Molecular Weights, Association, and Frictional Resistance of Bovine Liver Glutamate Dehydrogenase at Low Concentrations. Equilibrium and Velocity Sedimentation, Light-Scattering Studies, and Settling Experiments with Macroscopic Models of the Enzyme Oligomer*

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ABSTRACT: Equilibrium sedimentation results in the microgram range of concentrations show that the molecular weight of the active oligomeric hexamer of bovine liver glutamate dehydrogenase is close to 312,000, in good agreement with recent light-scattering results (Eisenberg, H., and Tomkins, G. M. (1968), *J. Mol. Biol. 31*, 37). The increase of the apparent weight-average molecular weight, M_w, with increasing enzyme concentration in 0.2 M phosphate buffer at pH 7 is identical when determined by either light scattering or equilibrium sedimentation, up to a concentration of 1.7 mg/ml, at 20°. The results may be compared with a reversible infinite discrete association mechanism with a single equilibrium constant, K. Up to a concentration of 0.4 mg/ml, thermodynamic nonideality does not interfere and the data are consistent with a value of K between 2.1 and 2.0 ml per mg. At higher concentrations deviations due to thermodynamic nonideality (virial coefficients) appear. The sedimentation coefficients, $s_{20,w}$, have also been determined in phosphate buffer, at low enzyme concentrations; with increasing concentration they increase faster at 20° than at 10°, due to slightly higher association at the higher temperature. The extrapolated value is in both cases identical, $s^0_{20,w} = 11.4$ S. In the presence of 10^{-3} M GTP, 10^{-3} M NADH the increase of $s_{20,w}$ with enzyme concentration is much reduced; it is even further reduced when the concentration of both these reagents is increased by a factor of five. In all cases the values of $s_{20,w}$ extrapolate to the same value ($s^0_{20,w} = 11.4$ S) at zero enzyme concentration. Settling experiments with macroscopic models of a structure of the enzyme recently proposed (Eisenberg, H., and Reisler, E. (1970), *Biopolymers 9*, 113) lead to translational frictional coefficients from which the value $s^0_{20,w} = 11.42$ S is calculated, in excellent agreement with the experimental results. Similar experiments with respect to viscosity and rotational diffusion have recently been reported. The translational frictional and the sedimentation coefficients are not sensitive enough to detect small changes in conformations or minor adjustments in the model for the structure of the enzyme proposed on the basis of other results.

We have shown recently (Eisenberg and Tomkins, 1968), on the basis of light-scattering studies, that the active oligomer of bovine liver glutamate dehydrogenase has a molecular weight close to 312,000 and is composed of six, apparently identical, subunits of mol wt 52,000 each.[1] With increasing concentration the oligomer associates to form long linear polymers. This indefinitely proceeding polymerization is strongly promoted by toluene (Eisenberg and Reisler, 1970; Reisler and Eisenberg, 1970) and is inhibited by a variety of low molecular weight effectors (Frieden, 1959; Yielding and Tomkins, 1961). We have, in our own work, studied the role of the reduced coenzyme NADH in conjunction with GTP in bringing about the depolymerization of the enzyme to the hexameric oligomer. The readily effected polymerization–depolymerization reaction is related to subtle changes in the substrate specificity of the active enzyme. It appears that the oligomer is capable of existing in at least two active forms with distinct substrate activity, but that only one of these forms is able to polymerize (Frieden, 1963; Tomkins *et al.*, 1963; Fisher *et al.*, 1965). The state and mechanism of aggregation of the enzyme are thus of great interest, particularly in view of the fact that the enzyme is believed to exist at high concentrations in its native and active form in the mitochondria of the liver.

Once the number of six subunits per active oligomer was firmly established we enquired into the physical structure involving the arrangements of these subunits in the oligomeric enzyme. Electron microscopy work of Valentine (1968) confirmed earlier observations (Horne and Greville, 1963) that the molecule has a triangular profile, suggesting two layers with three subunits in each layer of the oligomer. Recent low-angle X-ray-scattering work (Sund *et al.*, 1969) showed that the mass per unit length of GDH is constant in solution and the

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[1] When stating that the molecular weights are 312,000 and 52,000 we obviously refer to nominal values only (6 × 52,000 = 312,000); due to uncertainties in extrapolation to zero concentration, problems with absolute calibration, and the determination of accessory quantities (refractive index and density increments) in light scattering and equilibrium sedimentation, we estimate a possible deviation of about ±3 to 5% of the true molecular weight from this value. Such an uncertainty though is inconsequential for our further considerations.

radius of gyration of the cross section is independent of the state of association of the enzyme. On the basis of these and our own light-scattering results we proposed (Eisenberg and Reisler, 1970) that the oligomer is formed by two layers, each composed of three elongated subunits, approximated by prolate ellipsoids of rotation, arranged in triangular fashion; in the individual layers the major axes $2a$ = 66.5 Å of the ellipsoids point in the same direction. Two layers stacked on top of each other form an elongated oligomer (of length 133 Å), which can further polymerize, to polymers of indefinite length, in the direction of the major axes of the prolate ellipsoids. (This polymerization is affected by enzyme concentration and by a large number of reagents, but it is interesting to note that dissociation into biologically active half-oligomers containing three subunits each has so far not been achieved.) The minor axis $2b$ of the ellipsoidal subunits equals 43 Å. We postulated that, when viewed along the long axis of the oligomer, the two triangular layers are in the eclipsed configuration; a staggered configuration is equally likely, but such structural details cannot be distinguished in solution by light scattering, low-angle X-ray scattering, or hydrodynamic measurements, and may depend on sample preparation in electron microscopy. Recently Josephs (1970) confirmed, on the basis of a detailed electron microscopy study, the basic correctness of the model proposed above, and further suggested that, in his experiments, the subunits are arranged in the staggered configuration referred to above.

