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Sedimentation Equilibrium Studies on Glutamic Dehydrogenase*

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ABSTRACT: Sedimentation equilibrium experiments were performed on commercial preparations of beef liver glutamic dehydrogenase (GDH). Initial observations of a deviation from a simple associating system, as well as the presence of low ($<2 \times 10^5$) molecular weight materials were resolved by the discovery of proteolytic activity in the sample. Although native enzyme was not affected, denatured enzyme in 2–5 M guanidine hydrochloride was susceptible to proteolytic degradation. In higher concentrations of guanidine hydrochloride, the protease was inactivated. Pretreatment on gel filtration columns reduced protease activity, and served as well to make the GDH ultracentrifugally homogeneous.

Analysis at sedimentation equilibrium of enzyme treated on gel filtration columns indicated a monomer molecular weight of $3.2 \times 10^5 \pm 0.2 \times 10^5$. At high protein concentrations, this material polymerized to very large aggregates which slowly precipitated from solution. Computer analysis of molecular weight distributions at low protein concentrations indicated that a rapid equilibrium of the type monomer–dimer–tetramer or monomer–dimer–trimer–tetramer existed. The association–dissociation equilibrium had a maximum in the pH range 7.0–8.5. A Dixon plot of the association constants as a function of pH implicated two ionizing groups in the polymerization, one at pH 6–7 and one at pH 8.5–9.0. The 3.2×10^5 molecular weight monomer could be further dissociated in the presence of guanidine hydrochloride to produce polypeptide chains with a molecular weight of $5.7 \times 10^4 \pm 0.3 \times 10^4$. The monomers thus appear to be composed of 6 polypeptide chains.

It has been known for some time that glutamic dehyrogenase[1] (L-glutamate:NAD^+(P) oxidoreductase (deaminating), EC 1.4.1.3) isolated from bovine liver participates in a reversible association–dissociation equilibrium, resulting in the formation of high molecular weight aggregates (Olson and Anfinsen, 1952). This equilibrium can be shifted by a number of structurally diverse allosteric compounds. These ligands are also effective in either activating or inhibiting the enzyme (*e.g.*, Frieden, 1959, 1963a; Yielding and Tomkins, 1961; Wolff, 1962).

The parallel activation and stimulation of polymer formation, on the one hand, and inhibition together with polymer dissociation, on the other, led initially to the proposal that the enzymatically active species was the polymer (Frieden, 1959). However, later studies indicated that association was not required for activity (Fisher *et al.*, 1962; Frieden, 1963b). It thus appears that the altered enzymatic activity observed upon addition of allosteric effectors is not a consequence of changes in the state of aggregation of the enzyme. Rather, both phenomena seem to be the result of some conformational change in the enzyme (Frieden, 1963b).

The biological significance of the association behavior, and the metabolite effects thereon, are questions of considerable interest. An obvious prerequisite for the study of these problems is a knowledge of the subunit structure

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[1] Abbreviations used are: GDH, glutamic dehydrogenase; Gdn·HCl, guanidine hydrochloride.

of the enzyme. However, no consistent estimates of molecular weight have been obtained for either the monomeric or polymeric forms[2] (Frieden, 1959; Kubo *et al.*, 1959; Fisher *et al.*, 1962; Colman and Frieden, 1966; Eisenberg and Tomkins, 1968). Accordingly, in an attempt to resolve the discrepancies we have investigated the molecular weights of the subunits, monomer, and polymeric forms of bovine liver GDH. Irreproducible results obtained initially could be traced to the presence of proteolytic activity in the commercial preparation. Further purification removed the proteolytic activity and provided material which appeared homogeneous in the ultracentrifuge. In order to obtain the monomer molecular weight, we made measurements at the microgram level using the analytical ultracentrifuge in conjunction with a split-beam photoelectric scanner (Schachman and Edelstein, 1966). All the experiments were performed in the absence of added ligand. Studies were made of solutions having pH values which favored association as well as in regions of pH where the association was depressed. Attempts were made also to determine molecular weights of the polymeric species and of the polypeptide chains within the monomer.

Materials

Glutamate dehydrogenase was obtained from Calbiochem as a suspension in ammonium sulfate. The specific activity of the samples was measured as 8000 units/mg using the standard assay of Strecker (1955). Bio-Rad P-200 and P-25 resins were obtained from Calbiochem. Gdn·HCl was twice recrystallized from ethanol and stored under vacuum in a dessicator. All other chemicals were standard reagent grade.

Unless specifically noted otherwise, the samples were passed through a Bio-Rad P-200 column (1.5 × 35 cm) equilibrated against 0.05 M Tris buffer at the desired pH, containing 0.1 M NaCl, 0.128 M EtSH, and 10^{-3} M EDTA. The enzyme, which emerged in the void volume, was then placed on a Bio-Rad P-25 column (1.5 × 35 cm) equilibrated against the same buffer minus mercaptoethanol and EDTA. Enzyme concentrations were calculated from absorbance measurements on a Cary 14 spectrophotometer. The extinction coefficient (1 mg/ml) at 280 mμ was taken as 0.97 cm^{-1} (Frieden, 1963c).

Ultracentrifuge Methods. A Spinco Model E analytical ultracentrifuge equipped with an electronic speed control was employed for all sedimentation studies. Most experiments were performed with double-sector cells having a 12-mm light path, and fitted with sapphire windows. In some instances, the multichannel cell described by Yphantis (1964) was used. Column heights of 3 mm were employed routinely. Both Rayleigh optics and a split-beam photoelectric scanning apparatus (Schachman and Edelstein, 1966) were used. In order to permit sufficient light to reach the photomultiplier of the scanner, it was necessary to use wide window holders in the ultracentrifuge cells. The Rayleigh fringes were formed through the use of a narrow-slit, symmetrical upper aperture mounted on the condensing lens (Richards and Schachman, 1959).

All photographic plates were read on a Nikon microcomparator. For the high-speed experiments measurements were made up to concentrations corresponding to 2.0 fringes (about 0.5 mg/ml). The fringe separation at higher concentrations (and the centrifugal fields employed) was so small as to preclude accurate measurements. The uncertainty in determining fringe displacements was about ±5 μ corresponding to about 0.02 fringe.

Determinations of molecular weights using the high-speed sedimentation equilibrium technique (Yphantis, 1964) were performed at initial concentrations of 0.2–0.85 mg/ml. Checks for the attainment of equilibrium were made at 3–4-hr intervals near the end of the experiment until an equilibrium distribution was observed. The experiments were designed so that, at equilibrium, about one-third of the cell was depleted of solute. At the end of each experiment the speed was increased and the top of the solution column was monitored for possible changes in fringe position as a test to determine whether the region near the meniscus was indeed free of solute.

