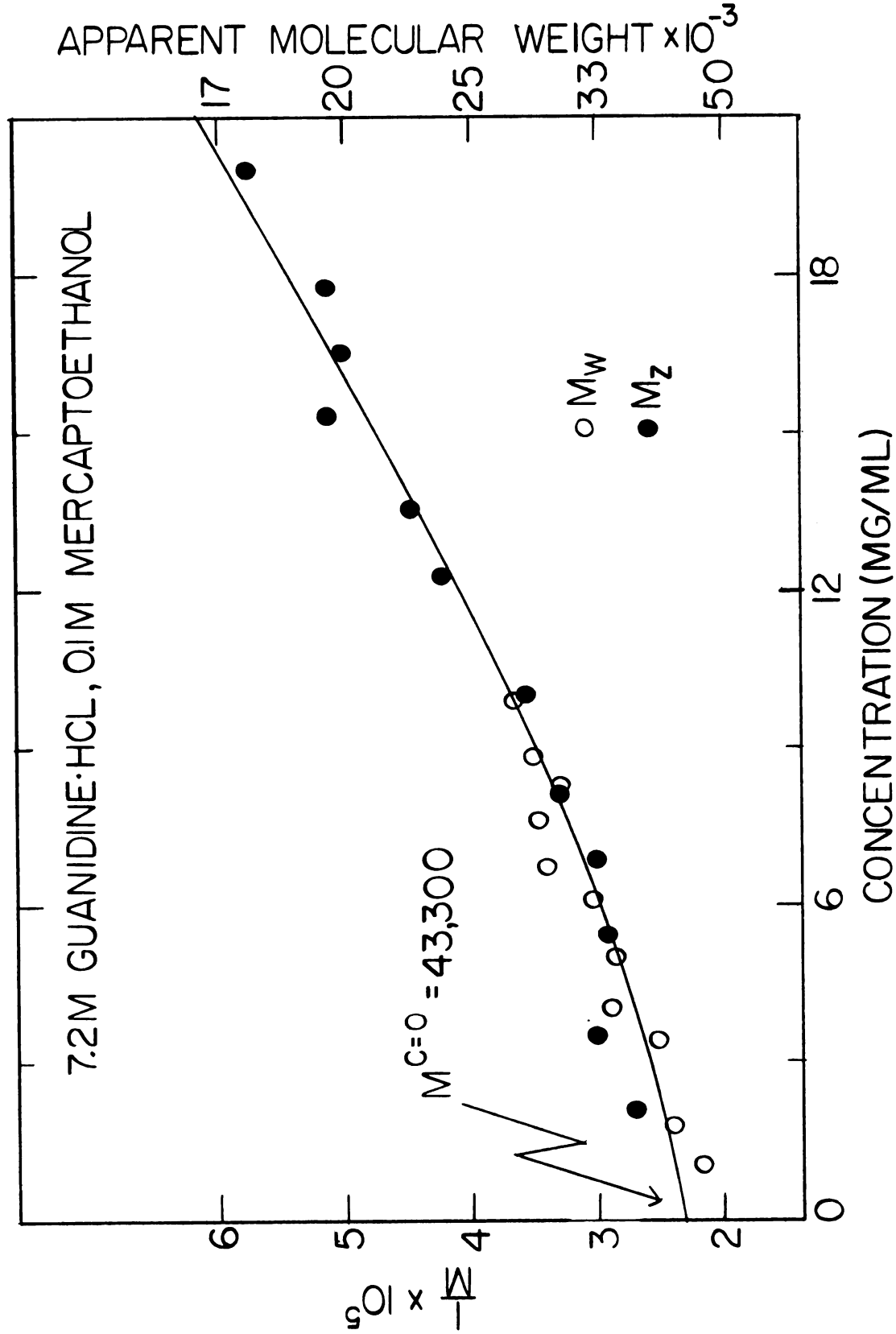
Sedimentation Equilibrium Studies of α-GDH Subunits

As mentioned earlier a series of sedimentation equilibrium experiments in 7M guanidine·HCl, 0.1M mercaptoethanol was simultaneously conducted with the sedimentation and diffusion experiments presented in the previous section. The same stock enzyme subunit solution was used for both. The results of the sedimentation equilibrium experiments are shown in Figure 13. A molecular weight of 43,300 was obtained by extrapolation of apparent weight average(M_w) and z-average molecular weights to zero protein concentration. This value is slightly greater than the value of 40,000 based on the sedimentation and diffusion coefficients; the equilibrium technique is somewhat more sensitive to aggregation than the other technique. However the individual schlieren patterns gave no suggestion of aggregation, suggesting that the aggregation was not pronounced. Further proof that there was not a great deal of aggregation was given by the fact that the z-average molecular weights lay on the same curve as the weight average molecular weights(see Figure 13).

The Requirement for Mercaptoethanol

Having obtained a reliable value for the subunit molecular weight, the next question was whether the mercaptoethanol was necessary to obtain stable subunits when the guanidine·HCl concentration was as great as 7M.

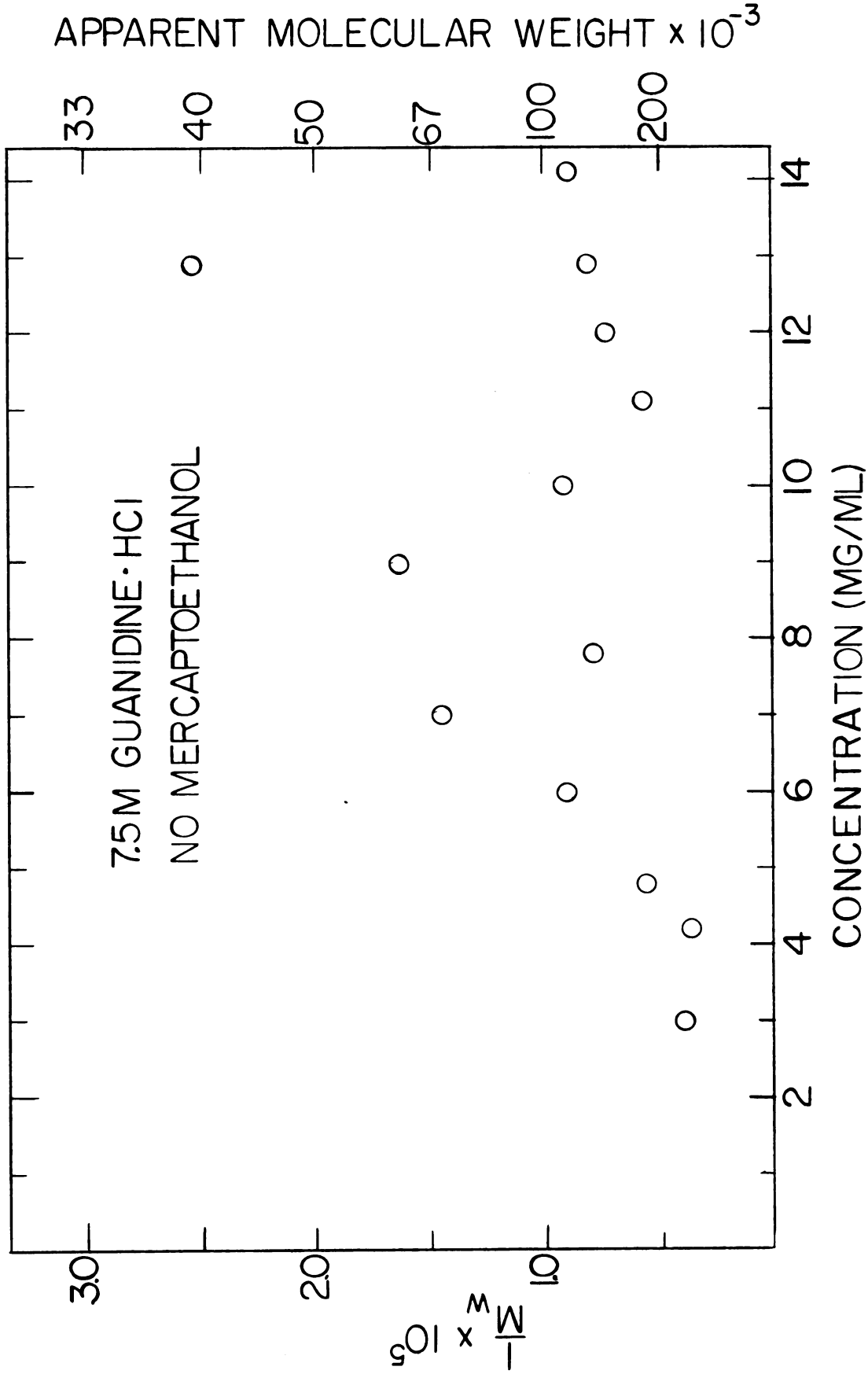
Figure 13. Extrapolation to zero protein concentration of the apparent weight-average(M_w) and z-average (M_z) molecular weights of α-GDH subunits in quanidine·HCl, mercaptoethanol. Apparent molecular weights were determined from sedimentation equilibrium experiments conducted at 5° with a rotor speed setting of 23,150 rpm. Concentrations were evaluated as $(C_m + C_b)/2$ for M_w and as $(C_m + C_b)$ for M_z. The protein was dialyzed for 24 hours prior to sedimentation equilibrium analysis against 7.2M guanidine·HCl, 0.2M NaCl, 0.1M Tris·HCl, 0.001M EDTA, 0.1M mercaptoethanol, pH 7.45. The initial concentration(C_o) of the stock solution was obtained by averaging the values obtained from synthetic boundary experiments performed on alternate samples. A partial specific volume of 0.746 cc/g was used for the calculations.