It is possible to test the basic features of the model proposed by hydrodynamic measurements. Whereas it is not possible to establish a model by such means, as it may not constitute a unique representation of the data, it is possible to test the consistency of viscosity and sedimentation data with a given model. We have recently studied (Reisler and Eisenberg, 1970) the viscosity of GDH solutions in phosphate buffer, in the concentration range 0.1–8 mg/ml. It was found that the reduced specific viscosity, η_{sp}/c, markedly increases with increasing enzyme concentration. In the presence of 10^{-3} M GTP and 10^{-3} M NADH the viscosity increase is much smaller and the results can be extrapolated to zero enzyme concentration to yield an intrinsic viscosity $[\eta]$ = 3.2 ml/g. In the presence of toluene η_{sp}/c increased very much more at much lower enzyme concentration. These observations are in good agreement with the hypothesis that the active oligomer of GDH may associate to form linear rodlike polymers of indefinite length. In conjunction with the viscosity measurements we also determined the transverse and axial rotary frictional coefficients of macroscopic bodies, similar to the physical model advanced for the structure of the oligomer of GDH, and calculated the viscosity corresponding to these frictional coefficients; this corresponded well to the measured values.

In the present work we have extended these investigations to sedimentation experiments in the ultracentrifuge. We show, from equilibrium sedimentation measurements at low concentrations, that good agreement exists between equilibrium sedimentation and light scattering, under similar conditions. We show that it is possible to describe the association of the GDH oligomer by an open sequential mechanism, with identical equilibrium constants. Formally this is identical with the purine stacking analyzed by Van Holde and Rossetti (1967). In the low concentration range analyzed in this work the contribution due to virial coefficients is small; we shall discuss the role of virial coefficients in the higher concentration range in a later work. Our own earlier data (Eisenberg and Tomkins, 1968) on the concentration-dependent association of GDH has been recently shown by Chun *et al.* (1969) and Chun and Kim (1969) to be consistent with this type of association. This conclusion is also confirmed by the results of Krause *et al.* (1970).

Sedimentation coefficients can be measured with good accuracy at very low concentrations in the ultracentrifuge equipped with an ultraviolet-scanning system. Unfortunately, in contradistinction to η_{sp}/c (which cannot be measured reliably at such low concentrations in the microgram range), sedimentation coefficients are related to translatory frictional coefficients (which depend on linear dimensions of the molecules) rather than to the rotary frictional coefficients (which depend on cubed dimension of the macromolecules). Sedimentation coefficients are therefore much more insensitive to changes in the parameters of the model than the corresponding viscosity measurements. Considering the convenience with which the concentration-dependent sedimentation coefficients can be obtained in the low concentration range (in which η_{sp}/c measurements cannot be satisfactorily performed), we have determined s both in phosphate buffer only and in the presence of GTP and NADH. We show that, independent of temperature and experimental conditions, the same value is obtained for $s^0_{20,w}$. We have also extended our macroscopic model experiments to settling experiments and show that the sedimentation coefficients, calculated from the properly averaged and scaled transverse and axial frictional coefficients of the macroscopic models, agree well with the measured value of GDH.

Experimental Section

Materials and Solutions. Bovine liver glutamate dehydrogenase, in the form of crystalline suspension in ammonium sulfate, was purchased from Boehringer und Soehne GmbH (Mannheim, Germany). Prior to experiments the enzyme was dialyzed in the cold room against phosphate buffer[2] for 2 days; the dialysis was allowed to take place at room temperature for the last few hours. Before use protein solutions and solvents were filtered through 0.45 μ Millipore filter. Protein concentration was determined by using an $\epsilon^{0.1\,cr}_{1\,cm}$ value of 0.97 at 279 mμ (Olsen and Anfinsen, 1952; *cf.* also Sund and Akeson, 1964). NAD and NADH were obtained from Sigma Chemical Co. and GTP from Mann Research Laboratories. All chemicals and reagents used were of the highest purity obtainable; NADH and GTP were freshly prepared before the sedimentation velocity measurements. Wherever possible the glassware and the cells were sterilized after cleaning.

Light-Scattering Measurements. Light-scattering experiments with vertically polarized light at wavelengths λ 436 and 546 mμ were made in the angular range 30–150°, at 20°, in a Wippler–Scheibling (1954) light-scattering photometer, manufactured by Fica, Paris.

Cylindrical (28 mm diameter) flat-bottom thin-walled cells provided with a glass joint cap were used. They were tightly closed to prevent creeping of toluene from the vat of the instrument into enzyme solutions. The solutions were prepared and

[2] Phosphate buffer is 0.2 M sodium phosphate, pH 7, containing 10^{-4} M EDTA.

clarified following the procedure of Eisenberg and Tomkins (1968).