Low-speed sedimentation equilibrium studies (Richards and Schachman, 1959; Richards *et al.*, 1968) were performed with solutions having an initial protein concentration of 2.0–3.0 mg/ml. All such experiments incorporated an overspeeding routine to reduce the time to reach equilibrium (Richards *et al.*, 1968). The concentration at the meniscus was determined by overspeeding the rotor at the end of the experiment until no further displacement of the fringes near the meniscus could be observed (LaBar, 1965). The fringe shift was then calculated to provide the meniscus concentration prior to overspeeding.

Molecular weights at high initial concentrations (4–7 mg/ml) of protein were also obtained using the method of Archibald (Archibald, 1947; Klainer and Kegeles, 1955; Ginsburg *et al.*, 1956). The temperature in these experiments was maintained constant at values between 10 and 13° by use of the standard RTIC unit. Calculations were made only for the top of the solution column.

Convection in the sedimentation equilibrium experiments was minimized by operating the ultracentrifuge at about 5°, near the temperature of the maximum density of the solvent. In addition the temperature was controlled by the refrigeration unit operating alone since it was found on occasion that convection resulted from the intermittent action of the heating coil when it was employed for temperature control (Gropper and Boyd, 1965).

Molecular weights of the polypeptide chains in Gdn·HCl were performed on solutions prepared by diluting the pretreated enzyme into solutions containing 2 to 7 M Gdn·HCl. Addition of 10^{-2} M EtSH to the Gdn·HCl enzyme solution did not alter the results.

No use was made of a layering fluid such as the fluorocarbon, FC-43, or silicone oil (Ginsburg *et al.*, 1956), to form a "false bottom." Preliminary experiments indicated that the presence of such substances resulted in the formation of large aggregates at the solution–fluid interface.

For the sedimentation experiments on concentrated solutions it was necessary to concentrate the enzyme after the gel filtration procedure. This was accomplished in a Microcell ultrafiltration apparatus fitted with a Diaflo membrane having a cutoff corresponding to substance of 5×10^4 molecular weight.

Data Analysis. The weight-average molecular weight, M_w, was calculated from the familiar equation

$$M_w = [2RT/(1 - \bar{v}\rho)\omega^2](\mathrm{d} \ln c/\mathrm{d}r^2) \quad (1)$$

where R is the gas constant, T is the absolute temperature, $\bar{v}$

[2] "Monomer" refers to the minimum molecular weight unit participating in the reversible equilibrium, while "polymer" refers to any aggregate of monomers whether or not it exists in equilibrium with lower molecular weight species. The "subunit" is the polypeptide chain(s) produced from the monomer by urea, acid, etc.

is the partial specific volume of the solute, ρ is the density of the solution, c is the concentration, and r is the distance from the axis of rotation.

For experiments involving native enzyme, the partial specific volume was taken to be 0.75 ml/g (Olson and Anfinsen, 1952; Eisenberg and Tomkins, 1968). In these experiments the plots of ln c *vs.* r^2 were nonlinear, and a computer program was used to evaluate the weight-average molecular weight as a function of radial position. The plots of ln c *vs.* r^2 were fitted to a three- or four-term polynomial, and the values of d ln c/dr^2 were obtained by taking the first derivative of the polynomial at each measured radial distance (Teller, 1965).

Since the plots of ln c *vs.* r^2 exhibited substantial curvature, efforts were directed toward distinguishing between polydisperse systems composed of molecules of different sizes, on the one hand, from solutions containing monomers and oligomers in a dynamic equilibrium, on the other hand. For the latter, the weight-average molecular weight at any level in the cell is a function only of the concentration at that level and is independent of the initial concentration, the acutal level and the rotor speed (Yphantis, 1964). Since M_w is proportional to d ln c/dr^2, the most convenient method for evaluating the data in terms of polydispersity or as a self-associating system is to compare directly plots of ln c *vs.* r^2. Experiments performed at different rotor speeds could be accommodated in single plots when the results were normalized by plotting ln c *vs.* $r^2\omega^2$ (Edelstein, 1967). For experiments in which the initial concentration, c_0, or column length differ (as in a six-chambered Yphantis cell), it is necessary to normalize all the plots by setting r^2 equal to zero at some arbitrary concentration. The data were thus plotted on a scale of ln c *vs.* $\omega^2\Delta r^2$. In this way the data from different experiments corresponding to varying initial concentrations and speeds should overlap if the system involves reversible associating–dissociating equilibrium. Nonideality was neglected since the protein concentrations did not exceed 0.5 mg/ml.

Analysis of Molecular Weight Distribution Curves. For an association–dissociation equilibrium of the type n(monomer) $\rightleftarrows$ (monomer)$_n$, Rao and Kegeles (1958) have developed an equation relating protein concentration and measured molecular weights, *e.g.*

$$c^{n-1} = \frac{((n-1)M_1)^{n-1}}{K_n}\frac{M_w - M_1}{(nM_1 - M_w)^n} \quad (2)$$

In this equation, n is the degree of polymerization, M_w the weight-average molecular weight at protein concentration c, K_n the dissociation constant, and M_1 the monomer molecular weight. The degree of polymerization is obtained by testing values of n until a linear plot of

$$c^{n-1} \text{ vs. } \frac{M_w - M_1}{(nM_1 - M_w)^n}$$

is obtained.

An alternative method of analysis employed a slight modification of the approach described by Adams (Adams and Williams, 1964; Adams and Filmer, 1966). If one neglects nonideality, an equation describing a monomer–dimer equilibrium can be given as

$$\left(2 - \frac{M_w}{M_1}\right)c = c_{1r}e^{\phi} \quad (3)$$

where M_w is the weight-average molecular weight at protein concentration c, M_1 is the monomer molecular weight, c_{1r} the monomer molecular weight at radial distance, r, from the axis of rotation, and ϕ is defined as $(M_1(1 - \bar{v}\rho)\omega^2/2RT)(r_2^2 - r_1^2)$. The term e^{ϕ} can be evaluated by use of the following relationships (van Holde *et al.*, 1969)

$$\frac{\text{d ln } c_1}{M_1} = A\text{d}r^2 = \frac{\text{d ln } c}{M_w} \quad (4)$$

where $A = (1 - \bar{v}\rho)\omega^2/2RT$). Since the distribution of monomer in the ultracentrifuge cell at equilibrium can be described by

$$c_{1r_2} = c_{1r_1}e^{MA(r_2^2 - r_1^2)} \quad (5)$$

we can modify eq 4 to give

$$\frac{c_{1,r_2}}{c_{1,r_1}} = e^{M_1 A\,dr^2} = e^{M_1\int_{c_{r_1}}^{c_{r_2}}\frac{\text{d ln } c}{M_w}} = e^{\phi} \quad (6)$$

Thus e^{ϕ} is determined by performing the appropriate integration.

