Biochemistry

Volume 10, Number 14 July 6, 1971

Bovine Liver Glutamate Dehydrogenase Association and Dependence of Association on Temperature*

Emil Reisler and Henryk Eisenberg†

ABSTRACT: Association constants, K, virial coefficients, A_2, and the dependence of the K's on temperature are discussed for the linearly associating system bovine liver glutamate dehydrogenase, in 0.2 M sodium phosphate buffer (pH 7)–10^{-4} M EDTA. The association exhibits a shallow maximum around 28°; heats and entropies of association are positive but decrease with increasing temperatures. Over most of the temperature range the association is entropy driven. In buffered solutions saturated with toluene the association is greatly enhanced but the K's decrease strongly with increasing temperatures. Entropies of the reaction are close to zero and the reaction appears to be enthalpy driven.

In a series of recent studies we have determined the molecular weight of the bovine liver glutamate dehydrogenase oligomer and shown that it is a hexamer which further associates to polymers of indefinite length (Eisenberg and Tomkins, 1968); we have proposed a model (Eisenberg and Reisler, 1970) for the enzyme oligomer which obeys D_3 dihedral symmetry. Dimensions of the oligomer model were tested with the help of viscosity (Reisler and Eisenberg, 1970) and sedimentation (Reisler *et al.*, 1970) measurements, and the mechanism of association was investigated (Reisler *et al.*, 1970) by equilibrium sedimentation and light-scattering determinations. The enhanced association of the enzyme in solutions saturated with toluene follows a linear polymerization pattern similar to that in the absence of this additive. Quantitative analysis of the angular dependence of scattering (Eisenberg and Reisler, 1971) confirmed the association to long rods, although the detailed conclusions with respect to the distribution of length of these rods exhibited apparent disagreement with conclusions derived from molecular weight determinations by light scattering and equilibrium sedimentation.

In the present communication we report calculations (based on some more extensive molecular weight determinations by light scattering) which emphasize the pitfalls inherent in the fitting of a constant second virial coefficient, A_2 (independent of the molecular size of the particles), and a single association constant, K (independent of the degree of association), for the description of the polymerization reaction. From the temperature dependence of the molecular weights in 0.2 M phosphate buffer and the derived equilibrium constants we calculate thermodynamic parameters ($\Delta G°$, $\Delta S°$, and $\Delta H°$) for the association and contrast these parameters with the temperature dependence observed in solutions saturated with toluene.

Experimental Section

Solutions of bovine liver glutamate dehydrogenase were prepared in phosphate buffer (0.2 M sodium phosphate buffer (pH 7)–10^{-4} M EDTA). Details of preparation of enzyme solutions and a description of the basic light-scattering technique have been given by Eisenberg and Tomkins (1968) and by Reisler *et al.* (1970).

Second Virial Coefficients and Association Constants. In our study (Reisler *et al.*, 1970) of the concentration-dependent association of glutamate dehydrogenase in 0.2 M phosphate buffer we have endeavored to derive association constants, K, at low enough concentrations, c, such that the term due to virial coefficients, A_2, in eq 1 could be neglected and the apparent weight-average molecular weights, M_w, could be identified with the true values $M_w°$. This applies for low enzyme concentrations up to about 0.4 mg/ml. The indefinite association of glutamate dehydrogenase in phosphate buffer, to yield open linear structures was represented by a stacking process with a single association constant, K. For such a process

$$\frac{1}{M_w} = \frac{1}{M_w°} + 2A_2c \qquad (1)$$

$$X_w^2 = 1 + 4Kc \qquad (2)$$

* From the Polymer Department, The Weizmann Institute of Science, Rehovot, Israel. *Received January 26, 1971.*