APPARENT MOLECULAR WEIGHT x10⁻³
17
20
25
33
50
7.2M GUANIDINE·HCL, 0.1M MERCAPTOETHANOL
○ M_w
● M_z
$M^{c=0}$ = 43,300
$\frac{1}{M} \times 10^5$
6
5
4
3
2
0
6
12
18
CONCENTRATION (MG/ML)

To answer this question a series of experiments was conducted in a manner identical to that just described, with the exception that mercaptoethanol was omitted from the dissociation solvent. With one single exception, the samples were very aggregated and gave molecular weights in the range of 100,000 to 250,000(see Figure 14). In the one case where dissociation occured the "subunits" were unstable and a repeat experiment conducted after several days yielded a schlieren pattern comparable with that of the other samples. At first appearance, this might seem to indicate the presence of disulfide bonds in the enzyme; but this does not explain the pronounced aggregation which occured. The aggregation made it impossible to tell whether dissociation had occured and was followed by aggregation, or whether the enzyme had simply aggregated without dissociating. The existence of the one "subunit" suggested that the former was the case. If this be the case, a further conclusion is that the sulfhydryl groups of α-GDH in an unfolded state, such as they experience in 7.2M guanidine·HCl, are extremely reactive, and that the presence of a reducing agent is necessary to prevent formation of disulfide bonds.

In order to further evaluate the possiblity that dissociation might occur in guanidine·HCl alone, sedimentation velocity experiments were performed on two

Figure 14. Extrapolation to zero protein concentration of the apparent weight average(M_w) molecular weights of α-GDH in in Guanidine·HCl with no mercaptoethanol. Apparent molecular weights were determined from sedimentation equilibrium experiments conducted a 5° and with a rotor speed setting of 12,600 rpm. Concentrations were evaluated as $(C_m + C_b)/2$. The protein was dialyzed for 24 hours prior to sedimentation equilibrium analysis against 7.5M guanidine·HCl, 0.2M NaCl, 0.1M Tris·HCl, 0.001M EDTA, pH 7.50. Mercaptoethanol was not present in the dissociating media. The initial concentrations(C_o) of the stock solution was obtained by averaging the values obtained from synthetic boundary experiments performed on the samples containing 15, 14, 13, 12 and 11 mg/ml.

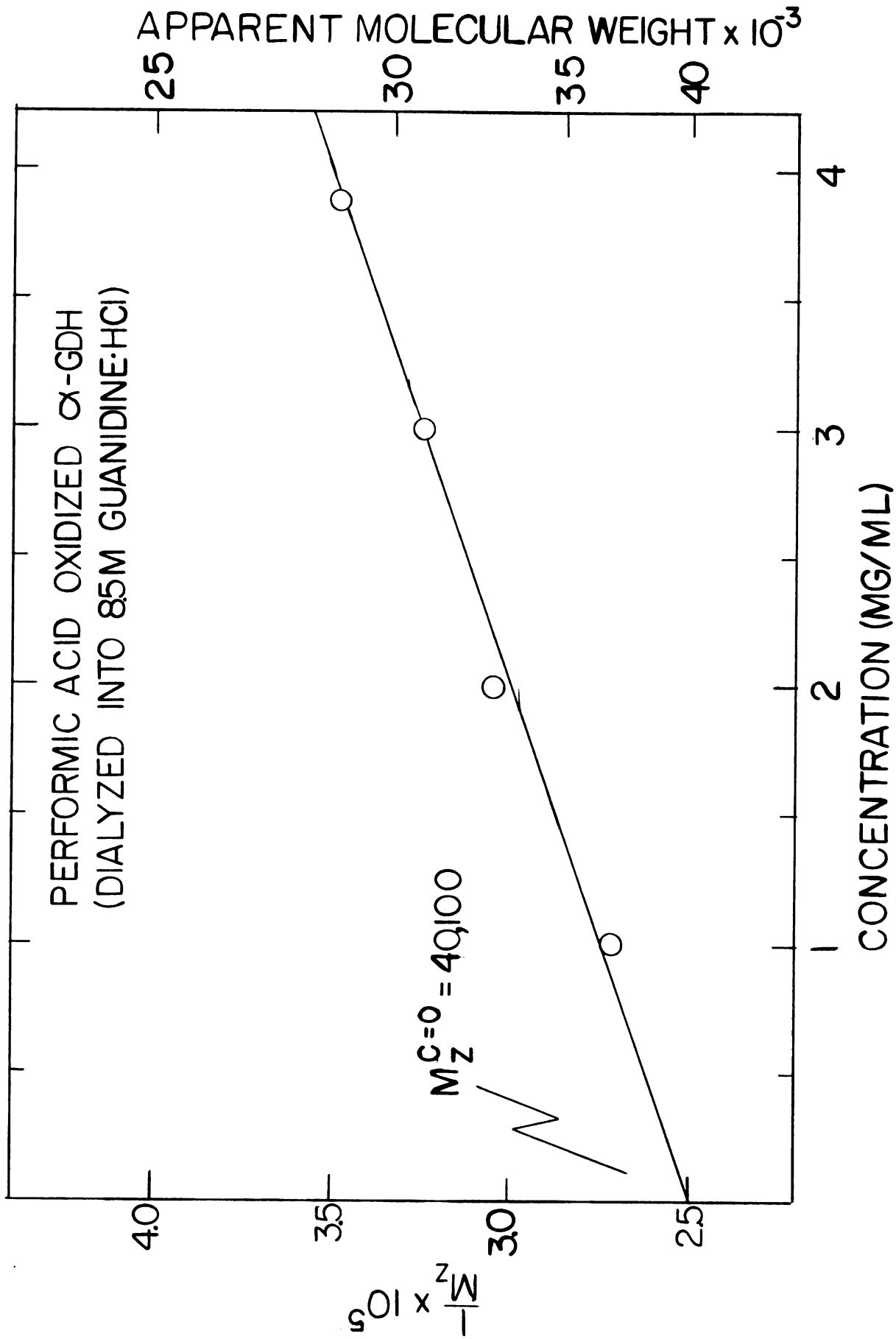

APPARENT MOLECULAR WEIGHT x 10^{-3}
33
40
50
67
100
200
7.5M GUANIDINE·HCl
NO MERCAPTOETHANOL
CONCENTRATION (MG/ML)
2
4
6
8
10
12
14
$\frac{1}{M_w} \times 10^5$
3.0
2.0
1.0

samples of α-GDH in 8.0M guanidine.HCl, 0.2M NaCl, 0.04M Tris.HCl, 0.001M EDTA, pH 7.42 at an enzyme concentration of 3 mg/ml. One of the samples contained 0.1M mercaptoethanol, while the other did not. There was no difference in the sedimentation coefficients of the reduced and unreduced samples; both had a value of $s^{0.3\%}_{20,w} = 1.57S$. These results suggested that dissociation occured in both cases and that in the mercaptoethanol system the reactive sulfhydryl groups remained reduced, while in the absence of mercaptoethanol they reacted to form random disulfide bonds.

Performic Acid Oxidation of α-GDH

Since α-GDH dissociated in the presence of 7.2M guanidine.HCl, 0.1M mercaptoethanol and aggregates occurred if the mercaptoethanol was not included, an experiment was designed where the formation of disulfide bonds was not possible. This experiment involved mild oxidation of α-GDH with performic acid. This process oxidized both cystine and cysteine to cysteic acid and oxidized methionine to methionine sulfone without cleavage of peptide bonds (Hirs, 1959). After oxidation, the protein was prepared for sedimentation equilibrium experiments by dialyzing against 7.5M guanidine.HCl, 0.2M NaCl, 0.04M Tris.HCl, 0.001M EDTA, pH 8.3 for 24 hours. The extrapolation of the z-average molecular weights to zero protein concentration (see Figure 15) yields a M_z =

Figure 15. Extrapolation to zero protein concentration of the z-average molecular weights(M_Z) performic acid-oxidized α-GDH. The enzyme was oxidized with performic acid and then dialyzed for 24 hours against 7.5M guanidine, 0.2M NaCl, 0.04M Tris·HCl, 0.001M EDTA, pH 8.3 prior to sedimentation epuilibrium analysis to remove the oxidizing reactants. Experiments were run at 24,630 rpm at 25°. A partial specific volume of 0.746 cc/g was used for the calculations.


APPARENT MOLECULAR WEIGHT x 10⁻³
25
30
35
40
PERFORMIC ACID OXIDIZED α-GDH
(DIALYZED INTO 8.5M GUANIDINE·HCl)
$M_z^{c=0} = 40{,}100$
$\frac{1}{M_z} \times 10^5$
4.0
3.5
3.0
2.5
1
2
3
4
CONCENTRATION (MG/ML)

40,100, which confirms the value of $M_w^o(s/D) = 40,000$ found with dissociated reduced α-GDH. This experiment thus showed that, for subunit studies, the best procedure for avoiding aggregation was to use performic acid oxidized enzyme. The question of whether disulfide bonds existed in the native protein still remained unanswered. The following experiment was designed to resolve this dilema; it conclusively demonstrated that the subunits in the native enzyme are not linked to one another by disulfide bonds.