Refractive index increments were determined at λ 436 and 546 mμ in a Rayleigh interference refractometer fitted with a thermostat bath. Measuring cells, from fused silica, of 3-, 1-, and 0.3-cm path length were used. Measurements made at 20 ± 0.05° yielded the values 0.176 for the refractive index increment $(\partial n/\partial c)_\mu$ at λ 546 mμ, in good agreement with Eisenberg and Tomkins (1968); the value 0.181 was found for $(\partial n/\partial c)_\mu$ at λ 436 mμ. Subscript μ signifies constant chemical potential μ of components diffusible through a semipermeable membrane.

Velocity Sedimentation. Sedimentation velocity experiments were performed in 12- and 30-mm double-sector cells, with a Beckman Model E ultracentrifuge equipped with ultraviolet optics, photoelectric scanner, multiplexer attachment, and temperature control unit.

Both schlieren and ultraviolet optics were used. Sedimentation velocity scans at low enzyme concentration were taken at 233 and 280 mμ. Cell concentrations were based upon 280-mμ absorption. Sedimentation of enzyme solutions in the presence of NADH and GTP was followed with schlieren optics at 10°; stability of the reagents was satisfactory at this temperature. All the experiments performed both at 10 and 20° were made at 30,000 rpm; no pressure dependence of a sedimentation constant was found up to 60,000 rpm. Before filling the centerpiece the sectors as well as the syringes were flushed twice with the appropriate solutions. Whenever a four-hole rotor was used one of the cells contained a bovine serum albumin solution which provided an internal check on the experiments.

The method of second moments (Goldberg, 1953), rather than the motion of the 50% concentration point in the boundary region, was used to determine the sedimentation velocity constants. This method, although time consuming, has the advantage of higher accuracy, in particular for an associating system. Ultraviolet scans and schlieren plates were analyzed with a Nikon Shadowgraph microcomparator. About 20 points/picture (or scan) were taken for the calculations of second moments.

The reported values of the sedimentation constants, measured at 10 and 20°, were corrected to water at 20°. A value of 0.749 was determined (the experimental procedure follows Reisler and Eisenberg, 1969) for the apparent volume, ϕ', in the expression $1 - \phi'\rho^0$; ρ^0 is the density of the solvent. Correction for radial dilution was also applied.

Velocity sedimentation experiments were carried out in Rehovot, except two values (*cf.* solid circles, Figure 5) obtained at the CRM in Strasbourg, at λ 230 mμ, with a Spinco Model E equipped with a photoelectric scanner constructed by one of us (Pouyet, 1966).

Equilibrium sedimentation experiments at 20° were carried out at the CRM in Strasbourg in the Model E ultracentrifuge described above. Measurements with 12- and 30-mm double-sector cells were made at λ 230, 280, and 295 mμ. Absorbancies of enzyme solutions recorded at these wavelengths were converted into concentrations in milligrams per milliliter by multiplication with suitable conversion factors. These factors were determined by measuring in the ultracentrifuge the optical densities at the three wavelengths of enzyme solutions of known concentrations.

In order to avoid problems of adsorption, especially at low protein loading concentrations, the centerpiece was filled and emptied twice before the final filling. No layering oil was used in these experiments.

Cell loading concentrations, in equilibrium sedimentation measurements, ranged from 0.04 to 0.6 mg per ml. Column heights of 4–6 mm were employed. The experiments were conducted at 4600- and 4900-rpm speeds, preceded by a short period (2–3 hr) of overspeed at 11,000–12,000 rpm. Scans were taken after 12 or more hr at speed. Equilibrium was achieved in 2–3 days as evidenced by scan pattern stability over 10- (or more) hr interval. Absorbancies, OD, and radial distances, r, obtained from scans were represented in the form of plots of ln OD *vs.* r^2. These plots exhibited a pronounced curvature (even at low enzyme concentrations) characteristic for an associating system. It was realized that for such systems the molecular weights cannot be obtained in a standard way, from the initial slope of the plots of ln OD *vs.* r^2.

The equilibrium runs were analyzed by the FORTRAN IV high-speed equilibrium ultracentrifugation computer program of Roark and Yphantis (*cf.* Roark and Yphantis, 1969). This program gives the various molecular weight moments, M_w, M_n, and M_z, with the associated error at each radial reading. Analyses of the final scans, and those taken 10 hr earlier, yielded essentially identical results. We are greatly indebted to Dr. Jamie Godfrey for introducing the use of this program at the WIS and for patient help in instructing us in its use.

Hydrodynamic Model Experiments. Translational friction experiments (*cf.* Broersma, 1960) were conducted in cylindrical vats 50 cm high and diameters of 21, 29, and 50 cm. The vats were filled with castor oil; the viscosity η = 43 P at 4° (in the cold room) of this solution was determined by dropping 2-, 3-, and 4-mm steel balls in the center of the vat. Stokes' law was obeyed throughout and viscosity calibrations were made before and after each translational experiment. The viscosity value as well as the Newtonian character of castor oil at 4° were checked with a Brookfield viscometer (Brookfield Eng. Lab. Inc., Stoughton, Mass.).