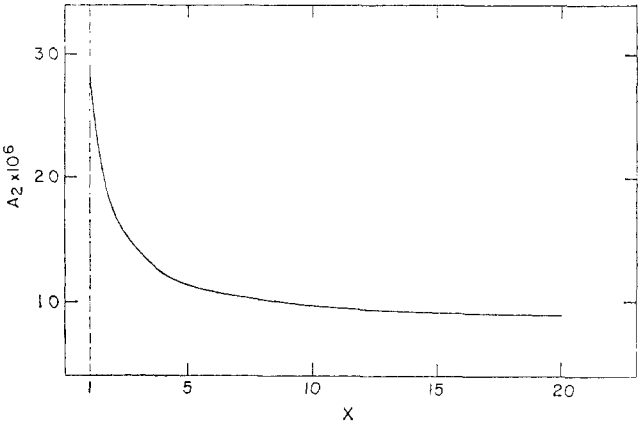

FIGURE 1: Calculation of second virial coefficient, A_2, *vs.* degree of stacking, X, of circular cylinder of length, l = 133 Å, constant radius r = 46.3 Å, and molecular weight, M_1 = 312,000; according to Ishihara and Hayashida (1950).

where $X_w = M_w°/M_1$ is the weight-average degree of polymerization. We have shown that a plot of $X_w^2 - 1$ is linear with c at concentrations below 0.4 mg/ml; downward curvature, apparently due to virial coefficients, appears at higher concentration. An alternate explanation could also be that the association constant, K, decreases with increasing degree of polymerization; this cannot be easily established because in a system in chemical equilibrium (in which particles associate in dependence of concentration) it is difficult to separate opposing influences due to either molecular association or positive virial coefficients. In enzyme solutions in 0.2 M phosphate buffer saturated with toluene a rather fortunate circumstance arises in view of the fact that high molecular weights are obtained at low concentrations of enzyme. Thus, over a considerable range of molecular weights, it is possible to neglect the second term on the right-hand side of eq 1 with respect to the first term and identify the apparent value M_w with the true value $M_w°$. We thus feel that it is possible, both in 0.2 M phosphate buffer, and in solutions saturated with toluene to derive association constants, K, at low enzyme concentrations which are independent of special assumptions with respect to A_2. In 0.2 M phosphate buffer, at 20°, K = 2.0 ± 0.1 ml/mg (Reisler *et al.*, 1970) and the uncertainty is due to the fact that measurements have to be carried to very low concentrations at which $X_w^2 - 1$ is rather sensitive to the experimental values of both M_w and M_1. In the case of solutions saturated with toluene, $X_w^2 - 1$, at comparable enzyme concentrations is 10–20 times higher and from linear plots of $X_w^2 - 1$ *vs.* c we derive an association constant K = 28.8 ml/mg at 20° (Reisler, 1971).

It has been customary, both in the case of purine stacking (Van Holde and Rossetti, 1967) and in an analysis by Chun and Kim (1969) of our own earlier data (Eisenberg and Tomkins, 1968) on glutamate dehydrogenase in 0.2 M phosphate buffer, 25°, to essentially combine eq 1 and 2 (the algebra is sometimes given in a more complicated form, but the procedure is basically the same) and to adjust A_2 in such a way that two constant parameters (K and A_2) describe the behavior of the system over the whole concentration range. In other cases procedures have been developed for the analysis of ultracentrifuge data (Roark and Yphantis, 1969) in which, by suitable combination of the apparent moments of the molecular weight distribution curve M_{z+1}, M_z, M_w, and M_n, A_2 was eliminated, and so called "charge-free" moments defined. We feel that these procedures must be viewed with caution both on theoretical and experimental grounds. For instance, in the case of glutamate dehydrogenase we believe that K is constant at low enzyme concentrations. On the other hand, we do not know whether curvature in the plot $(X_w^2 - 1)$ with c at higher concentrations is due to slight decrease in K or to the influence of nonideality terms. As long as, for instance, virial coefficients cannot be directly determined by the determination of M_w on particles for which $M_w°$ does not depend on concentration, the uncertainty will persist.