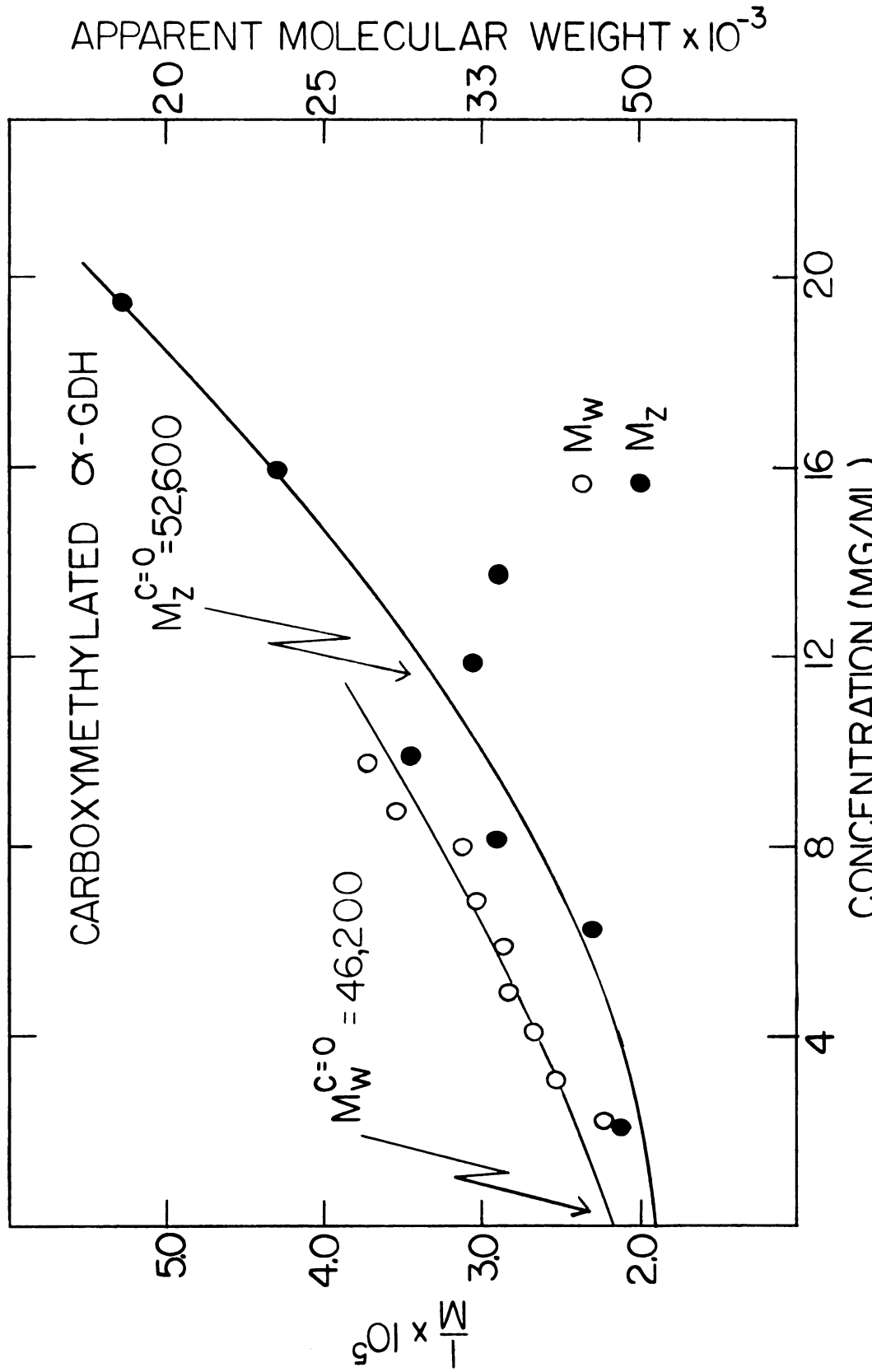
Molecular Weight of Unreduced, Carboxymethylated α-GDH

If the aggregation of unreduced α-GDH in guanidine·HCl was due to the oxidation of sulfhydryl groups to disulfide bonds, it seemed possible that it might be prevented by dissolving the protein in a carboxymethylating dissociation solvent; i.e. a solvent containing the iodoacetic acid in the dissociation solvent before the native enzyme is dissolved in it. This is in contrast to the usual procedure of dissolving the enzyme in a solvent containing guanidine·HCl only, and then adding the iodoacetic acid to this solution. With this simultaneous carboxymethylation dissociation the sulfhydryl groups would become carboxymethylated as soon as they became exposed to the media and therefore they could not form disulfide bonds. The subunit samples for this series of sedimentation equilibrium experiments were prepared by dissolving the ammonium sulfate pellet containing native α-GDH in a dissociation carboxymethylating

solvent containing 7.5M guanidine·HCl and 0.2M iodoacetic acid(for other details see legend of Figure 16). Extrapolation of the weight-average and z-average molecular weights to zero protein concentration gave molecular weights of 46,200 and 52,600 respectively. This indicated that dissociation had been achieved with this enzyme despite the fact that it was not reduced. Although slightly higher molecular weight values were obtained for the subunits, there was no doubt that this molecular weight value represented subunits and was slightly high because of aggregation. The individual sedimentation equilibrium schlieren patterns showed a small amount of aggregate at the bottom of the cell. Additional evidence for the existence of aggregated protein is shown by inspection of Figure 16 where the z-average molecular weights are greater than the weight average molecular weights at corresponding protein concentrations. As a result the weight average molecular weight of 46,200 is expected to be high. Indeed based on previous experience with aggregation, it was predicted that some aggregation would occur.

It would be difficult to overestimate the importance of the results from this series of experiments. These results directly, clearly, and conclusively prove that the polypeptide chain subunits of native α-glycerophosphate dehydrogenase are not linked by disulfide bonds.

Figure 16. Extrapolation to zero protein concentration of the apparent weight average(M_w) and z-average (M_z) molecular weights of α-GDH simultaneously carboxylmethylated and dissociated. Apparent molecular weights were determined from sedimentation equilibrium experiments conducted at 5° and with a rotor speed setting of 25,980 rpm. Concentrations were evaluated as $(C_m + C_b)/2$ for M_w and as $(C_m + C_b)$ for M_z. The protein was dissolved in 7.5M guanidine·HCl, 0.14M Tris·HCl, 0.003M EDTA, 0.2M iodoacetic acid, pH 8.5. After 45 minutes the protein was dialyzed against 7.5M guanidine·HCl, 0.2M NaCl, 0.1M Tris·HCl, 0.001M EDTA, pH 7.50 for 24 hours. The initial concentration(C_o) of the stock solution was obtained by averaging the values obtained from synthetic boundary experiments performed on samples containing,10, 9, 8, 7, and 6 mg/ml.


APPARENT MOLECULAR WEIGHT x 10⁻³
20
25
33
50
CARBOXYMETHYLATED α-GDH
$M_Z^{c=0}$=52,600
$M_W^{c=0}$ = 46,200
o M_W
● M_Z
$\frac{1}{M}$ x 10^5
5.0
4.0
3.0
2.0
4
8
12
16
20
CONCENTRATION (MG/ML)

Summary of the Molecular Weight Analysis of the α-GDH Subunit

Table VII gives a summary of the molecular weight analysis of the subunits of α-GDH

Table VII. Summary of the molecular weight analysis of the α-GDH subunit

Treatment	Molecular Weight M_w^o	$M_w^o(s/D)$	M_z^o
7.2M guanidine·HCl 0.1M mercaptoethanol	43,300	40,000	43,300
Performic acid oxidized			40,100
7.5M guanidine·HCl	40,000 to 250,000		
carboxymethylation in 7.5M guanidine·HCl	46,200		

Renaturation of α-GDH

Upon dissociation of α-GDH into stable subunits it was of interest to see if active enzyme could be reformed by removal of the dissociating agent. It was hoped that the knowledge gained from these experiments would yield information concerning the conditions necessary for the folding of the newly synthesized polypeptide chains into active enzyme molecules. Therefore a study was undertaken to determine the conditions necessary for maximum recovery of enzyme activity. These conditions included pH, protein concentration, the presence of the substrates

and products, and the effect of reducing agents.

Preliminary attempts to reverse the dissociation of α-GDH by removing the dissociation agent were immediately successful. When an aliquot of denatured α-GDH was diluted directly into the assay mixture there was a lag of a few seconds followed by a dramatic increase in enzymatic activity as the enzyme recovered its catalytically active conformation. The following experiments were then designed to determine conditions necessary for a maximum recovery of catalytic activity. The enzyme was prepared for analysis by dissolving a pellet of native α-GDH, centrifuged from an ammonium sulfate suspention, into the dissociating solvent which contained the following: 7.5M guanidine·HCl, 0.1M Tris·HCl, 0.001M EDTA, pH 7.46. The final protein concentration was 1 mg/ml. This stock dissociated enzyme was allowed to sit in this dissociation mixture for 2 hours. The denatured protein was diluted (1:40) from this stock solution into tubes containing 1 ml of the reversal solvent. The optimum reversal conditions were: 0.1M Tris·HCl, 0.2M mercaptoethanol, 0.001M EDTA, pH 7.42 and a final protein concentration of 0.025 mg.ml. It is important to note that mercaptoethanol was not included in the reversal solvents since its effect was discovered later. After 15 minutes at room temperature 10 µl of the renatured enzyme was assayed in a volume of 0.3 ml. For the experiments to to follow, all variables (except mercaptoethanol) were at

optimum conditions except the one under study. In order to accurately determine the % recovery, the activity of the stock enzyme solution was determined with each series of experiments. Although the specific activity of the native enzyme varied from one stock solution to another, the relative percent did not. It was constant for a given condition, regardless of the specific activity of the stock enzyme. The native enzyme control was treated exactly as the dissociated sample with the single exception that the dissociating agent was not present. All dilutions of the denatured enzyme into the reversal media were performed in duplicate.