From eq 3 it can be seen that a plot of $(2 - (M_w/M_1))c$ *vs.* e^{ϕ} will be linear for a system of the type $2M_1 \rightleftarrows M_2$. Similar equations can be generated for any interacting system.

Assay for Proteolytic Activity. Determinations of proteolytic activity were made by the method of Kunitz (see Laskowski, 1955). Casein (100 mg) was boiled in 19 ml of solution containing 0.05 M Tris (pH 8.4)–0.1 M NaCl. One milliliter of this casein solution was then mixed with 1 ml of enzyme and the solution incubated at 37°. At selected time intervals, 3 ml of 5% trichloroacetic acid was added. The precipitate was spun down and the absorption of the supernatant fluid was measured at 280 mμ.

Results

Characterization of the Enzyme Preparation. Initial experiments on the associating system were performed with enzyme preparations which had been exhaustively dialyzed against the appropriate buffer. Determinations of M_w as a function of concentration were poorly reproducible from experiment to experiment.

In order to remove this apparent heterogeneity in the preparation, the sample was prefractionated on Bio-Rad P-200 columns. This treatment was designed primarily to remove low molecular weight fragments. The presence of such materials was suggested by the existence, in untreated samples, of solute having molecular weights of less than 2×10^5. Since the enzyme emerged in the void volume of the P-200 columns, these low molecular weight contaminants could be effectively separated. Precautions were taken to ensure that no irreversible aggregates were produced. Thus, the addition of EDTA was primarily to remove extraneous Zn^{2+} since preliminary experiments had demonstrated that added Zn^{2+} resulted in the formation of very large aggregates which slowly precipitated from solution (M. Cassman, unpublished results, 1968). The mercaptoethanol was added to prevent disulfide interchange. In addition, it was observed that at the concentration of EtSH used, the association was repressed. This is presumably due to disruption of noncovalent bonding by the ethanol moiety of the EtSH, since

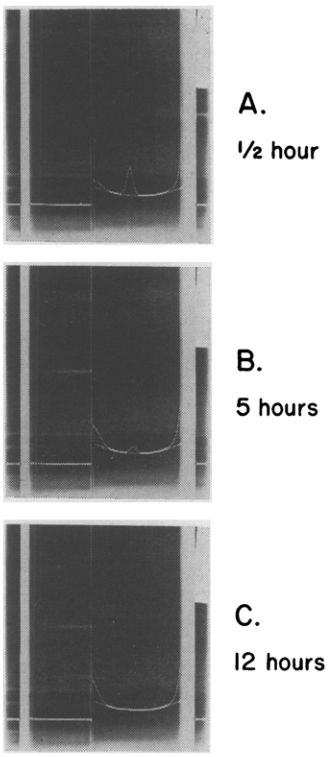

FIGURE 1: Sedimentation velocity patterns of GDH after incubation in 2 M Gdn·HCl. The initial concentration of the untreated commercial GDH was 10 mg/ml, in 0.01 M sodium acetate (pH 5.4) + 0.1 M NaCl × 2 M Gdn·HCl. The rotor speed was 60,000 rpm and the temperature was maintained at 8°. The schlieren patterns were photographed at a phase-plate angle of 80°. Pictures were taken between 12 and 16 min after the rotor had reached the operating speed. The patterns from top to bottom correspond to 30 min, 5 and 12 hr, respectively, after the enzyme had been exposed to Gdn·HCl.

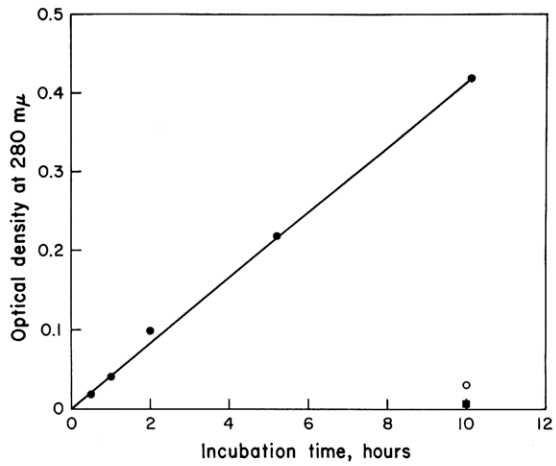

FIGURE 2: Determination of proteolytic activity in untreated commercial samples of GDH. Activity was followed by measuring the optical density of the supernatant at 280 mμ following treatment with 5% trichloroacetic acid. Denatured casein was used as substrate. The GDH concentration in the incubation mixture was 0.5 mg/ml. All values were corrected for controls run in the absence of enzyme. The ordinate represents the optical density at 280 mμ, and the abscissa gives the time of incubation in hours. The data indicated by ● were obtained with untreated GDH, while the single point given as ○ is the result of an incubation with GDH fractionated on gel filtration columns. The point marked as ■ was obtained from an incubation mixture containing untreated GDH, but in the absence of casein.

the effect could be mimicked by a variety of alcohols (M. Cassman, unpublished results, 1968). Centrifugation was begun within 4 hr after fractionation to avoid any possible deterioration of the sample upon storage. After such treatment, the molecular weights were reproducible to within 10% over the entire concentration range measured. As will be shown in the following section, this procedure resulted in a preparation which obeyed the requirements for a homogeneous associating system.

The origin of the low molecular weight contaminants was illuminated during the course of experiments on the enzyme in dilute Gdn·HCl solutions. Preliminary studies of the enzyme in 2–3 M Gdn·HCl solutions using preparations that had been dialyzed (and not pretreated on gel filtration columns) gave plots of ln c *vs.* r^2 which were distinctly nonlinear. Further, the weight-average molecular weights evaluated near the meniscus indicated the presence of very low molecular weight material, having M_w about 10^4. Surprisingly, these results were observed only for solutions at concentrations of Gdn·HCl below 5 M. Sedimentation velocity studies performed on enzyme incubated with low concentrations of Gdn·HCl indicated that extensive degradation of protein occurred. Figure 1A is the schlieren pattern observed at 30 min after exposure of the enzyme to 2 M Gdn·HCl. Although this picture shows a symmetrical boundary, even at this time the gradient at the top of the cell indicated that considerable amounts of low molecular weight material were present. By 5 hr after incubation in 3 M Gdn·HCl, the sedimenting boundary had diminished markedly in amount (Figure 1B), while after 12 hr it had completely disappeared (Figure 1C).