Second virial coefficients may depend on molecular size and asymmetry. Ishihara and Hayashida (1950) have calculated the virial coefficients for various geometrical forms in particular for ellipsoid and cylindrical shapes. For a cylinder of radius r, and length L, they find

$$A_2 = \frac{4N_A V}{M^2}\,\frac{\pi r^2 + (\pi + 3)rL + L^2}{8rL} \qquad (3)$$

where N_A is Avogadro's number and $V = \pi r^2 L$ is the volume of the cylinder; eq 3, for long cylinders of constant radius, becomes independent of M because L is proportional to M,

$$A_2 = \frac{\pi N_A r L^2}{2M^2}$$

a result already found by Zimm (1946) and used by us previously (Eisenberg and Tomkins, 1968). We note that these calculations take into account positive deviations from ideality due to geometric volume exclusion only, and not the more complicated excluded volume problem (Berry and Casassa, 1970) in which attractive or repulsive forces between molecules are taken into account as well. Once more, in a system in chemical equilibrium, it seems that a clear separation of association constants and nonideality terms is rather difficult to achieve. We present below (Figure 1) as an example a calculation of A_2 according to eq 3, for a structure approximating glutamate dehydrogenase polymers; l = 133 Å, r = 46.3 Å (this is the cylindrical envelope of the proposed enzyme structure, *cf.* Reisler and Eisenberg, 1970, Figure 4), $L = Xl$. We have plotted A_2 as a function of X, the degree of polymerization, and see that A_2 decreases from $A_2 = 28 \times 10^{-6}$ (mole ml)/g^2 at X = 1 to a constant value $A_2 = 8 \times 10^{-6}$ (mole ml)/g^2 at high values of X.

An attempt at calculating K as a function of c, for various assumed values of A_2, is shown in Figure 2. Indeed, at low concentrations of glutamate dehydrogenase (data below 1 mg/ml have not been drawn, for the sake of clarity), all values of K converge to 2 ml/g, as previously derived (Reisler *et al.*, 1970) for this system (0.2 M phosphate buffer, 20°) from both light scattering and sedimentation equilibrium. At higher concentrations light-scattering data only have been used. It does not appear possible to fit a unique set of values of K and A_2 to the data. We must conclude that we have at present no good way of telling whether K indeed remains constant at high enzyme concentrations and what precise value A_2 assumes at each particular value of c.

It is instructive to consider the data over a limited concentration range (between 3 and 8 mg per ml) shown in the stippled area in Figure 2. In this region it is possible to fit such a value of A_2 that K remains constant in this range. (The value obtained for K by this procedure is lower (~1.6 ml/mg) than the value previously obtained (~2.0 ml/mg) at very low values of enzyme concentration.) This is in essence the procedure

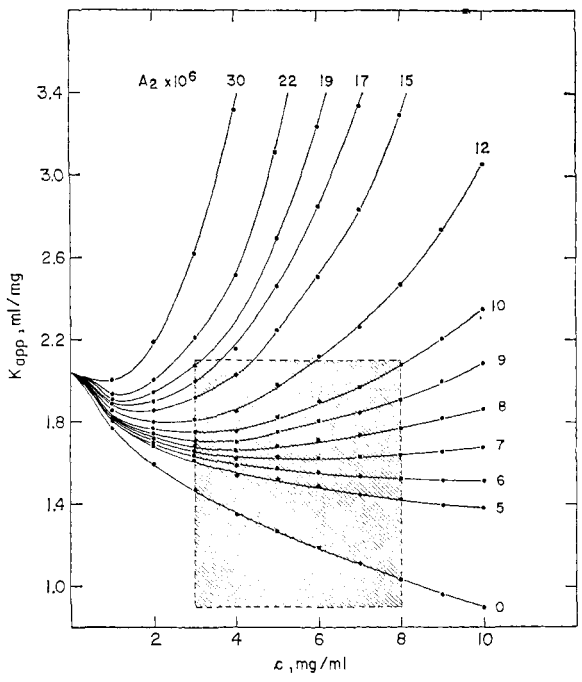

FIGURE 2: Variation of apparent equilibrium constants, K_{app}, for various assumed values of the second virial coefficient, A_2; based on light-scattering measurements in solutions of glutamate dehydrogenase at 20° in 0.2 M phosphate buffer; for details, see text.