Cylindrical aluminium rods (length 2.4 and 4 cm, axial ratio p = 10; length 3.2 and 4.8 cm, p = 20) were dropped sidewise in the vats; their velocity ranged from 0.3 to 1.2 cm per sec. Settling velocities of macroscopic models of the enzyme covered the same velocity range. It was concluded from measurements performed with the cylinders in vats of different diameters that only in the big vat (diameter 50 cm) no correction for finite distance to the walls was required; identical frictional coefficients were obtained for rods of the same axial ratio, when scaled to equal size. An empirical wall correction (*cf.* Results) was successfully applied in the smaller vats.

The macroscopic enzyme models used in these experiments were described previously (Reisler and Eisenberg, 1970). Volumes of the two models, required for calculation of the buoyant force in the settling experiments, were determined by immersing the models in a glass vessel filled with methanol and supplied with a side capillary neck. The volume change, due to immersion of the models, was found by following the height of the liquid in the capillary with a cathetometer. Transverse and axial translational frictional coefficients of the two size enzyme models were identical in the big vat. Measurements performed in smaller vats, corrected for the wall effect, again yielded the same frictional coefficients.

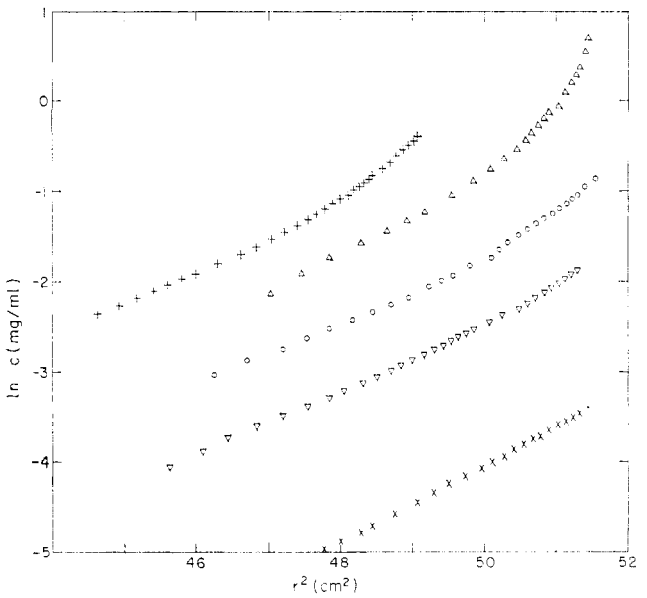

FIGURE 1: Sedimentation equilibrium plots of ln c *vs.* r^2 over a concentration range from about 0.015 to 1.7 mg per ml. The optical density was recorded at 230 mμ at low concentrations and at 280 and 295 mμ at the higher concentrations; $t = 20°$.

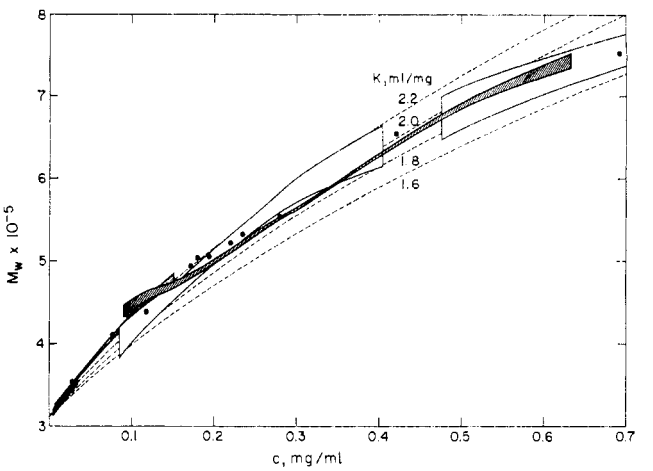

FIGURE 2: Weight-average molecular weight (M_w) *vs.* concentration (up to 0.7 mg/ml) in phosphate buffer; boxes, computed values from equilibrium sedimentation runs of Figure 1, with estimated error (see text); filled circles, light-scattering results, also at 20°; curves, calculated according to reversible infinite linear association (stacking) with single value of association constant K.

The average translational frictional coefficient was calculated from the experimentally established transverse and axial coefficients.