These observations prompted us to consider the possibility that a proteolytic contaminant was present in the commercial GDH preparation. An assay for proteolytic activity using casein as a substrate, and in the absence of Gdn·HCl, showed a linear increase of acid-soluble material with time when untreated GDH was added (Figure 2). In the absence of casein, acid-soluble material was not produced, indicating that endogenous protein is not a substrate for the proteolytic contaminant. Similarly, untreated enzyme, in the absence of Gdn·HCl, does not lose activity when stored in the cold for several weeks. Sedimentation velocity patterns of such samples observed on successive days showed no loss of material, even when the samples were stored at room temperature.

These results demonstrated that the native enzyme is not sensitive to the action of the contaminating proteolytic enzyme, while the observations in solutions containing 5 and 7 M Gdn·HCl indicate that at these concentrations the proteolytic contaminant was inhibited or inactivated. The digestion of high molecular weight materials observed in Figure 1 must reflect a set of circumstances whereby the Gdn·HCl is present at a level sufficient to denature the GDH, but not sufficient to inactivate the proteolytic enzyme. Since the native enzyme is not susceptible to the action of the protease, the low molecular weight material found in the unfractionated commercial GDH presumably arises from the degradation of partially or wholly denatured enzyme. The lack of reproducibility between experiments could thus be attributed to limitations on the proteolytic activity due to the precise conditions of dialysis, pH, time of storage, etc., prior to analysis in the ultracentrifuge.

The proteolytic activity is decreased markedly by pretreating the enzyme with EtSH and EDTA, together with passage through gel filtration columns as described above.

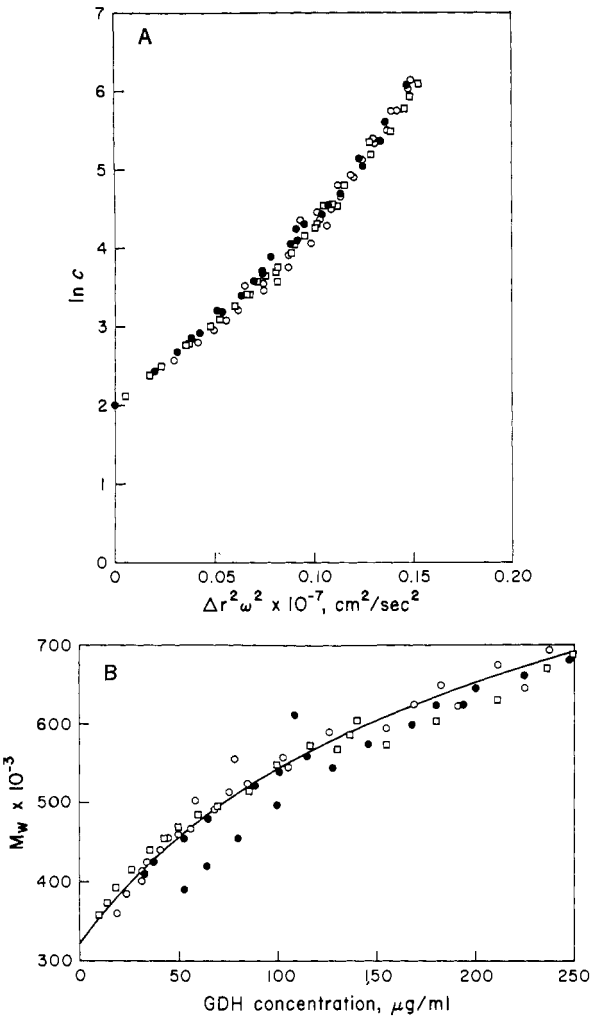

FIGURE 3: (A) Sedimentation equilibrium of GDH at different initial concentrations. The experiments were performed in a six-chambered Yphantis cell under conditions which left the meniscus depleted of solute. The GDH had been fractionated on gel filtration columns and was in a solvent of 0.05 M Tris–0.1 M NaCl (pH 7.25). The experimental points represent measurements made at 8000 and 10,000 rpm, at a temperature of 4°, using both the interference and the absorption optical systems. The photoelectric scanner was operated at wavelengths of 280 and 235 mμ. The ordinate represents the logarithm of concentration in micrograms per milliliter, and the abscissa gives $\omega^2\Delta r^2 \times 10^{-7}$ cm²/sec². The symbols refer to different initial concentrations of GDH: ○, 0.25 mg/ml; ●, 0.45 mg/ml; □, 0.65 mg/ml. (B) Dependence of molecular weight of GDH on concentration. Evaluated from the curves of part A as described in text. The ordinate is weight-average molecular weight. The abscissa is the concentration of GDH in the cell, in units of micrograms per milliliter. The symbols refer to different initial concentrations of GDH. ○, 0.25 mg/ml; ●, 0.45 mg/ml; □, 0.65 mg/ml.

Such pretreated preparations of GDH were virtually free of proteolytic activity as shown by the absence of a decrease in M_w even after several days of incubation in 2 M Gdn·HCl; moreover, little proteolytic activity was observed with denatured casein as substrate (Figure 2).

Monomer Molecular Weight. Following the pretreatment by gel filtration it was observed that the resulting enzyme preparation had lost no catalytic activity and appeared homogeneous upon ultracentrifugation. Figure 3A presents the results for samples studied at 0.25, 0.45, and 0.65 mg per ml initial concentrations. The plot includes data obtained using both the Rayleigh and the absorption optical system,

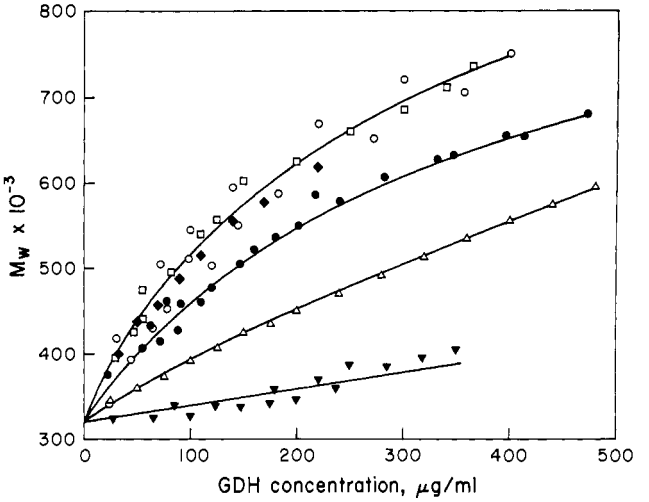

FIGURE 4: The pH dependence of the association equilibrium. GDH was fractionated on gel filtration columns equilibrated at the requisite pH. The solutions contained 0.05 M Tris and 0.1 M NaCl. The points represent pooled data from experiments performed over an initial concentration range 0.25–0.65 mg/ml. Rotor speed was kept constant at values between 9000 and 13,000 rpm. In all experiments, the solute concentration at the meniscus was determined to be zero at equilibrium. Both the interference and the absorption optical systems were used. The photoelectric scanner was operated at 280 and 235 mμ. The temperature was kept constant in the range 3–6°. The ordinate gives the weight-average molecular weight, and the abscissa presents the concentration of protein in micrograms per milliliter. The pH values at which the experiments were performed are indicated by ▼, pH 5.8; ●, pH 6.65; ○, pH 7.2; □, pH 8.15; ◆, pH 8.5; and △, pH 9.0.

at speeds of 8000 and 10,000 rpm. The molecular weight dependence on concentration shown in Figure 3B was determined by evaluating d ln c/dr^2 from the data given in Figure 3A. The major deviations from a smooth continuous curve in Figure 3B come from the extrema of a set of points, especially when a single experiment covers a wide range of concentrations. These deviations are most probably due to the curve-fitting procedure, since the polynomial is least accurate in fitting the points at the ends of the curve.