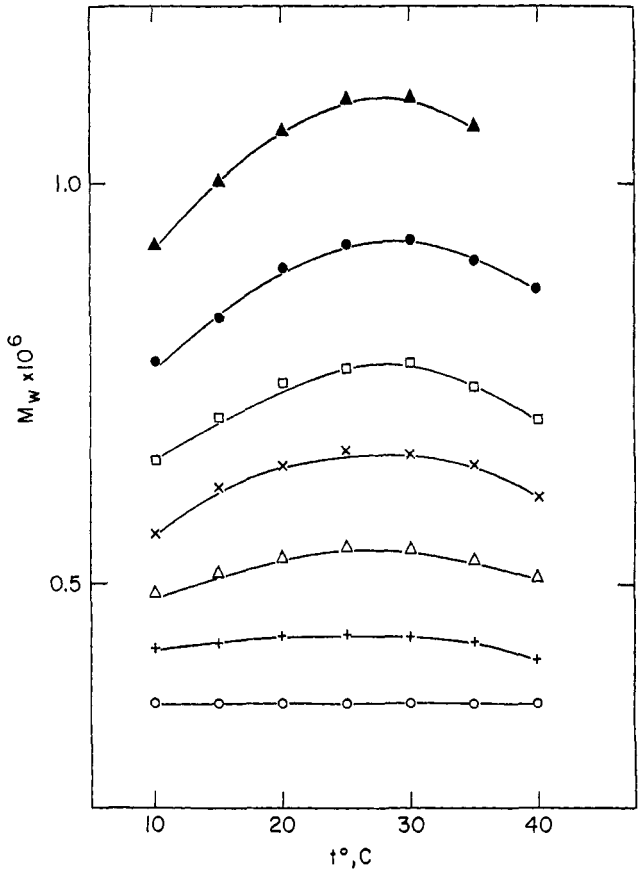

FIGURE 3: Temperature dependence of molecular weight, M_w, of glutamate dehydrogenase in 0.2 M phosphate buffer; concentrations (in milligrams per milliliter) in ascending order: 0.027, 0.094, 0.23, 0.44, 0.69, 1.10, 1.30.

used by Chun and Kim (1969); compare their Table II. They have rejected all data below enzyme concentrations of 3 mg/ml (compare blank spaces in their table), in which region both K and A_2 ($2A_2M_1 \equiv BM_1$ in their nomenclature) are changing to other values. The value of K at 25° (at which temperature the data of Eisenberg and Tomkins (1968) were obtained) derived from measurements at low concentrations is about 2.1 ml/g. We hope to have shown clearly that description of the molecular weight dependence on concentration in associating enzyme systems by parametric fitting of a unique association constant and nonideality term is fraught with danger.

Temperature Dependence of Association. The reversible temperature dependence of M_w in 0.2 M phosphate buffer, at various concentrations in the temperature range 10–40°, is shown in Figure 3. At low enzyme concentrations the dependence of M_w on temperature is very slight; at higher concentrations M_w increases with increasing temperature at low temperatures, reaches a maximum around room temperature, and decreases again with further increasing temperature.[1] This is to be contrasted with the behavior in low ionic strength (0.05 M) phosphate buffer (Frieden, 1962) and in Tris (0.05 M containing 0.1 M NaCl) buffer (Cassman and Schachman, 1971),[2] in which M_w continuously decreases with increasing temperature in the range 11–33 and 4–19°, respectively. The decrease of M_w in Tris buffer can be accounted for by the considerable dependence of the pH of the buffer on temperature. The pH of phosphate buffer, on the other hand, is almost constant in the temperature range 10–35°. We may therefore ascribe the change in molecular weights in phosphate buffer with temperature to a shift in association constants at constant pH. The change observed in the dependence of M_w on temperature in 0.2 M phosphate buffer is paralleled in that both intrinsic viscosity (Reisler and Eisenberg, 1970) and velocity sedimentation coefficients (Reisler *et al.*, 1970) increase between the two temperatures 10 and 20°.