Results

Equilibrium Sedimentation. Altogether five equilibrium sedimentation runs, with loading concentrations ranging between 0.04 and 0.6 mg per ml, will be presented. The classical plots of ln c *vs.* r^2 are shown in Figure 1. It is seen that the curves curve away from the r^2 axis, indicating negative nonideality, *i.e.*, association; the apparent molecular weights increase with increasing concentration. The data have been analyzed with the help of the Roark and Yphantis program and the apparent number-average M_n, weight-average M_w, and z-average M_z molecular weights obtained. The results are shown in Figures 2–4; the curves have been calculated in accord with a simple treatment for indefinite association described in the Discussion. In Figure 2 we show (boxed areas) the molecular weights for five runs, in the low concentration range 0.01–0.7 mg per ml, with the error as estimated by the computer program. We attempt here a novel presentation of the results. The computed values of M_w are halfway between the upper and lower curves running along the concentration axis and representing the estimated molecular weight error in the computation. The overlap between the various runs is also indicated. Light-scattering results (also at 20°), in good agreement with the equilibrium sedimentation, are shown as black circles. It is seen that the extrapolation to vanishing concentration confirms the previously established value of close to 312,000 for the molecular weight of the active oligomeric hexamer. In Figure 3 we show similar agreement for M_w between light scattering and sedimentation in a slightly higher (up to 1.7 mg per ml) concentration range. The fact that the values of M_w obtained by both methods show good agreement is highly significant. It engenders renewed confidence in both light scattering and in equilibrium sedimentation (with the ultraviolet absorption and photoelectric scanner technique) under difficult experimental conditions. Moreover, these results were not obtained on identical stock solutions, at identical times, but rather over considerable period of times with different enzyme lots, albeit from the same manufacturer (light-scattering results (E. Reisler, unpublished data) at 25° agree well with the light-scattering results of Eisenberg and Tomkins (1968) with a crystalline enzyme obtained in 1965 from Sigma Corp). Thus, while this does not necessarily indicate the purity of the samples, it reflects the reproducibility of association results obtained with a number of instruments and methods, with thoroughly dialyzed solutions of the crystalline enzyme, over a number of years. In Figure 4 we show the apparent values of M_n and M_z obtained from equilibrium sedimentation by the above mentioned procedure of Roark and Yphantis. The consistency with respect to M_z is somewhat worse, but still satisfactory, in view of the experimental uncertainty in the raw data of Figure 1, and the loss in precision when calculating higher molecular weight moments from ultracentrifuge data. We have calculated M_z from the individual runs, and not from the smoothed M_w *vs.* c curve. The calculated curves will be discussed below.

Velocity Sedimentation. Figure 5 shows the sedimentation coefficients in phosphate buffer, in the low concentration range, as a function of enzyme concentration, at two temperatures. The data have been obtained in 1967 at the WIS with the Beckman commercial scanner, except for the two black circles, measured (after a 2-year interval) with the scanner constructed by Pouyet at the CRM. Again the agreement is good. Both sets of data (obtained at 10°, but recalculated in both cases to $s_{20,w}$) extrapolate to the same value $s^0_{20,w} = 11.4$ S for the oligomer; the curve for $s_{20,w}$ at finite concentrations for the data obtained at 10° is below that for the data of 20°, because of lower association (at finite concentration) of the enzyme at the lower temperature (E. Reisler and H. Eisenberg,

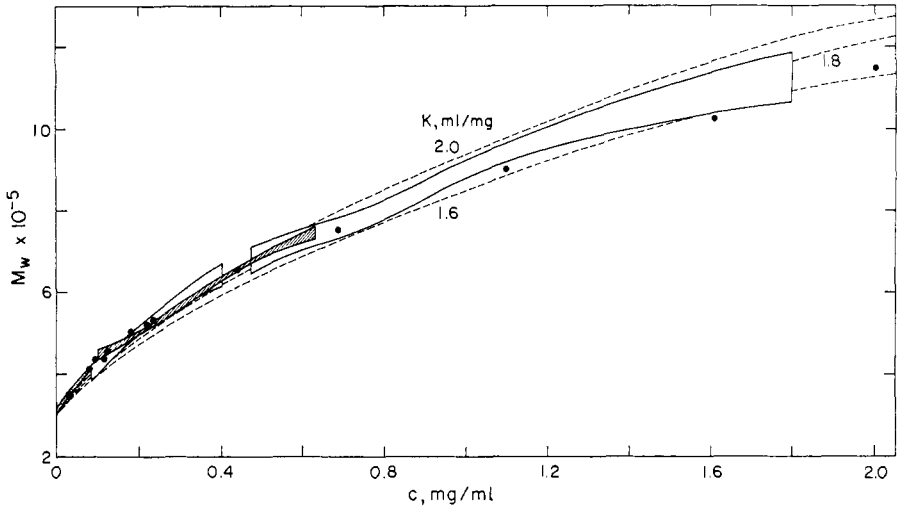

FIGURE 3: Weight-average molecular weight (M_w) *vs.* concentration (up to 1.7 mg/ml) in phosphate buffer; curves and symbols as in Figure 2.

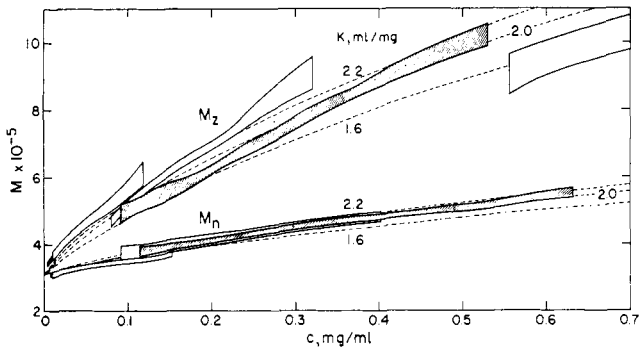

FIGURE 4: *z*-average (M_z) and number-average (M_n) molecular weights from equilibrium sedimentation; curves and symbols as in Figure 2.