In general, the evaluated molecular weights for a given pH at a given concentration in the cell were reproducible to within 10% and usually to within 5%; moreover they were independent of initial concentration or rotor speed.

The rapid increase in molecular weight at low concentrations, under the conditions given in Figure 3, make an accurate extrapolation to infinite dilution very difficult; thus, at pH 8.15, the molecular weight increased from 3.95×10^5 to 5.6×10^5 in the concentration range from 30 to 130 μg per ml. A better estimate of the molecular weight at infinite dilution was obtained from examination of a family of curves obtained at different pH values. A study of the pH dependence of the association–dissociation reaction was made in solutions containing 0.05 M Tris and 0.1 M NaCl. Tris was used over the entire pH range so as to maintain the ionic environment as constant as possible. The results are shown in Figure 4. It is clear that all the curves converge to essentially the same intercept, evaluated as $3.2 \times 10^5 \pm 0.2 \times 10^5$. There is, however, still some ambiguity in the extrapolation to infinite dilution, since, as previously noted, the curve-fitting procedure may introduce curvature at the extremes of a data set. An attempt was made therefore to determine the weight-average molecular weight under condi-

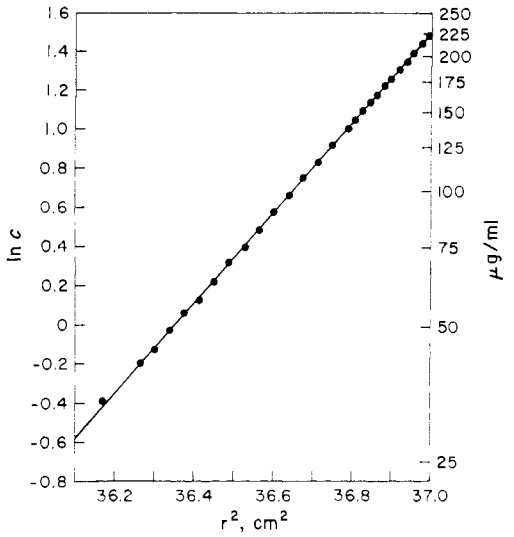

FIGURE 5: Determination of GDH monomer molecular weight at pH 5.8. The experimental conditions as given in the legend for Figure 3A. Measurements were made with the absorption optical system at 280 mμ. Rotor speed was 11,000 rpm and the temperature 5°. Initial concentration of GDH was 0.4 mg/ml. The abscissa is distance from the center of rotation in cm². The left ordinate is the natural logarithm of the protein concentration, with c measured as centimeters chart deflection, while the right ordinate gives the protein concentration in micrograms per milliliter. 1-cm chart deflection = 50 μg/ml.

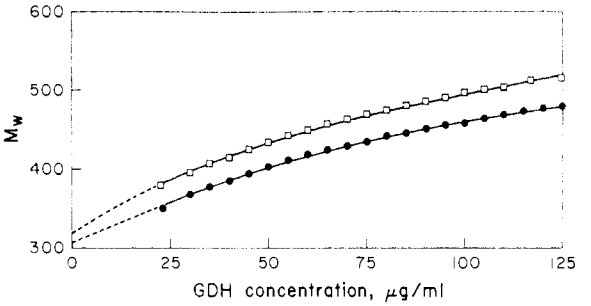

FIGURE 6: Temperature dependence of the association–dissociation equilibrium. The experiments were performed in Tris-NaCl buffer, pH 6.5 (at 4.8°). The absorption optical system was used at 280 mμ. The rotor speed was 10,000 rpm and the initial protein concentration was 0.45 mg/ml. The symbol □ indicates results obtained at 4.1°, while ● represents data taken at 18.8°. The ordinate gives the weight-average molecular weight, while the abscissa is the concentration at protein in the cell in units of micrograms per milliliter.

tions in which monomer was effectively the only species present. With the absorption optical system, measurements were obtained in the concentration range 25–200 μg/ml at pH 5.8. The plot of ln c *vs.* r^2 was linear in this region of concentration, and M_w was calculated as 3.24×10^5 (Figure 5).

Measurements were also made to determine whether any temperature dependence of the association–dissociation equilibrium could be observed. In these studies, the enzyme was brought to equilibrium at a given temperature, the temperature in the chamber altered, and the sample allowed to reequilibrate. All comparisons were thus made on the same sample under identical conditions of rotor speed and initial concentration. Since the buffer used was Tris, it was however necessary to consider the temperature dependence of the ionization constant. A typical measurement made at pH 6.5 showed a slight decrease in molecular weight over the range of concentrations examined, when the temperature was increased from 4.1 to 18.7° (Figure 6). This increase in temperature would be expected to result in a drop of 0.43 in the pK_a of ionization of Tris. As can be seen from Figure 4, at pH 6.5 a decrease in pH results in a reduction of the degree of GDH association. An estimate of the change in M_w expected due to the decrease in pH indicates that it would be sufficient to account for the difference in M_w observed in Figure 6; for this calculation it was assumed that the error was approximately 5% in the determination of M_w. Other studies of this type gave comparable results, indicating that there is little or no temperature dependence for the interacting system.

Subunit Molecular Weight. Numerous studies have demonstrated that the GDH monomer is composed of more than one polypeptide chain (Jirgensons, 1961; Marler and Tanford, 1964). In contrast to the readily reversible, concentration-dependent association of the monomer to higher aggregates, dissociation of the monomer to its constituent polypeptide chains can be accomplished only in the presence of some reagent such as Gdn·HCl or urea, or at extremes of pH.