In Figure 4 we show the equilibrium association constants, K, as a function of temperature and the standard free energy, $\Delta G°$, for the association process, as derived from the K's (converted into units of liter per mole of oligomer) by the standard thermodynamic relation; the association constants, K, were obtained, as previously described (Reisler *et al.*, 1970)

[1] Temperature studies in this buffer to higher temperatures indicate that at about and above 43° prolonged heating causes slow denaturation and aggregation of the enzyme. An enzyme solution at a concentration of 0.5 mg/ml was heated for 2 hr at 45°. The scattering and scattering dissymmetry increased strongly and irreversibly, as evidenced by unchanged scattering when the solution was cooled back to room temperature. Almost all (90%) of the biological activity was maintained. It was possible to remove the highly scattering material by centrifugation for one hour at 15,000 rpm, and without further loss in activity. Thus it became clear that, even after prolonged heating at 45°, only a small amount of material undergoes denaturation. In general it was observed that solutions of glutamate dehydrogenase were more stable to heat treatment (when tested for biological activity) at lower concentrations. Enzyme association thus apparently lowers the resistence to denaturation. We are grateful to Mrs. Mira Kedar-Blake for these measurements.

[2] We were apparently extremely fortunate to use the 0.2 M phosphate buffer described above in all our measurements. Phosphate buffer protects and stabilizes the enzyme solutions (Di Prisco and Strecker, 1966), and reversible equilibrium conditions are swiftly achieved. Tris buffers, on the other hand (and to some extent low ionic strength phosphate buffers), which have been used by other research groups destabilize the enzyme solutions (Di Prisco and Strecker, 1966) and lead to slow aggregation and precipitation. This may be one of the reasons for largely divergent results obtained in the past in physicochemical studies of glutamate dehydrogenase solutions.

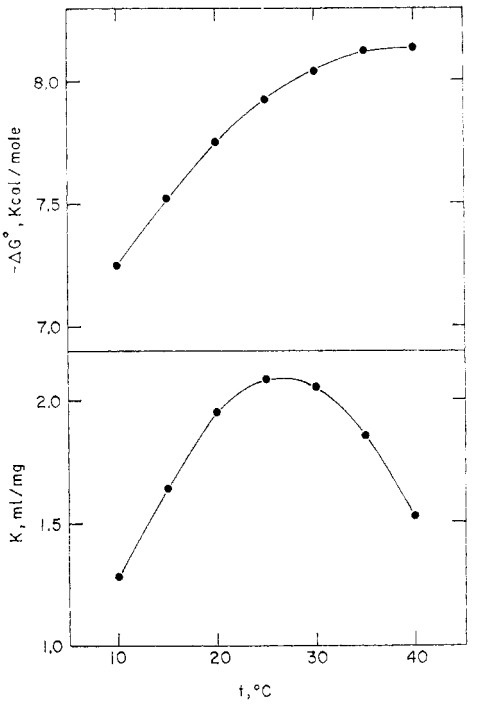

FIGURE 4: Equilibrium constant, K, and standard free energy, $\Delta G°$, of glutamate dehydrogenase association reaction, calculated from light-scattering data.

from light-scattering measurements at concentrations up to 0.44 mg/ml at various temperatures. In Figure 5 we present the standard free enthalpy, $\Delta H°$, and standard free entropy, $\Delta S°$, for the association derived from the temperature dependence of $\Delta G°$.

Many efforts have been invested in the study of nature of bonds and forces responsible for the aggregation of proteins. Yet until a detailed atomic structure of the protein is known, these investigations are mostly of speculative character, and rarely can they bring a decisive evidence in favor of one of the postulated mechanisms.