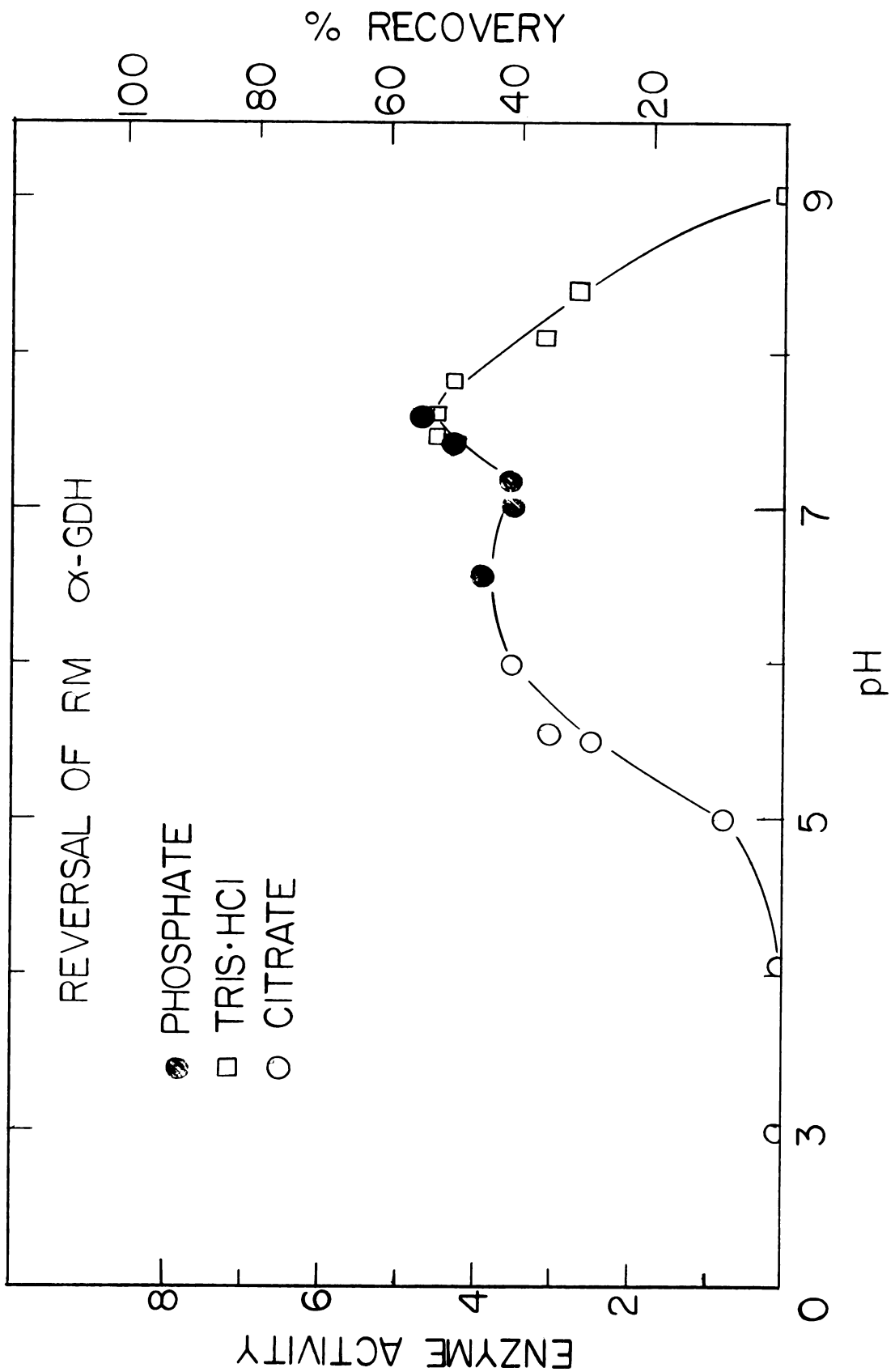
Effect of pH on Renaturation

Experiments were designed to examine the effect of the pH of the renaturation solvent on the degree of renaturation. As shown in Figure 17 the amount of renaturation is very dependent on pH; two maxima were obtained at pH's 6.8 and 7.5. At pH 7.5 the activity recovery was in the range of 55%. The amount of renaturation did not appear to be dependent on the type of buffer used. For example, at pH 7.4 the amount of recovery obtained with a phosphate buffer and that obtained with a Tris·HCl buffer differed by only 2%.

Effect of Protein Concentration on Renaturation

As previously mentioned it was suspected that the concentration of protein in the renaturation media might

Figure 17. Effect of pH on renaturation of -GDH. The protein (1 mg/ml) was denatured for 2 hours in 7.5M guanidine·HCl, 0.1M Tris·HCl, 0.1M mercaptoethanol, 0.001M EDTA, pH 7.50. The enzyme was diluted into buffers of various pH ranging from 3.0 to 9.0. After 15 minutes at room temperature a portion of the enzyme was assayed for enzymatic activity (see text for experimental details). Specific activity is plotted on the left ordinate and % recovery is plotted on the right ordinate.


% RECOVERY
100
80
60
40
20
REVERSAL OF RM α-GDH
PHOSPHATE
TRIS·HCl
CITRATE
ENZYME ACTIVITY
0
2
4
6
8
pH
3
5
7
9

affect the amount of recovery of enzymatic activity. The results of a series of experiments where the concentration of protein in the reversal mixture was varied are shown in Figure 18. This experiment was designed so that the amount of guanidine•HCl in the renaturing mixture was always constant. A maximum recovery of 55% was obtained at 0.025 mg/ml. Renaturation at concentrations of 0.1 mg/ml and higher was not possible, for the protein precipitated immediately upon dilution into the renaturation media.

Effect of Metabolites on Renaturation

Since it has been shown by many investigators(Chilson et al., 1965; Hill et al., 1964; Beychok et al., 1959) that coenzymes or substrates may have an effect on the amount of renaturation, the effects of the α-GDH substrates and products were tested. With the exception of α-glycerophosphate, none of the substrates or products had any effect on the degree of renaturation in the concentration range tested (1×10^{-6} M to 1×10^{-2} M). The results of a series of experiments in which the concentration of α-GP was varied from 1×10^{-7} M to 3×10^{-2} M are shown in Figure 19. It is seen that at concentrations of α-GP greater than 5×10^{-5} M the renaturation of α-GDH is inhibited; for example, only 30% recovery of enzyme activity was observed at a α-GP concentration of 3×10^{-3} M. The possibility that lower

Figure 18. Effect of protein concentration upon renaturation. A stock solution of enzyme (50 mg/ml) was allowed to denature for 2 hours in 7.5M guanidine·HCl, 0.1M Tris·HCl, 0.001M EDTA, o.1M mercaptoethanol, pH 7.46. An aliquot was than diluted into 1.0 ml of 0.1M Tris·HCl, 0.001M EDTA, pH 7.43 to give the desired protein concentration. After 15 minutes a portion of the enzyme was assayed. Measured amounts of guanidine.HCl were added to each reversal sample in order that the final guanidine·HCl concentration was the same in all cases. Renaturation was performed at room temperature. Specific activity is plotted on the left ordinate and % recovery is plotted on the right ordinate.