When the enzyme was diluted into 2, 3, 5, or 7 M Gdn·HCl and brought to sedimentation equilibrium, the resulting plots of ln c *vs.* r^2 were linear. The measured slopes are proportional to $M_e(1 - \bar{v}_e\rho)$, where the molecular weight and partial specific volume refer to protein plus any preferentially bound solvent component. Values of $M_e(1 - \bar{v}_e\rho)$ obtained at different Gdn·HCl concentrations were plotted *vs.* ρ, the density of the solvent (Schachman and Edelstein, 1966). The results from the individual experiments fit a straight line which extrapolated to an intercept of 1.36 g/ml corresponding to zero redistribution of the polypeptide chains. This extrapolated value represents the density at which $\rho = 1/\bar{v}_e$, *i.e.*, the density at which no solute redistribution occurs; it corresponds therefore to the density of the hydrodynamic unit. The reciprocal obtained is 0.735 ml/g. Since this value is only slightly less than 0.75 ml/g, the partial specific volume of the native protein (Olson and Anfinsen, 1952; Eisenberg and Tomkins, 1968), it appears that there is no preferential binding of either Gdn·HCl or H_2O to the GDH subunit. The difference between the two values (−0.015 ml/g) can be attributed to a decrease in $\bar{v}$ arising from denaturation. Such a decrease has been observed previously in the denaturation of a number of proteins (Kielley and Harrington, 1960; Marler *et al.*, 1964). From these results we conclude that M_e corresponds to M_w. As seen in Table I the results from the experiments at varying Gdn·HCl concentrations are internally consistent.

The molecular weight of the polypeptide chains is $5.7 \times 10^4 \pm 0.4 \times 10^4$. This value combined with the molecular weight of the monomer indicates that the monomer is composed of six polypeptide chains.

Polymer Molecular Weight. Attempts were made to determine the limiting molecular weight of the polymers formed in the reversible association–dissociation equilibria. The necessity of obtaining molecular weights at high protein concentrations required that either low-speed sedimentation equilibrium or Archibald techniques be applied. In the initial low-speed sedimentation equilibrium experiments measurements made for times as long as 4 days indicated that sedimentation equilibrium was not achieved. The calculated time required to reach equilibrium was between 12 and 18

TABLE I: Molecular Weight of GDH in Guanidine Hydrochlorine.

Gdn·HCl Concn (M)	M_w[a]
2	5.7×10^4[b]
3	5.8×10^4
5	5.8×10^4
7	5.6×10^4

[a] The molecular weights were calculated using a value of 0.735 ml/g for the apparent specific volume, as described in the text. [b] Each molecular weight represents an average of at least three experiments. The deviations from the average were $\pm 0.2 \times 10^4$. The measurements were made at temperatures between 15 and 19°, and in the speed range 26,000–34,000 rpm.

TABLE II: Test of Association–Dissociation Equilibrium Models.[a]

pH	6.65	7.2	8.15	8.5	9.0
Degree of polymerization[b] (*n*)	3	3	–	–	–
Models for association–dissociation equilibrium[c]	1–3 and 1–2–3	1–3	–	–	–

[a] Values of M_w and c for each pH examined were obtained from the data plotted in Figure 4. [b] The values given for the degree of polymerization (n) are those for which a linear plot of the data is obtained from eq 2. Where no value is given, no value of n tested gave a linear plot. [c] The models listed are those to which the data could be fit, employing an equation analogous to eq 3 in the text. Where no model is listed, no such equation fit the data.

hr. A continuous depletion of material occurred during the course of the centrifugation as observed by continuous changes in concentration throughout the cell. This observation presumably resulted from the formation of large aggregates and the simultaneous readjustment of the remaining material to conform to the requirements of chemical and sedimentation equilibrium.

The relatively long times required to reach equilibrium at low speeds left open the possibility that irreversible aggregates were forming during the course of the experiment. In order to reduce the likelihood of such an occurrence, the Archibald technique was used. Measurements were made during the approach to equilibrium, within 0.5 hr after the start of centrifugation. The sample was examined immediately after fractionation on the gel filtration column. The weight-average molecular weights, evaluated at the meniscus, varied between 1.8 and 2.4×10^6. These molecular weights corresponded to concentrations in the cell of 1.5–2.5 mg/ml. No particular trend of molecular weight with concentration in this concentration range was noted.

These results, together with the inability to reach equilibrium at high initial protein concentrations, indicated that with increasing concentration the preparation became polydisperse through the formation of high molecular weight material which was irreversibly aggregated.

Association Mechanism and Equilibrium Constants. Initial attempts were made to analyze the curves of Figure 3 using the methods of Rao and Kegeles (1958) and of Adams (Adams and Williams, 1964; Adams and Filmer, 1966). The Rao–Kegeles approach discriminates between association–dissociation processes having the apparent mechanism $n(\text{monomer}) \rightleftarrows (\text{monomer})_n$. If the system to be examined is of this type, use of the appropriate value of n in eq 2 generates a straight line. Inappropriate values of n inserted into eq 2 produces nonlinear plots. If the interacting system contains significant amounts of species intermediate between monomer and n-mer, *all* values of n tested will give nonlinear plots. The only linear plots obtained were at pH values of 6.65 and 7.1, for degree of polymerization, $n = 3$. Data obtained at other pH values did not fit eq 2 for any values of n tested. The results are summarized in Table II.

The method of analysis outlined by Adams permits the testing of models having more than one polymeric species such as dimer and trimer. The derived formulation for each model can be plotted in a manner which will yield a linear plot, when the experimental data are inserted into the appropriate equation. The results of such a treatment were inconsistent, in that more than one model could be fit to the data at a given pH value, and no one model could be fit to the data at all pH values. These results are also summarized in Table II.

Another approach to the analysis of interacting systems involves use of a computer program for curve fitting. The program, designed by Dr. Robert Dyson (personal communication; van Holde *et al.*, 1969), provides the best fit to the experimental data for the model to be tested. The models examined were monomer–trimer, monomer–dimer–trimer, monomer–dimer–tetramer, and monomer–dimer–trimer–tetramer. The ouput presents, for each model tested, the overall root-mean-square deviation of the calculated *vs.* the experimental molecular weights, the appropriate equilibrium constants, and the calculated molecular weights at each value of concentration. The summarized results are presented in Table III. At any given pH, *e.g.*, pH 7.1, the root-mean-square deviations may be so close for all models tested that none can be eliminated. However, the best fit to the data at all the pH values tested was provided by the monomer–dimer–tetramer model. The monomer–dimer–trimer–tetramer model was almost as satisfactory, since a small change in the value of the monomer molecular weight significantly improves the fit to the data at some of the pH values examined. The monomer–trimer and monomer–dimer–trimer models can be ruled out on the basis of high root-mean-square deviations. In addition, the use of root-mean-square deviations as an exclusive criterion of goodness of fit is misleading when applied to these two models. An examination of the calculated molecular weights as compared to the experimental values at each concentration evaluated shows that the overall root-mean-square error presented comes from consistently high values of the calculated weight-average molecular weights in one region of concentration taken together with consistently low values of M_w's in another concentration range. In contrast, the root-mean-square deviations for the monomer–dimer–tetramer and monomer–dimer–trimer–tetramer models arsie from random error over the observed concentration range.