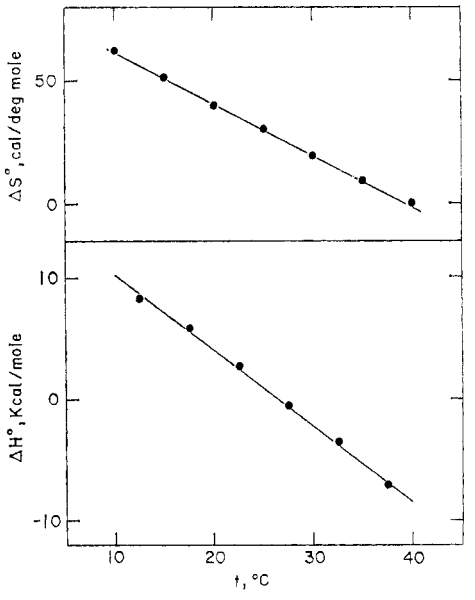

FIGURE 5: Standard free enthalpy, $\Delta H°$, and entropy, $\Delta S°$, of glutamate dehydrogenase association reaction, calculated from light-scattering data.

The bulk of the studies performed runs along three lines: derivation of thermodynamic parameters of the association process (temperature dependence of the aggregation), consideration of electrostatic forces involved in the reaction (pH dependence of the association), and influence of various solvents and additives on the association state of the protein. With respect to glutamate dehydrogenase the last two approaches have been utilized in past to probe into the question of forces governing the association reaction. The observed pH dependence of the association of glutamate dehydrogenase (Frieden, 1962; Sund *et al.*, 1970; Cassman and Schachman, 1971) and the instability of the enzyme in low ionic strength buffers seem to support participation of electrostatic interactions in the aggregation mechanism. On the other hand, it was found that dioxane dissociates the enzyme (Churchich and Wold, 1963) and that D_2O enhances the polymerization reaction (Henderson *et al.*, 1970). Both effects are usually attributed to the presence of hydrophobic interactions (Kauzman, 1959; Lee and Berns, 1968), yet we cannot exclude the possibility that they are but secondary products of conformational changes brought about by these solvents.[3]

In this work we present a study of temperature dependence of the association reaction followed in 0.2 M phosphate buffer. The increase of association constants with increase in temperature (Figure 4), in the range 10–28°, seems to support the prevalence of hydrophobic contributions. Hydrophobic bonds are believed to be stabilized at higher temperatures. The tendency of association constants to decrease above 28° with a further increase in temperature indicates an opposing tendency which may be ascribed to charged group interactions. The last notion is also supported by the increased liability to heat denaturation[1] of the associated form of the enzyme.

The free energies, $\Delta G°$ (Figure 4), are negative and increase toward a limiting value with increasing temperature. Inspection of Figure 5 reveals that the association reaction is entropy driven at low temperatures and the increase in $T\Delta S$ outweighs the unfavorable increase in ΔH. As a result of polymerization only (without participation of solvent) a loss of entropy should be expected. The favorable entropy change is presumably due to electrostatic or hydrophobic interactions with solvent (Steinberg and Scheraga, 1963). The enthalpy changes from positive to negative values as the temperature rises above 28° and at the same time $T\Delta S$ drops to values close to zero; thus in this temperature range the association is driven by the favorable enthalpy change. In 0.05 M phosphate buffer, Frieden (1962) observed a continuous decrease in M_w with increasing temperature (in the range 11–33°) which may be due to an enhancement of electrostatic interactions at the lower ionic strength.

Association of glutamate dehydrogenase is greatly enhanced upon saturation of enzyme solutions with toluene. In the presence of toluene, at the temperatures 10, 20, and 25°, the association constants, K, decrease from 52 to 28.8 to 21.7 ml per

[3] The release of water molecules believed to be associated with the creation of a hydrophobic bond in aqueous solutions should produce a measurable increase in volume. Direct measurements of the volume change upon glutamate dehydrogenase dissociation to oligomers have been attempted by Willick (1967); no measurable volume change was observed. We have determined sedimentation coefficients of glutamate dehydrogenase in 0.2 M phosphate buffer, at 20°, at concentrations 3–5 mg/ml, in the velocity range 30,000–60,000 rpm and could not observe any influence (due to changes in volume) of the pressure gradient on the sedimentation coefficients.

mg; from the standard relationship we derive that $\Delta G°$ is almost constant (and equal to -9.3 kcal/mole), $\Delta S°$ is zero within experimental error, and $\Delta H°$ is equal to $\Delta G°$. It thus appears that the reaction is driven by a favorable enthalpy term, which corresponds to the behavior in the branch above 28° in the enzyme system in 0.2 M phosphate buffer only.