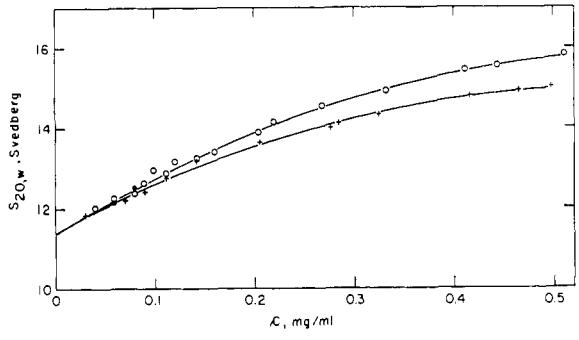

FIGURE 5: Sedimentation coefficients $s_{20,w}$ *vs.* concentration, in phosphate buffer; ○, measured at 20°; +, measured at 10°; ●, measured at 20° at CRM, Strasbourg.

unpublished results). At higher concentrations (Figure 6) schlieren and scanner data have been combined; data in the presence of GTP and NADH have been obtained with schlieren optics only (this is because of the strong light absorption of these latter reagents). We see that the extrapolation to zero concentration in phosphate buffer only and in the much more dissociated form (in the presence of GTP and NADH) leads to the same results, in good agreement with previous conclusions from molecular weight results (Eisenberg and Tomkins, 1968) and viscosity data (Reisler and Eisenberg, 1970).[3]

We have also measured the sedimentation coefficients in Tris buffer (0.05 M Tris, 0.1 M NaCl, 10^{-4} M EDTA, and 10^{-3} M phosphate, pH 6.0) at 20° and find the same value $s^0_{20,w}$ = 11.4 S; the concentration dependence of $s_{20,w}$ is only very slightly different from the values reported above in phosphate buffer.

Macroscopic Model Experiments. The axial $f_{||}$ and transverse $f_\perp$ translational frictional coefficients of macroscopic models of the enzymes (*cf.* Experimental Section) were derived from $g(m - V\rho)(\eta_{20,w}/\eta)/v\alpha$, where g is the acceleration constant due to gravity, m is the mass of the model, V its volume, ρ the density of the castor oil, η its viscosity, and $\eta_{20,w}$ the viscosity of water at 20°; v is the settling velocity of the model and α is the scale factor which transforms to molecular dimensions. The average frictional coefficient f_{av}, required in the calculation of s, is given by (for bodies with symmetry of rotation) $3/f_{av} = (1/f_{||}) + (2/f_\perp)$. From f_{av} we calculate $s^0_{20,w}$ which is equal to $10^{13}M(1-\phi'\rho^0)/N_{Av}f_{av}$; here N_{Av} is Avogadro's number.

In cases in which a wall correction was required, we used an empirical correction for finite walls, of the form

$$f = f_{app}(1 + ar/R)^{-1}$$

where f_{app} is the measured apparent coefficient, a is a constant of order unity, and r/R is the ratio between the radial extension

[3] The fact that both in the presence of 10^{-3} M GTP, 10^{-3} M NADH, and in the presence of five times higher concentrations of these reagents (lowest curve in Figure 6) the initial slope of $s_{20,w}$ *vs.* c is positive indicates that GTP and NADH, even at high concentrations, do not completely dissociate the enzyme at finite enzyme concentrations. The decrease of $s_{20,w}$ at high enzyme concentrations in buffer only (upper curve, Figure 6) represents the normal decrease of $s_{20,w}$ with increasing protein concentration and shows that at high concentration this effect overrides the increase due to enzyme association. This effect has previously been reported by Olson and Anfinsen (1952) and also by Sund (1963).

TABLE I: Results of Hydrodynamic Experiments with Macroscopic Models (Scaled to Molecular Dimensions).

Diameter Vat (cm)	50		29		21	
$f \times 10^7$	app	corr (a = 0)	app	corr (a = 1)	app	corr (a = 1.2)
$f_{\parallel}$, small model	0.99		1.08	0.99	1.13	0.98
$f_{\parallel}$, large model	1.00		1.16	1.01	1.27	1.02
$f_{\perp}$, small model	1.23		1.37	1.21	1.48	1.22
$f_{\perp}$, large model	1.20		1.47	1.20	1.68	1.24
$f_{\parallel}$, mean	1.00					
$f_{\perp}$, mean	1.22					
f_{av}	1.135					

of the model and the radius of the vat. We found that in the 50-cm vat no correction for finite walls was required, in the 29-cm vat a = 1, and in the 21-cm vat a = 1.2. The results of the hydrodynamic experiments, scaled to the molecular dimensions given by Eisenberg and Reisler (1970) for the proposed structure of glutamate dehydrogenase, are summarized in Table I. Hydration has not been taken into consideration in the evaluation of the frictional properties. Water of hydration may be immobilized in the voids between the subunits without causing additional frictional resistance.

Discussion

Concentration-Dependent Association. The weight-average molecular weights of glutamate dehydrogenase derived from light scattering (Eisenberg and Tomkins, 1968) (and values derived from sedimentation equilibrium in this work) are apparent values. From these the true M_w^0 and thermodynamic nonideality, as expressed (to a first approximation) by the second virial coefficient, A_2, in the equation

$$\frac{1}{M_w} \equiv \frac{1}{M_w^0} + 2A_2c \qquad (1)$$

cannot be experimentally determined in an associating system without further assumptions. A number of computations have been recently published (Chun and Kim, 1969; Krause *et al.*, 1970), in which a constant A_2 is ascribed to the system[4] in conjunction with a simplified model of the association process, with one equilibrium constant K.

Whereas for elongated rods A_2 may (for constant mass per unit length) indeed be independent of the length of the rods (Zimm, 1946), in the transition region from the globular oligomer to the higher linear polymers, A_2 may not necessarily be constant. Computations based on a unique and constant association constant K and a constant virial coefficient A_2 may thus not be representative of the physical situation.