TABLE III: Test of Association-Dissociation Models by Curve-Fitting Procedures.[a]

	pH	6.65	7.2	8.15	8.5	8.0
Monomer–trimer	RMS[b]	14,000	8300	10,500	16,100	42,000
	K_1[c]	1.4×10^6	1.8×10^6	3.1×10^6	2.2×10^6	5.5×10^6
Monomer–dimer–trimer	RMS	8604	7900	8500	9100	32,000
	K_1	3.7×10^2	7.5×10^1	2.7×10^2	5.9×10^2	neg[d]
	K_2	1.0×10^1	1.2×10^1	2.1×10^1	1.5×10^1	1.7
Monomer–dimer–trimer–tetramer	RMS	1733	7815	5582	8790	28,600
	K_1	1.3×10^3	8.5×10^1	6.0×10^2	6.5×10^2	neg[d]
	K_2	3.3	1.2×10^1	2.2×10^1	1.4×10^1	1.8
	K_3	4.1×10^{-2}	neg[d]	9.2×10^{-3}	3.2×10^{-3}	2.2×10^{-4}
Monomer–dimer–tetramer	RMS	3000	6000	10,300	4000	13,500
	K_1	1.6×10^3	1.5×10^3	2.4×10^3	1.5×10^3	2.0×10^2
	K_2	8.2×10^{-2}	1.1×10^{-1}	2.6×10^{-1}	1.4×10^{-1}	3.8×10^{-3}

[a] The curve fitting was performed using a computer program designed by Dr. R. Dyson (personal communication; van Holde *et al.*, 1969). A value of $M_1 = 3.2 \times 10^5$ is assumed. [b] RMS refers to the root-mean-square deviation of the experimental *vs.* the calculated molecular weights. RMS is defined as $\sqrt{[\Sigma(M_w(\text{expt}) - M_w(\text{calcd})^2/(N + 1)]}$, where M_w(expt) and M_w(calcd) are the experimental and calculated weight-average molecular weights, respectively, while N is the number of data points used. [c] K_1, K_2, and K_3 are the molar association constants of the first, second, and third equilibria (if required), respectively, for the association–dissociation model examined. [d] The comment "neg" indicates that the best fit to the data required that a negative quantity be added of the polymeric species involved. Values are not reported, since a negative equilibrium constant is physically meaningless. Such constants can arise since there are no external restrictions on the computer to pick positive terms in obtaining the best fit to the data.

Discussion

The present series of investigations indicate that the monomer molecular weight of beef liver glutamic dehydrogenase is $3.2 \times 10^5 \pm 0.2 \times 10^5$, and that this monomer is probably composed of six polypeptide chains. The association–dissociation equilibria, when examined at high protein concentrations, results in the continuous formation of large aggregates which slowly precipitate from solution. At lower protein concentrations, the self-associating system appeared stable and reversible and is most compatible with either a monomer–dimer–tetramer or monomer–dimer–trimer–tetramer model. Initial irreproducibility in the results and heterogeneity in the preparation were related to the presence of a proteolytic contaminant. This contaminant could be removed or inactivated during a prefractionation procedure on gel filtration columns.

The value for the monomer molecular weight could not be determined with any degree of assurance until the system was found to be homogeneous. For a self-associating system involving reversible equilibria, the question of homogeneity becomes quite complex. At least three types of phenomena can give rise to operationally observable heterogeneity, (1) more than one species of protein is present, *e.g.*, heterologous protein, proteolytic fragments, etc.; (2) part of the sample cannot participate in the equilibrium, producing "incompetent" monomers and/or oligomers; and (3) there exists more than one equilibrium constant governing the interconversion of two molecular weight species. This third type of heterogeneity can be inferred from the data if the other two possibilities are eliminated through experiment. More than one equilibrium constant for two molecular weight species could arise if, for example, a portion of the associating material had a tightly bound low molecular weight component. Even if this contaminant is not large enough to be detected by its contribution to the molecular weight, it could produce an apparent heterogeneity by modifying the association behavior of the protein to which it is attached.

The molecular weight for the associating monomer, $3.2 \times 10^5 \pm 0.2 \times 10^5$, falls midway in the range of values previously reported. These vary from 2.5×10^5 (Frieden, 1959) to more recent estimates of 4.0×10^5 (Colman and Frieden, 1966). The lower values may be attributable to the presence in the preparations of substantial amounts of low molecular weight contaminants. Such contaminants may have arisen from the action of endogenous proteolytic activity in the GDH preparations. Additional uncertainties in the earlier determinations of the molecular weight stem from the difficulty in evaluating the infinite dilution molecular weight by extrapolation of the data from experiments at comparatively high protein concentrations. This extrapolation is most hazardous for studies on solutions having pH values from 6.5 to 8.5 because the molecular weight increases markedly over the concentration range from 0 to 100 μg per ml. It is interesting to note that our value for the monomer is consistent with the values obtained by Eisenberg and Tomkins (1968), of 316,000, and of Kubo *et al.* (1959) of 350,000. Both of these are obtained from light-scattering studies at low protein concentration. Since no fractionation occurs in such experiments and the technique yields weight-average values, small amounts of low molecular weight contaminants will have a small effect on the observed molecular weight. Willick (1967) has also reported a value of $3.6 \times 10^5 \pm 0.2 \times 10^5$ for the monomer. This result was obtained from sedimentation equilibrium experiments. However, the preparation appeared to be quite heterogeneous.

Our results are also consistent with molecular weights of enzymes obtained from species whose GDH do not participate in a reversible equilibrium. Two such enzymes are dogfish liver GDH, having an M_w of 3.3×10^5 (Corman

et al., 1967), and rat liver GDH, with an $M_w = 3.5 \times 10^5$ (Sedgewick and Frieden, 1968).

Analysis of studies performed in 2, 3, 5, and 7 M Gdn·HCl resulted in an estimated molecular weight of the subunit of $5.7 \times 10^4 \pm 0.4 \times 10^4$ and a $\bar{v} = 0.735$. Uncertainty in the partial specific volume due to preferential interactions is the major difficulty in obtaining accurate molecular weights in systems containing high concentrations of salts (Casassa and Eisenberg, 1960, 1961). Although the approach used by us to obtain v_c is capable of a high degree of precision, it is complicated by the implicit assumption that the degree of preferential binding is constant at all concentrations of Gdn·HCl at which dissociation occurs. If the extent of preferential binding is a function of the Gdn·HCl concentration the values of the calculated molecular weight will vary with the concentration of Gdn·HCl. It should be noted that the value of v_c extrapolated from the sedimentation data is in excellent agreement with the directly measured value of 0.726 ml/g for the protein in 5.7 M Gdn·HCl (Eisenberg and Tomkins, 1968). Their value for the molecular weight of the polypeptide chains is $5.35 \times 10^4 \pm 0.15 \times 10^4$. Earlier measurements by Marler and Tanford (1964), using sedimentation equilibrium, have been evaluated by Eisenberg and Tomkins on the basis of $\bar{v}_c = 0.726$ ml/g to give an M_w of 5.2×10^4. These results for the molecular weight of the polypeptide chains taken together with that for the monomer lead to the conclusion that the monomer consists of six polypeptide chains.