It is worth noting that the extent of association enhancement by toluene does not parallel the solubility of toluene in enzyme solutions. Toluene, as well as other hydrocarbons (Bohon and Claussen, 1951), exhibits a solubility minimum near 18° corresponding to zero enthalpy of solution. Had the association enhancement been a function of toluene solubility we would expect a higher association constant at 25° than at 20°. We find the opposite at higher temperatures the association constant, K, is further decreased. Hydrocarbon solubility though may play a role in the association process because benzene, which is somewhat more soluble than toluene in aqueous solutions, lead to higher association than the latter (Reisler, 1971).

In summary a great deal more could be said about hydrophobic and other interactions, and about forces which bring protein molecules together, and hold them apart. Unfortunately much of what could be stated at this stage would not withstand sober criticism. We hope that further work along the lines developed here and also recent sequence studies (Smith *et al.*, 1970), and maybe future X-ray diffraction investigations, may permit analysis of this interesting system on a quantitative level. We might for instance learn why the glutamate dehydrogenase oligomer, which is formed to two superimposed triangular layers (Eisenberg and Reisler, 1970) does not dissociate into trimers, and in what way the polarity of the subunits determines the association reaction. We have recently encountered (Eisenberg and Reisler, 1971) some unexpected difficulties in the interpretation of the angular distribution of the scattered light, and the various phenomena may be intimately connected.

References

Berry, G. C., and Casassa, E. F. (1970), *Macromol. Rev. 4*, 1.

Bohon, R. L., and Claussen, W. F. (1951), *J. Amer. Chem. Soc. 73*, 1571.

Cassman, M., and Schachman, H. K. (1971), *Biochemistry 10*, 1015.

Chun, P. W., and Kim, S. J. (1969), *Biochemistry 8*, 1633.

Churchich, J. E., and Wold, F. (1963), *Biochemistry 2*, 781.

Di Prisco, G., and Strecker, H. J. (1966), *Biochim. Biophys. Acta 122*, 413.

Eisenberg, H., and Reisler, E. (1970), *Biopolymers 9*, 113.

Eisenberg, H., and Reisler, E. (1971), *Biopolymers* (in press).

Eisenberg, H., and Tomkins, G. M. (1968), *J. Mol. Biol. 31*, 37.

Frieden, C. (1962), *J. Biol. Chem. 237*, 2396.

Henderson, R. F., Henderson, T. R., and Woodtin, B. M. (1970), *J. Biol. Chem. 245*, 3733.

Ishihara, A., and Hayashida, T. (1950), *J. Phys. Soc. Jap. 6*, 40.

Kauzmann, W. (1959), *Advan. Protein Chem. 14*, 1.

Lee, J. J., and Berns, D. S. (1968), *Biochem. J. 110*, 465.

Reisler, E. (1971), Ph.D. Thesis, The Weizmann Institute of Science, Rehovot, Israel.

Reisler, E., and Eisenberg, H. (1970), *Biopolymers 9*, 877.

Reisler, E., Pouyet, J., and Eisenberg, H. (1970), *Biochemistry 9*, 3095.

Roark, D. E., and Yphantis, D. A. (1969), *Ann. N. Y. Acad. Sci. 164*, 245.

Smith, E. L., Landon, M., Piszkiewicz, D., Brattin, W. J., Langley, T. L., and Melamed, M. D. (1970), *Proc. Nat. Acad. Sci. U. S. 67*, 724.

Steinberg, I. Z., and Scheraga, H. A. (1963), *J. Biol. Chem. 238*, 172.

Sund, H., Markau, K., Minssen, M., and Schneider, J. (1970), *Wenner Gren Symp., Stockhomm, Abstr.*

Van Holde, K. E., and Rossetti, G. P. (1967), *Biochemistry 6*, 2189.

Willick, G. E. (1967), Ph.D. Thesis, University of Illinois, Urbana, Ill.

Zimm, B. H. (1946), *J. Chem. Phys. 14*, 164.