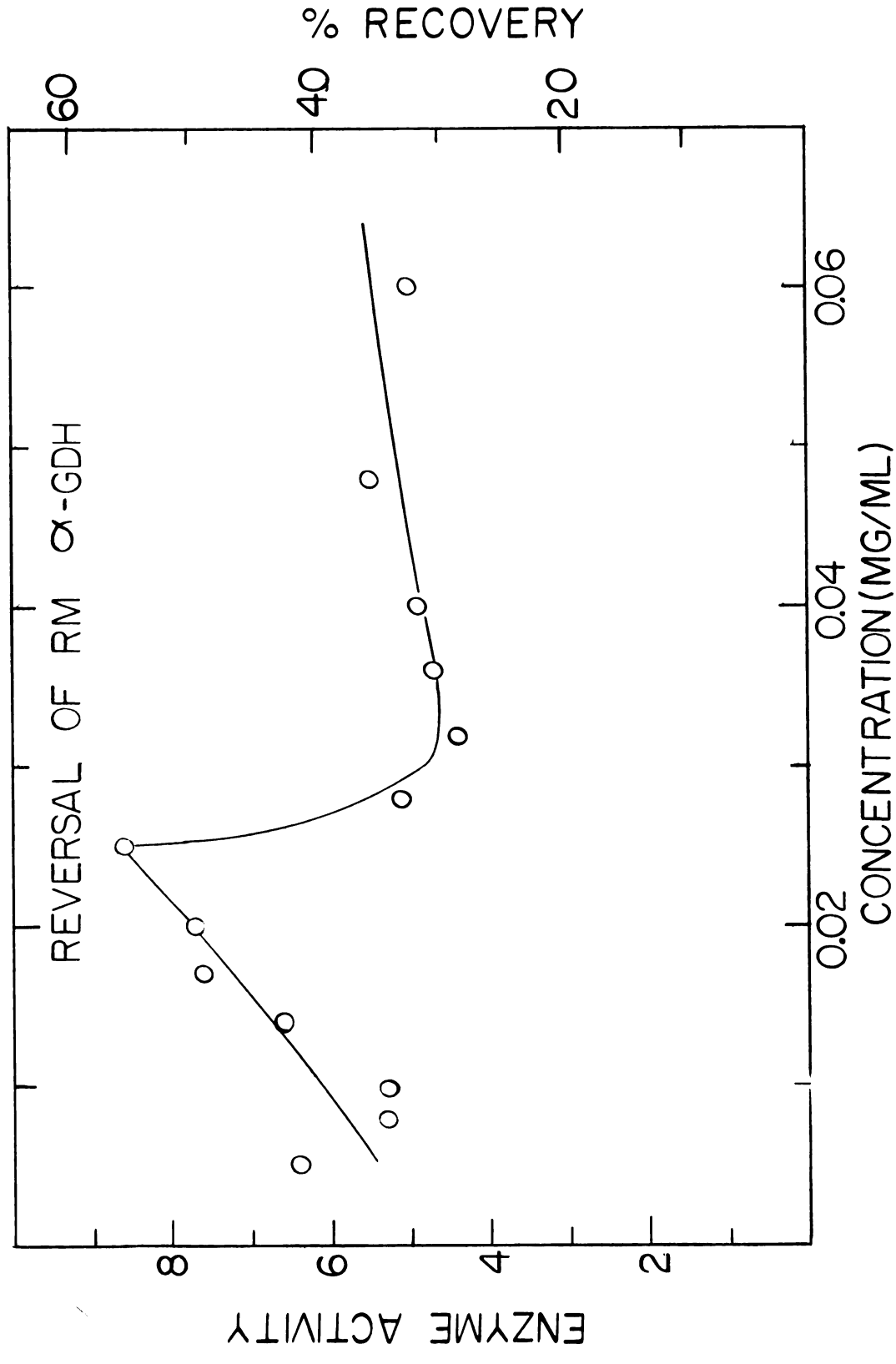
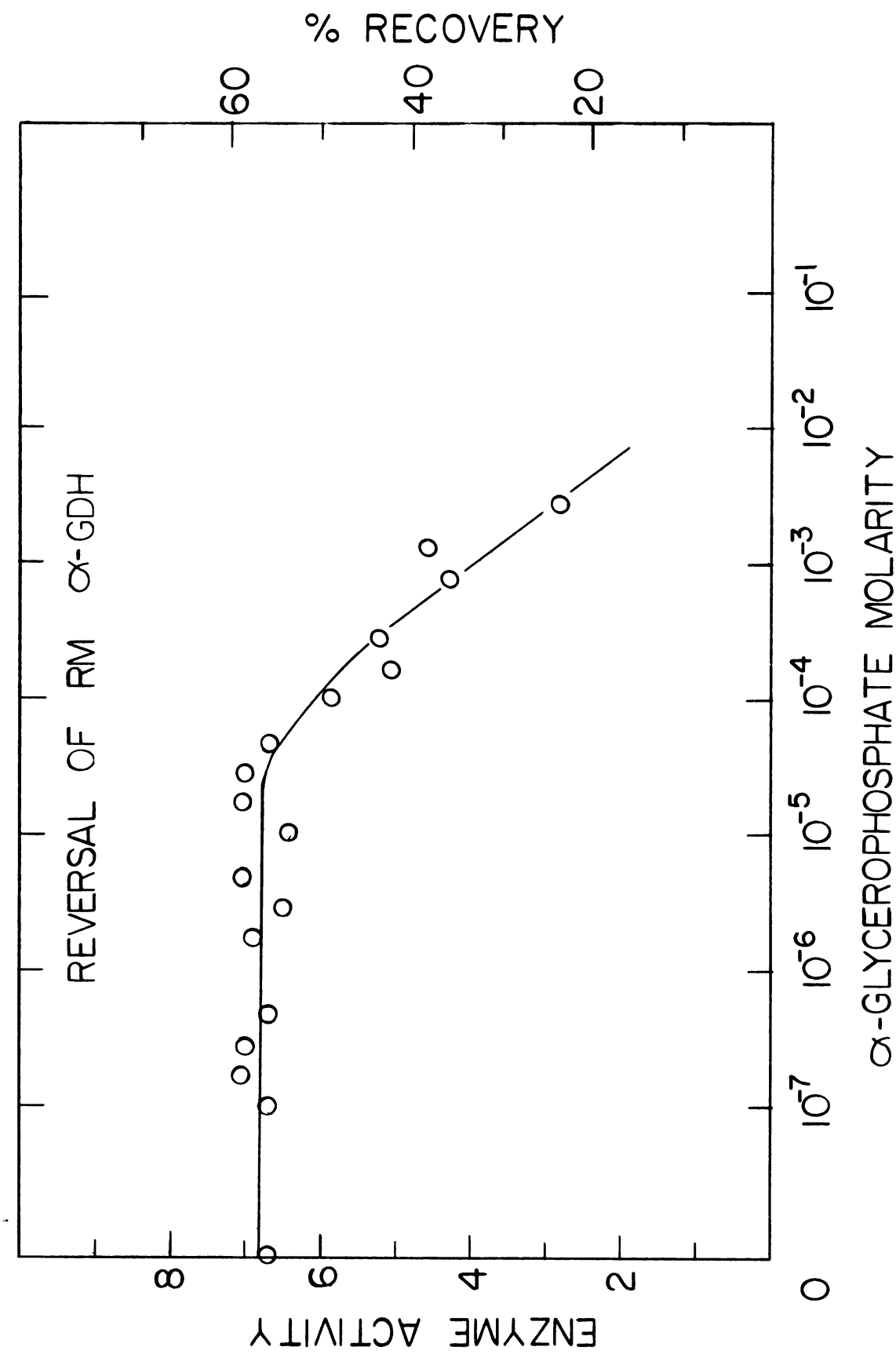

% RECOVERY
60
40
20
REVERSAL OF RM α-GDH
ENZYME ACTIVITY
8
6
4
2
0.02
0.04
0.06
CONCENTRATION (MG/ML)

Figure 19. Effect of α-glycerophosphate upon renaturation. α-GDH, denatured in 7.5M guanidine·HCl, 0.001M EDTA, 0.1M Tris·Hcl, pH 7.42, 0.1M mercaptoethanol for two hours was diluted into 0.1M Tris·HCl, 0.001M EDTA, pH 7.42 which contained the designated concentration of α-GP. The final protein concentration was 0.025 mg/ml. After 15 minutes at room temperature, 10 lambda of the renatured enzyme was diluted into 0.3 ml of assay media. Specific activity is plotted on the left ordinate and % recovery is plotted on the right ordinate.


% RECOVERY
60
40
20
REVERSAL OF RM α-GDH
ENZYME ACTIVITY
8
6
4
2
0
10^{-7}
10^{-6}
10^{-5}
10^{-4}
10^{-3}
10^{-2}
10^{-1}
α-GLYCEROPHOSPHATE MOLARITY

recovery of catalytic activity might be due to product inhibition of active enzyme in the assay had to be considered. This could occur for example as a result of the contribution of α-GP from the reversal sample to the total α-GP concentration in the assay. Since the dilution of the reversal mixture into the assay mixture is 1:30, the assay mixture concentration of α-GP will be 1/30 that of the reversal mixture. For example, a concentration of 3×10^{-3} M α-GP in the renaturation mixture will yield a concentration of 1×10^{-4} M in assay mixture. Thus, from these experiments the only conclusion possible was that α-GP was inhibiting; (1) either the refolding of the dissociated enzyme in the reversal mixture or, (2) the expression of activity of active enzyme in the assay.