Roark and Yphantis (1969) have shown that from extremely precise equilibrium sedimentation experiments not only apparent number, weight, and z averages of the molecular weight may be determined, but also molecular weight averages in which the virial coefficients do not appear (to be quite precise this is only true if the correctly weighted averages of the virial coefficients are equal, an assumption which may not apply to an associating polydisperse system). Our own evaluation of M_n and M_z (*cf.* Figure 4) from equilibrium sedimentation experiments with ultraviolet absorption optics at low enzyme concentrations is not precise enough for such calculations. We are now attempting the use of interference optics at somewhat higher protein concentrations. In the present work we restrict our quest to relatively low concentrations (up to 1.7 mg/ml) only—in the range 0.01–0.5 mg/ml the contributions due to virial coefficients are within experimental error of either light-scattering or equilibrium sedimentation determinations and may be disregarded. We can therefore at this stage probe into the likely mode of association, and the associated equilibrium constants, at low enzyme concentration, unencumbered by the contribution of thermodynamic nonideality.

The reversible association of glutamate dehydrogenase in phosphate buffer may be represented in the same way as the purine stacking process described by Ts'o *et al.* (1963). We

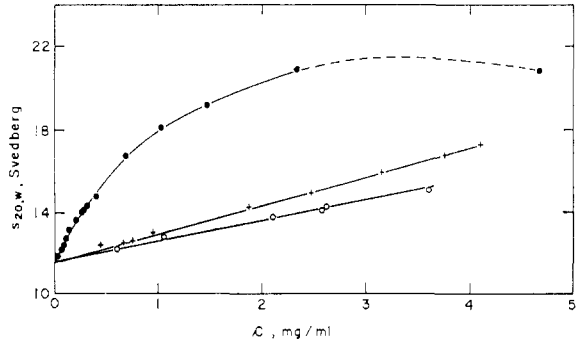

FIGURE 6: Sedimentation coefficients $s_{20,w}$ *vs.* concentration at 10°; ●, in phosphate buffer; +, in phosphate buffer plus 10^{-3} M NADH, 10^{-3} M GTP; ○, in phosphate buffer plus 5×10^{-3} M NADH, 5×10^{-3} M GTP.

[4] The value of $A_2 \simeq 8 \times 10^{-6}$ mole ml/g^2 calculated by us at high concentrations (Eisenberg and Tomkins, 1968), on the basis of Zimm's (1946) theoretical evaluation of the excluded volume of elongated rods, is in agreement with the value of $A_2 = 8 \times 10^{-6}$ mole ml/g^2 recently evaluated by Krause *et al.* (1970) from computations of experimental data at concentrations up to 56 mg/ml, and with the value $A_2 \simeq 10 \times 10^{-6}$ calculated by Chun and Kim (1969) in conjunction with our previous results (Eisenberg and Tomkins, 1968).

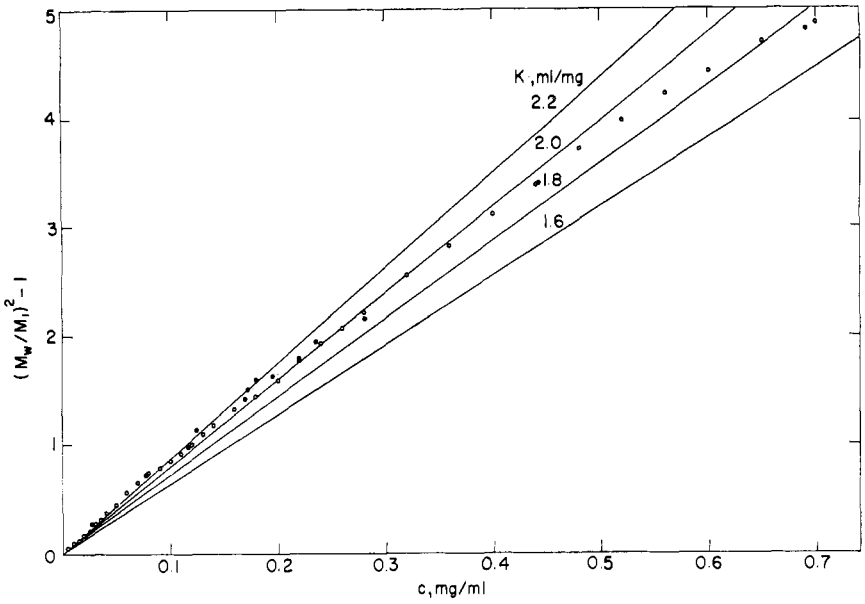

FIGURE 7: Plots of $[(M_w/M_1)^2 - 1]$ *vs.* c; M_w values averaged at each concentration from data from overlapping runs (*cf* Figure 2), M_1 = 312,000; curves as in Figure 2.

shall try to analyze it in analogy to the analysis of Van Holde and Rossetti (1967) for purine stacking in terms of a simple reversible polymerization with one equilibrium constant K and thermodynamic nonideality. The equations have also been derived by Adams and Lewis (1968) in relation to studies on lactoglobulin A. We consider the set of reactions

$$P_i + P_1 \rightleftharpoons P_{i+1};\ K_i = c_{i+1}/c_i c_1$$

where P_i is a polymerized species of weight M_i, i runs from 1 to n, c_i is the concentration of species P_i in mg/ml, and all $K_i = K$ (ml/mg) are considered to be equal. As long as $Kc_1 < 1$, which is almost always true, and when contributions due to A_2 may be neglected (at low enzyme concentrations), a simple calculation shows that

$$c = c_1/(1 - Kc_1)^2$$

and

$$M_w^0 = M_1(1 + 4Kc)^{1/2} \qquad (2)$$

where c is the total concentration (in milligrams per milliliter) Σc_i of the enzyme. We have in Figures 2, 3, and 7 drawn the calculated curves for K equaling 2.2, 2.0, 1.8, and 1.6 ml per mg.