The results obtained from low-speed equilibrium and Archibald experiments indicate the presence of very large aggregates ($M_w = 2 \times 10^6$). These apparently do not participate in the reversible equilibrium. The formation of these irreversible aggregates appeared to be very slow, as indicated by the small changes in molecular weight distributions over a 3- to 4-day period in the low-speed equilibrium experiments. This would account for the ability to attain equilibrium in the high-speed experiments at low initial concentrations (0.2–0.8 mg/ml) and relatively short times (10–20 hr). Although the concentration at the bottom of the cell in the high-speed experiments reached 3–7 mg/ml, it is likely that very little irreversible aggregation occurred during the time required to reach equilibrium. The samples examined at a given pH under different conditions of initial concentration and rotor speed all came to equilibrium and showed no observable heterogeneity, as indicated by the overlap of the molecular weight *vs.* concentration curves.

On the basis of our data we cannot distinguish between the formation of linear aggregates, which eventually reach the limit of solubility, and the existence of a reversible equilibrium involving a few molecular weight species, followed by a slow irreversible aggregation to a polydisperse mixture. Eisenberg and Tomkins (1968) have proposed from light-scattering data that the GDH polymer is a linear extended aggregate. The existence of the polymeric forms as linear aggregates is confirmed by the X-ray studies of Sund *et al.* (1969) and the viscosity studies of E. Reisler and H. Eisenberg (personal communication). Reisler *et al.* (1970) have recently reported light-scattering and sedimentation equilibrium studies on GDH in 0.2 M phosphate (pH 7). They obtained a monomer molecular weight of 312,000 and were able to fit the polymerization process to a reversible infinite discrete association model with a single equilibrium constant. The computer analysis of high-speed equilibrium experiments strongly indicates that only the initial monomer–dimer association is favored, with all the larger aggregates having much lower association constants. Until additional data are available the values for the equilibrium constants must be considered tentative; nonetheless these values can be employed in an attempt to account for the interesting pH dependence. Dixon's method (see Dixon and Webb, 1958) was used to evaluate the nature of the ionizing groups in the pH-dependent equilibria. The equilibrium constants for the monomer–dimer–tetramer model were used. The plots of pK *vs.* pH for values from 6.6 to 9.0 are shown in Figure 7. The intersection of the extrapolated curves gives the pK of those ionizing groups which affect the association. Since the system at pH 5.8 showed so little aggregation over the concentration range measured, no reliable estimate of an equilibrium constant could be obtained. However, it can be seen from Figure 7 that one pK exists between pH 6 and 7, and another at pH 8.5–9.0, for both the monomer–dimer and dimer–tetramer equilibria. Since not enough points are available, no reliable estimate of the equilibrium constant could be obtained. The slopes of the curves in Figure 7 below pH 6.55 are therefore represented by dotted lines to indicate the direction of change.

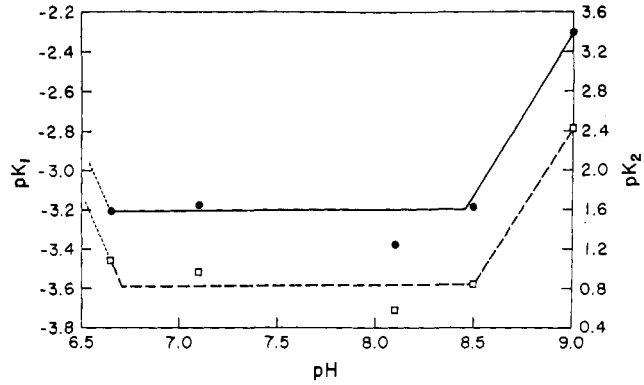

FIGURE 7: Variation with pH of pK_1 and pK_2 in the monomer–dimer–tetramer model. The values of pK_1 and pK_2 are calculated from the association constants given in Table II. The symbol ● represents the data for pK_1 while □ indicates the results obtained for pK_2. The abscissa gives the pH while pK_1 and pK_2 are presented on the left and right ordinates, respectively.

The presence of an ionizing group involved in the association with a pK near 9 is particularly intriguing since Colman and Frieden (1968) have demonstrated that the reversible association could be inhibited by blocking a single lysine group on each monomer. Another likely candidate for such an ionizing group would be $Zn(H_2O)_2^{2+}$, which Tanford and Epstein (1954) have shown to have a pK of approximately 8.0. Adelstein and Vallee (1958) have demonstrated the presence of 3.4 ± 1.0 M $Zn^{2+}/10^6$ of GDH.

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Nuclear Magnetic Resonance Studies of Bovine Carbonic Anhydrase. Binding of Sulfonamides to the Zinc Enzyme*

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ABSTRACT: Nuclear magnetic resonance line broadening of sulfonamide inhibitors upon their binding to the zinc bovine carbonic anhydrase has been studied at various temperatures and two frequencies. Competition of different inhibitors for the same site was demonstrated. Comparison of the binding constants ratio determined by nuclear magnetic resonance technique with that determined by enzymatic activity confirmed that the broadening effect was due to specific binding to the active site. The dissociation rate constant of the enzyme–inhibitor complexes was found to be greater than 200 sec^{-1}. Frequency dependence of the broadening indicated a dipolar relaxation mechanism rather than a chemical shift dependent mechanism.

The rotational motion of the aromatic rings of several sulfonamide inhibitors was found to be close to that of the whole protein molecule, while that of methyl groups is much faster. Furthermore, it was concluded that, for the methyl protons, the main contribution to the dipolar relaxation is the intramolecular proton–proton interaction, and for the aromatic protons, the interaction with closely adjacent protein protons. The significance of these results to our understanding of the mode of binding is discussed.

Sulfonamides are known to be potent and specific inhibitors of carbonic anhydrase (carbonate hydrolyase, EC 4.2.1.1.) (Mann and Keilin, 1940; Maren, 1967). All the strongest known sulfonamide inhibitors have an unsubstituted SO_2NH_2 group attached to aromatic or heterocyclic residue. Some N^1-substituted and aliphatic sulfonamides also inhibit the

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