Product Inhibition of α-GDH by α-glycerophosphate

In order to distinguish between the two possibilites presented by the previous results the following experiment was performed. The effect of 1×10^{-3} M α-GP was measured on two samples; (1) α-GDH was renatured in the presence of α-GP, (2) native α-GDH activity was assayed in the presence of αGP. In the former case the concentration of α-GP in the assay mixture was 3×10^{-5} M while it is 1×10^{-3} M in the latter case. Examination of Table VIII shows that inhibition was greater when α-GDH was reversed in 1×10^{-3} M than when α-GDH was assayed at the same α-GP concentration. Two conclusions could

Table VIII. Effect of α-GP on α-GDH

	Concentration of α-GP in assay	% inhibition
Renatured[a] enzyme	3×10^{-5} M	40 (inhibition of reversal)
Native enzyme	1×10^{-3} M	30

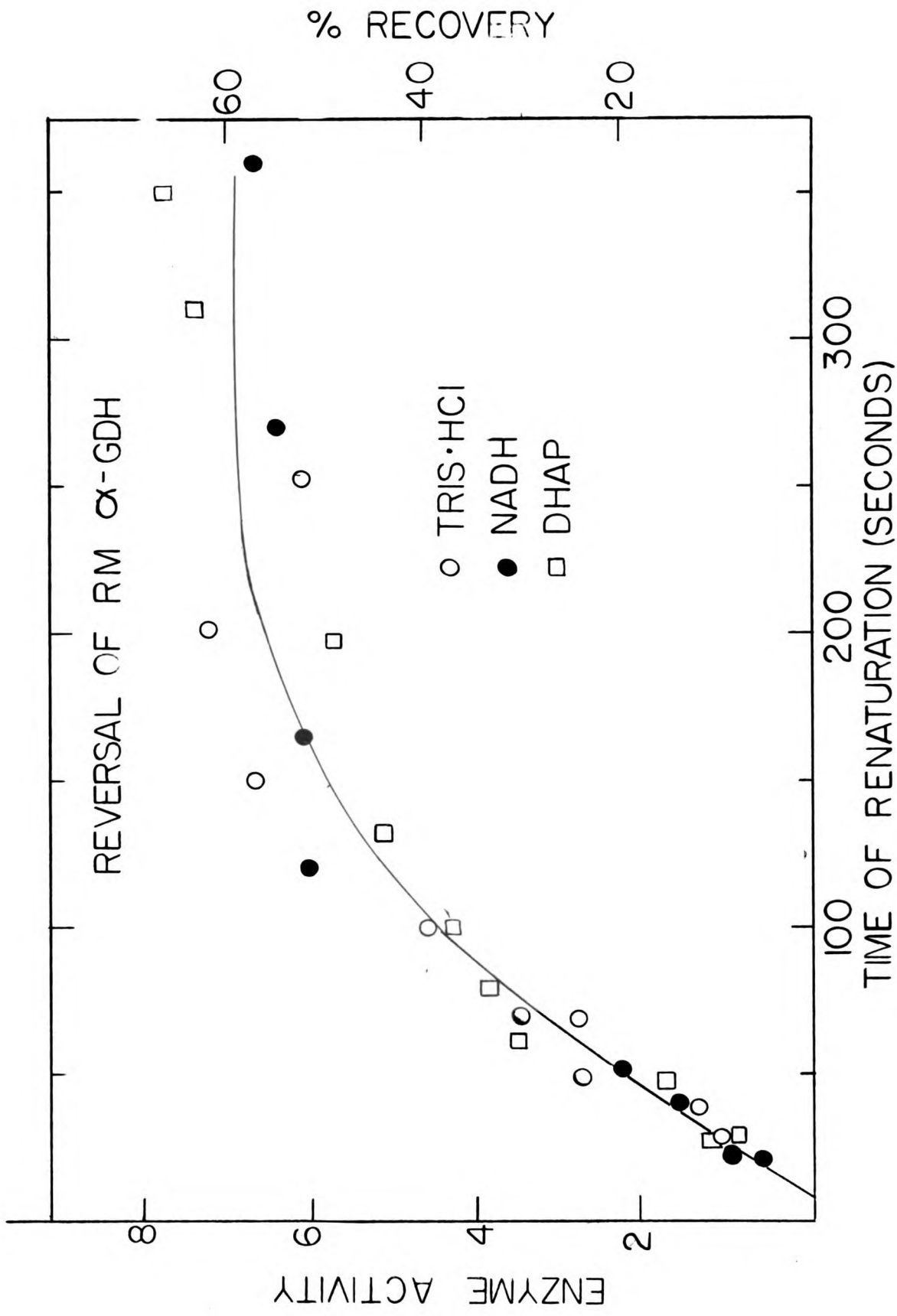
[a]Renatured in the presence of 1×10^{-3} M α-GP. Therefore α-GP concentration in the assay was 3×10^{-5} M.

be drawn from these experiments, (1) 1×10^{-3} M α-GP inhibited the refolding of the dissociated enzyme and (2) 1×10^{-3} M α-GP inhibited the native enzyme.

Kinetics of Renaturation in the Presence of NADH or DHAP

Although the substrates and products of the α-GDH reaction showed no affect on the extent of renaturation, it was possible that they might effect the rate of renaturation. To test for this effect, the amount of recovery of enzymatic activity after various periods of time of incubation in the reversal mixture was measured. α-GDH was dissolved in 7.5M guanidine·HCl, 0.1M Tris·HCl, 0.001M EDTA, 0.001M mercaptoethanol, pH 7.52 to give a protein concentration of 1 mg/ml. After two hours in this solvent 25 µl was diluted into 1.0 ml of 0.1M Tris·HCl, 0.001M EDTA, pH 7.42. Aliquots of 10µl were withdrawn after the indicated time period and assayed for enzymatic activity. Examination of Figure 20 suggests that the presence of NADH or

Figure 20. Effects of NADH or DHAP upon the renaturation of α-GDH. The protein was denatured for 2 hours in 7.5M guanidine·HCl, 0.1M Tris.HCl, 0.001M EDTA, pH 7.42. For the studies of renaturation in the presence of NADH or DHAP, these substances were present at 2.5×10^{-4}M and 1×10^{-3}M respectively. After the indicated time interval a portion of the renatured enzyme was assayed. Specific activity is plotted on the left ordinate and % recovery is plotted on the right ordinate.


% RECOVERY
60
40
20
REVERSAL OF RM α-GDH
○ TRIS·HCl
● NADH
□ DHAP
100
200
300
TIME OF RENATURATION (SECONDS)
ENZYME ACTIVITY
8
6
4
2

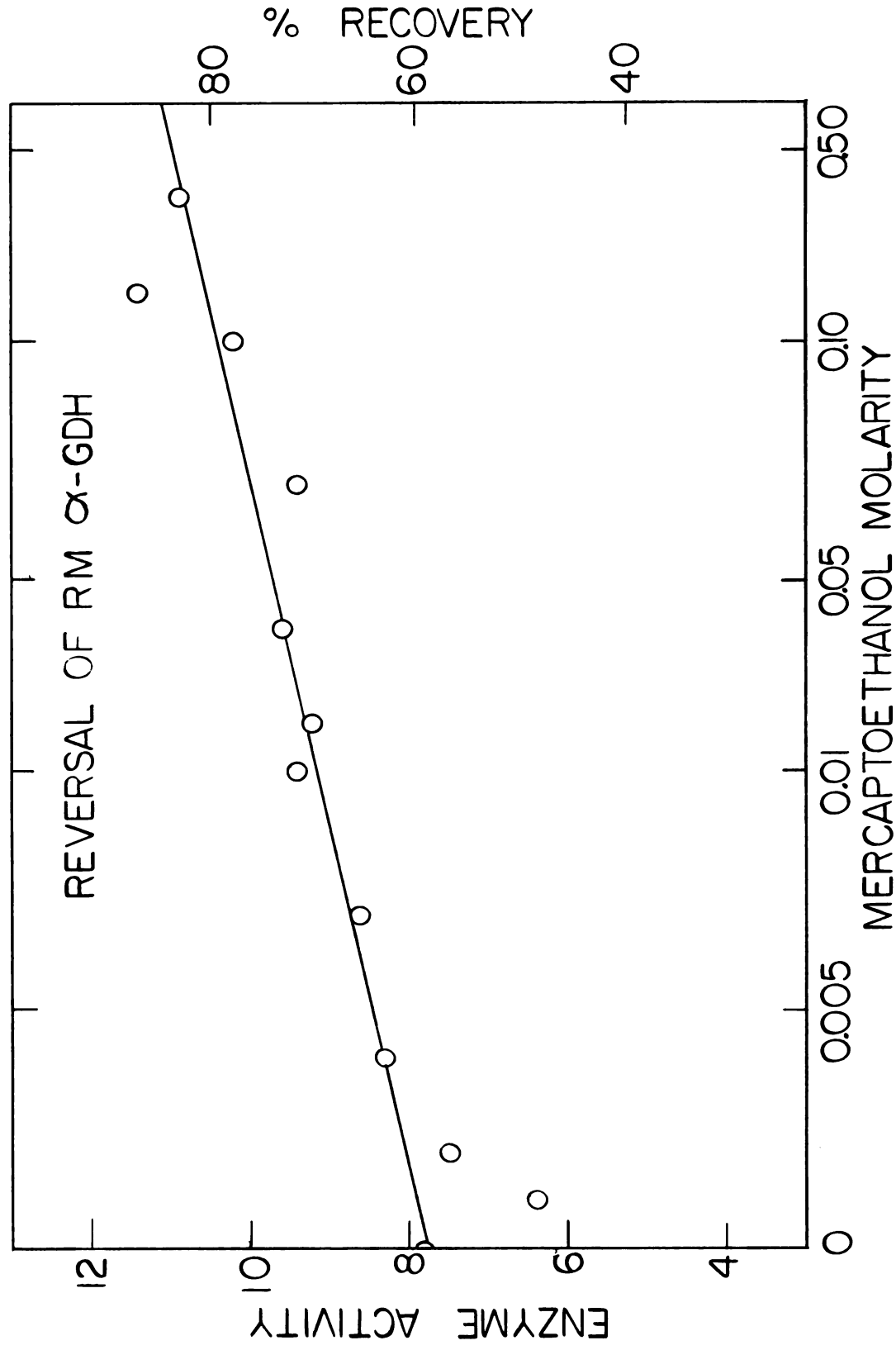
DHAP had no effect on the rate of renaturation. Maximum recovery was in the range of 60%. The half life of renaturation, i.e., the time required for 50% recovery, was in the vicinity of 70 seconds.