We have plotted the equilibrium sedimentation values of M_w in Figure 7 in the form $[(M_w/M_1)^2 - 1]$ to give straight lines *vs.* c (with slope $4K$) for the calculated ideal curves (*cf.* eq 2).[5] The values of M_w have been obtained by averaging the values from the various runs (Figure 2) at each concentration, wherever overlap with respect to concentration existed. For M_1 the value 312,000 was taken. At low concentrations (below 0.4 mg/ml) the experimental results to M_w are best fitted by K between 2.1 and 2.0 ml per mg. Because of the insufficient accuracy of the experimental data at the low concentration end, we do not consider the slight shift toward lower K values in this range to be real. At higher concentrations (above 0.4 mg/ml) the apparent values of M_w deviate from the calculated curves as the contribution due to a positive virial coefficient increases; to establish meaningful statements with respect to the constancy of the association constants and the values of the virial coefficients, precise analysis in the higher concentration range is required. In Figure 4 we show that the same value of K between 2.1 and 2.0 ml per mg also describes the concentration dependence of M_n and M_z, derived from the equilibrium sedimentation runs. For the calculation we use, on the basis of the model assumed for the association, the correlations $2M_n = M_w + M_1$ and $2M_z = 3M_w - M_1^2/M_w$ for the region in which virial coefficients need not be considered. Although M_w is the primary quantity usually obtained from equilibrium sedimentation, we see that both M_n and M_z (albeit with lower accuracy) are also reasonably well decribed by the same reversible linear association model with a single equilibrium constant. It is possible to write down, if required, a complete distribution of all species present in solution at each concentration, if the description by a single equilibrium constant K is considered to be an acceptable working hypothesis.

Frictional Parameters of Enzyme Oligomer. From the average frictional parameter f_{av} (measured with the use of macroscopic models) at the bottom of Table I, we calculate $s^0_{20,w}$ = 11.42 S, which agrees with the value obtained by extrapolation to zero concentration of the sedimentation coefficients in Figures 5 and 6. We would like here to qualify the good agreement between the experimental $s^0_{20,w}$ and the value determined from the model by stating that the sedimentation coefficient

[5] If nonideality is considered, then M_w^0 in eq 2 must be replaced by M_w (eq 1), to give $M_w/(1 - 2A_2M_wc) = M_1(1 + 4Kc)^{1/2}$. From the above equation the limiting slope $d[(M_w/M_1)^2 - 1]/dc$ when $c \to 0$ is not equal to $4K$, but rather to $4(K - A_2M_1)$; as $K \approx 2000$ ml/g, $A_2 \approx 10^{-5}$ mole ml/g^2, and $M_1 \approx 3 \times 10^5$ g/mole, $A_2M_1 \approx 3$ is negligible when compared with K. Thus for all practical purposes and for this system, the nonideal term may be disregarded in the limit slope.

is rather insensitive to precise dimensions of the model and that, in general, no far reaching conclusions can be reached on a given model, from such model studies. We find thus that, if we substitute the ellipsoids of our model by a molecular model of the oligomer composed of six spheres (of radius 24.9 Å) in close contact, in the form of a triangular antiprism (*cf.* Josephs, 1970), the value of $s^0_{20,w}$ calculated from model experiments drops to 11.2 S only; this is still close enough to be considered on the fringe of the experimental extrapolation. Conformational changes due to binding of enzyme substrate or effectors are thus also not likely to be detected by sedimentation velocity. Intrinsic viscosity on the other hand is a more sensitive parameter. We have shown recently (Reisler and Eisenberg, 1970) that for the model proposed by us we calculate a viscosity of 3.20–3.26 ml/g, rather close to the experimentally obtained value 3.20 ml/g. The determination of the rotational frictional coefficient of a macroscopic model composed of spheres (as described above) yields an intrinsic viscosity $[\eta] = 2.85$ ml/g which, we feel, is beyond the range of the error of the experimental extrapolation. Hydrodynamic methods are limited in scope but may be helpful if judiciously applied. We believe that significantly high values of $s^0_{20,w}$ for glutamate dehydrogenase which have been reported are not likely to be correct, and are probably due to the difficulty of extrapolation at extremely low concentrations or in the presence of coenzyme and effectors (Rogers *et al.*, 1965; Sund and Burchard, 1968).

An additional conclusion that may be obtained from both the velocity and the equilibrium experiments is that, under the conditions studied, namely in phosphate buffer (between 10 and 25°) and also in the presence of up to 5×10^{-3} M GTP, NADH, the oligomeric hexamer (mol wt 312,000) is stable and does not dissociate into further subunits.

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