Renaturation in Mercaptoethanol

It was of interest to test the effect of mercaptoethanol on reversal for two reasons. First it was thought that the presence of mercaptoethanol in the renaturing mixture might prevent aggregation thus yielding a greater degree of recovery. Secondly, if renaturation could be accomplished in the presence of mercaptoethanol, indirect proof would be obtained for the absence of disulfide bonds in the catalytic unit. Figure 21 illustrates the dramatic effect that mercaptoethanol had on the extent of α-GDH renaturation. The extent of renaturation increased with increasing mercaptoethanol concentration, with an approximate recovery of 90% being obtained at 0.2M mercaptoethanol. This increase in activity was not due to an activation of the native protein, for the enzymatic activity of native α-GDH did not increase when incubated with 0.2M mercaptoethanl.

The ability of the enzyme to renature in the presence of 0.2M mercaptoethanol provides further indirect evidence that disulfdie bonds are not necessary for catalytic activity of the enzyme.

Figure 21. Renaturation of α-GDH in the presence of various concentrations of mercaptoethanol. The protein was denatured for two hours prior to the renaturation experiments in 7.5M guanidine·HCl, 0.1M Tris·HCl, 0.001M EDTA, 0.1M mercaptoethanol at pH 7.50. An aliquot was diluted into 1.0 ml of 0.1M Tris·HCl, 0.001M EDTA, pH 7.42 which contained the indicated amounts of mercaptoethanol. Renaturation was performed at room temperature. Specific activity is plotted on the left ordinate and % recovery is plotted on the right ordinate.


% RECOVERY
80
60
40
REVERSAL OF RM α-GDH
ENZYME ACTIVITY
12
10
8
6
4
0
0.005
0.01
0.05
0.10
0.50
MERCAPTOETHANOL MOLARITY

DISCUSSION

In this investigation the values for the following physical properties of native rabbit muscle α-glycerophosphate dehydrogenase were obtained: $s^{o}_{20,w}$ = 4.86S, $D^{o}_{20,w}$ = 6.20 x $10^{-7}cm^2/sec$, f_o/f = 1.23, $\bar{v}$ = 0.746 cc/g and $M^{o}_{w}(s/D)$ = 74,400. Van Eys et al. (1959) also measured these quantities and found the following values, $s^{o}_{20,w}$ = 4.9S, $D^{o}_{20,w}$ = 5.1 x $10^{-7}cm^2/sec$, f_o/f = 1.44, $\bar{v}$ = 0.70 cc/g and $M^{o}_{w}(s/D)$ = 78,000. Young and Pace (1958a) found a value of 0.70 cc/g for $\bar{v}$, and a value for f_o/f of 1.4. Thus the same values were obtained by these groups for $s^{o}_{20,w}$ and $M^{o}_{w}(s/D)$, but different values were obtained for f_o/f, $D^{o}_{20,w}$, and $\bar{v}$.

While the value for f_o/f is somewhat secondary in interest it is necessary to know the correct $D^{o}_{20,w}$ and $\bar{v}$ values, since both affect the molecular weights.

The diffusion coefficient will be considered first. If the diffusion coefficient values obtained in this research are compared with typical results for other globular proteins (see Figure 6) with known molecular weights and diffusion coefficients, it is seen that our value of $D^{o}_{20,w}$ = 6.20 x $10^{-7}cm^2/sec$ lies on the empirical curve (---) expected for a protein of molecular weight ca. 75,000 while the value of Van Eys et al. (1959) of $D^{o}_{20,w}$ = 5.1 x $10^{-7}cm^2/sec$ falls far below the line. This suggests that the diffusion coefficient value of Van Eys et al. (1959) may be in error. Pur-

suing this further, it is of interest that the value of $s^{o}_{20,w}$ = 4.9S found by both groups does lie near the empirical line (---) for the sedimentation coefficients of a globular protein of molecular weight 75,000 (see Figure 5). Thus, our values for both $s^{o}_{20,w}$ and $D^{o}_{20,w}$ lie on the empirical line for typical globular proteins while the Van Eys et al. (1959) value for the $s^{o}_{20,w}$ lies on the typical line but the $D^{o}_{20,w}$ value does not.

As mentioned before, the value of f_o/f measured by Van Eys et al. (1959) is greater by 20% than that expected for most globular proteins (Tanford, 1961) thus raising the possibility that this value is also in error. This 20% variation in f_o/f is directly attributable to the low value of the diffusion coefficient, for f_o/f is directly related to the diffusion coefficient as seen below.

$$D = \frac{kt}{f} \qquad \text{and} \qquad \frac{D}{D_o} = \frac{f_o}{f}$$

where the subscript, $_o$, refers to a sphere, k is Boltzmann's constant, t is absolute temperature and f is the frictional coefficient. Now, f_o/f is also dependent on $\bar{v}$ but this dependence is small since f_o/f depends on the 1/3 power of $\bar{v}$ (see appendix). Thus the use of either of the two values for $\bar{v}$ (0.70 and 0.746 cc/g) would give an f_o/f differing by less than 2%.

If we assume that the correct value of $D^{o}_{20,w}$ is 6.20 x 10^{-7} cm^2/sec and use the values of $s^{o}_{20,w}$ and $\bar{v}$ calculated by Van Eys et al. (1959) a value of ca. 64,000 is calculated for the molecular weight of native α-GDH.

The fact that the value of 0.70 cc/g found by Van Eys et al. (1959) and indicated by the data of Young and Pace (1958a) is probably in error is suggested by the following; (1) the value of 0.70 cc/g is lower than that expected for typical globular proteins which have $\bar{v}$ values in the range 0.73 to 0.74 cc/g (Tanford, 1961) and (2) using this value for $\bar{v}$ and the correct value for $D^{o}_{20,w}$ a molecular weight of 64,000 is obtained for α-GDH which is much lower than expected for a protein with a sedimentation coefficient of 4.9S (see Figure 5), and (3) the value of 64,000 in conjunction with the subunit molecular weight of 40,000 does not yield a whole number for the the number of polypeptide chains present in α-GDH.

There are three reasons for the confidence placed in the value of 0.746 cc/g obtained in this work for the $\bar{v}$ of α-GDH; (1) extensive direct measurement (falling drop method) and empirical calculations (based on amino acid composition) yielded identical results, (2) the value of 0.746 cc/g is close to that expected for globular proteins and (3) the use of 0.746 cc/g in molecular weight calculations gave values for the native enzyme and for subunits which yielded an integral number of subunits in the enzyme. There is no obvious explanation for the discrepancy in our results and those of Van Eys et al. (1959) and of Young and Pace (1958a). One possible explanation is the limited scope of the experiments of Van Eys et al. (1959) and the unusual conditions (1.0M ammonium sulfate) used by Young and Pace (1958a).

The precise value used for the partial specific volume in the guanidine•HCl has a large effect on the molecular weight results because of the high solvent density(7M guanidine•HCl = 1.195). There are two problems which occur in the use of the correct value for the $\bar{v}$ for the enzyme, (1) whether the $\bar{v}$ is the same for the folded(native) and unfolded(subunits) protein molecule and (2) if there is preferential binding of one of the solutes(water or guanidine•HCl) to the protein molecule.

The results reported in the literature are conflicting as to the effect of guanidine•HCl on partial specific volume. Kielley and Harrington (1960) reported that the $\bar{v}$ of myosin in 5M guanidine•HCl decreased by 0.01 cc/g relative to the value obtained for the native protein in aqueous salt solution. Marler et al.(1960) showed similiar effect for γ-globulin. On the other hand, Reithel and Sakura(1963) have found little change in $\bar{v}$ for a number of proteins. Calculation of the $\bar{v}$ of a protein by summation of the partial specific volume contributions of the individual amino acids (Cohn and Edsall, 1943) would seem to us to be the best approximation of the $\bar{v}$ for an unfolded protein. The fact that the $\bar{v}$ obtained from amino acid analysis is identical to the experimentally determined value argues for the conclusion that the partial specific volume of unfolded α-GDH is the same as that for the native molecule.

Schachman and Edelstein(1966) have made calculations for the preferental interaction of water with the denatured

protein and found a substantial contribution of water to the partial specific volume of aldolase.

It was assumed in the calculations used in this report that the partial specific volume was the same for the native and unfolded protein and therefore no corrections were made for the effect of guanidine·HCl on the partial specific volume of α-GDH.

The chemical and physical evidence presented here is compatible with the theory that α-GDH is composed of two polypeptide chains. This was supported by the following experiments: (1) the molecular weight of the subunit obtained from sedimentation and diffusion experiments is approximately one half that of the native enzyme($M_w^o(s/D) = 74,400$, (2) digestion of α-GDH with carboxypeptidase-A released 2.0 moles of methionine per mole of protein.

The question of whether the polypeptide chains of α-GDH were held together by disulfide bonds was a very perplexing problem which was solved only after great difficulty. The presence of disulfide bonds in the native protein was suggested in several ways: (1) the necessity of 0.1M mercaptoethanol in the dissociating mixture in order to observe stable subunits, (2) the production of stable subunits when the protein was oxidized with performic acid, (3) failure to obtain the maximum number of carboxymethyl cysteines when the enzyme was carboxymethylated but not reduced. There are two obvious explanations for these results: (1) disulfide bonds are an integral part of the native α-GDH molecule or (2) disulfide bonds are not an integral part of the native

molecule but are formed upon treatment and handling of the enzyme.

Fortunately, evidence was obtained which conclusively proved that the latter was the case and that disulfide bonds were not an an integral part of the native molecule. This experiment involved dissolving the unreduced protein in a dissociating-carboxymethylating solvent, which prevented sulfhydryl groups from being oxidized to disulfide bonds. Under these conditions dissociation did occur. A second proof for the absence of disulfide bonds in the catalytic unit was the observation that the dissociation process could be reversed in the presence of 0.2M mercaptoethanol.

SUMMARY

(1) It has been shown that native α-GDH has the following properties.

a. $D^{o}_{20,w} = 6.20 \times 10^{-7} cm^2/sec$ (new value: cf. Van Eys et al., 1959)

b. $s^{o}_{20,w} = 4.86S$ (confirming Van Eys et al., 1959)

c. $f_o/f = 1.23$ (new value: cf Van Eys et al., 1959)

d. $\bar{v} = 0.746$ cc/g (new value: cf Van Eys et al., 1959; Young and Pace, 1958a)

e. $M^{o}_{w}(s/D) = 74,400$

(2) A partial specific volume of 0.746 cc/g was found for the native enzyme by two independent methods; (a) indirectly from the amino acid composition and (b) by direct measurement using the falling drop method.

(3) The values obtained for the $s^{o}_{20,w}$ and $D^{o}_{20,w}$ fell on a line drawn for typical globular proteins of known molecular weights.

(4) There are 21 moles of half-cystine per mole of protein; determined as carboxymethyl cysteine and as cysteic acid

(5) Two moles of C-terminal methionine were found per mole of α-GDH, showing that α-GDH is composed of two subunits.

(6) Fingerprinting of carboxymethylated α-GDH suggests that α-GDH is composed of a maximum of three identical polypeptide chains.

(7) The complete amino acid analysis has been performed (confirming Van Eys et al., 1964)

(8) The enzyme dissociates into subunits in 7.2M guanidine·HCl, 0.1M mercaptoethanol. The subunits have the following properties.

a. $s^{o}_{20,w} = 1.70S$

b. $D^{o}_{20,w} = 4.1 \times 10^{-7} cm^2/sec$

c. $M^{o}_{w}(s/D) = 40,000$

d. M^{o}_{w} (sedimentation equilibrium) = 43,300. This value is high due to slight aggregation

(9) The enzyme aggregates if mercaptoethanol is not included in the dissociating solvent.

(10) The inclusion of iodoacetic acid in the dissociating solvent results in the production of subunits thus proving that the subunits are not linked by disulfide bonds.

(11) Reversal of the dissociating process is accomplished upon dilution of the dissociating agent.

a. A maximum recovery of 90% is obtained.

b. The optimal conditions for renaturation are 0.1M Tris·HCl, pH 7.42, 0.2M mercaptoethanol, at 25° at a final protein concentration of 0.025 mg/ml.

c. NADH, NAD^{+}, DHAP and d-GP do not affect renaturation

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APPENDIX

Table I. Values for the diffusion and sedimentation coefficients used in Figures 5 and 6

Protein	Molecular Weight	$s_{20,w}$	$D_{20,w}$	Reference
RNAse	13,683	1.64	11.9	Smith, E., Moore, S., and Stein, W., J. Biol. Chem., 238, 227(1963).
Papain	20,700	2.42	10.27	Smith, E., Kimmel, J., and Brown, D., J. Biol. Chem., 207, 533(1964).
Chymotrypsinogen	24,744	2.54	9.5	Schwert, G.W., J. Biol. Chem., 190, 799(1951).
β-Lactoglobulin	35,000	2.83	7.82	Cecil, R., Ogston, A., J. Biochem. 44, 33(1949).
Ovalbumin	44,000	3.55	7.76	Tanford, C., Buzzell, J., Rands, D., and Swanson, S., J. Am. Chem. Soc. 77, 6421(1955).
Hemoglobin	64,450	4.31	6.90	Schroeder, W., Ann. Rev. Biochem., 32, 301(1963).
α-glycerophosphate dehydrogenase	74,400	4.86	6.30	See this thesis
α-amylase	96,900	6.47	5.72	Fischer, E.H., Proc. 4th Int. Congress Biochem. 8, 124(1958).
Enolase	82,000	5.7	6.3	Winstead, J.A., and Wold, F., Biochemistry, 4, 2145(1965).
Aldolase	150,000	7.9	4.63	Tanford, C., Biochemistry, 5, 1578(1966). Schachman and Edelstein (1966).
Fumarase	194,000	9.04	4.01	Kanarek, L., Hill, R.L., J. Biol. Chem. 239, 4207(1964)
Pyruvate kinase	240,000	10.04	3.96	Steinmetz, M.A., and Deal, W.C., Biochemistry, 5, 1399(1966).
Xanthine oxidase	290,000	11.4	3.6	Avis, P.G., J. Chem. Soc., 1212(1956).
Catalase	225,000	11.3	4.5	Samejima, T., and Shibata, K., Arch. Biochem. Biophys., 93, 407(1961).
Lactic Dehydroganase	135,000	7.0	5.3	Nielands, J.B., J. Biol. Chem., 199, 373 (1952).
Phosphorylase	495,000	13.2	2.6	Keller, P.J. and Cori, G.T., Biochim. Biophys. Acta, 12, 235(1953).

The theoretical values for the sedimentation coefficients of spheres as a function of of molecular weight(see Figure 5) were calculated in the following manner.

The frictional coefficient(f) for a sphere is given by Stokes Law as,

$$f_o = 6\pi\eta r_o \qquad 1)$$

η = viscosity of solvent at 20°, in this case water(0.0100 poise)

r = radius of a sphere

subscribt, $_o$, refers to a sphere

The radius of a sphere is defined as,

$$r_o = \left(\frac{V_o 3}{\pi 4}\right)^{1/3} \qquad 2)$$

V_o = volume of sphere

The volume of a sphere of a given molecular weight is given as,

$$V = \frac{\bar{v} M}{N} \qquad 3)$$

$\bar{v}$ = partial specific volume

M = molecular weight of sphere

N = Avogadro's number (6.023×10^{23})

Combination of equations 1), 2) and 3) yields the following for the frictional coefficient of a sphere

$$f_o = 6\pi\eta\left(\frac{\bar{v} M 3}{N 4}\right)^{1/3} \qquad 4)$$

The sedimentation coefficient, s, is defined as

$$s = \frac{M(1 - \bar{v}\rho)}{N f} \qquad 5)$$

ρ = density of solvent

Substitution of the value for the frictional coefficient, equation 4), into equation 5) yields a value for the sedimentation coefficient of a sphere.

$$s_o = \frac{M(1 - \bar{v}\rho)}{6\pi N\eta} \left(\frac{4N\pi}{3\bar{v}M}\right)^{1/3} \qquad 6)$$

Rearrangement of equation 6) gives,

$$s_o = \frac{M^{2/3}}{(\pi N)^{2/3}\, 6\eta} \left(\frac{4}{3}\right)^{1/3} \left(\frac{1 - \bar{v}\rho}{(\bar{v})^{1/3}}\right) \qquad 7)$$

Substitution of the known constants into equation 7) yields the following relationship.

$$s_o = (M)^{2/3} \frac{(1 - \bar{v}\rho)}{(\bar{v})^{1/3}} \times 1.199 \times 10^{-15} \qquad 8)$$

For the calculations of the values used in Figure 5, $\bar{v}$ was set equal to 0.73 cc/g.

The theoretical values for the diffusion coefficients of spheres as a function of molecular weight(see Figure 6) were calculated in the following manner

The diffusion coefficient is defined as follows

$$D = \frac{kt}{f} \qquad 1)$$

k = Boltzmann's constant (1.38×10^{-16})

t = absolute temperature

f = frictional coefficient

The frictional coefficient for a sphere is given by Stokes law as,

$$f_o = 6\pi\eta r_o \qquad 2)$$

r = radius of sphere

η = viscosity of solvent in this case water at 20^o (0.01005 poise)

subscribt, $_o$, refers to a sphere

Substitution of of the value for f_o in equation 2) into equation 1) yields,

$$D_o = \frac{kt}{6\pi\eta r_o} \qquad 3)$$

The radius of a sphere is defined as

$$r_o = \left(\frac{v\,3}{\pi\,4}\right)^{1/3} \qquad 4)$$

v = volume of sphere

The volume of a sphere of a given molecular weight is defined as

$$V = \frac{\bar{v}M}{N} \qquad 5)$$

$\bar{v}$ = partial specific volume

M = molecular weight of sphere

N = Avogadro's number ($6.02_3 \times 10^{23}$)

Combination of equations 3), 4) and 5) yields an equation which can be used to calculate the diffusion coefficient of a sphere of a given molecular weight.

$$D_o = \frac{kt}{6\pi\eta}\left(\frac{4\pi N}{3}\right)^{1/3}\left(\frac{1}{\bar{v}M}\right)^{1/3} \qquad 6)$$

Substitution of the known constants into equation 6) gives the following,

$$D_o = 2.905 \times 10^{-5}\left(\frac{1}{\bar{v}M}\right)^{1/3}$$

For the calculations of the theoretical diffusion coefficients listed in Figure 6, $\bar{v}$ was set equal to 0.73 cc/g.