Equilibrium Sedimentation of Proteins in Acid Solutions. Dissociation of Aldolase by Aqueous Acetic Acid†

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ABSTRACT: The molecular weight of native aldolase at neutral pH and at moderate ionic strengths has been found to be $(161.0 \pm 3.3) \times 10^3$ using high-speed equilibrium ultracentrifugation. As has been reported previously, aldolase dissociates in 0.05 M HCl–0.01 M NaCl (pH 2.0). However, the resultant solutions are so heterogeneous that the resultant nonideality of these solutions is not seen but rather is masked by heterogeneity. Aqueous acetic acid from 0.1 to 10 M, with no added supporting electrolyte, has been used to dissociate aldolase into subunits. Aldolase shows only small heterogeneity in 1 M acetic acid, provided that care is taken in the selection and preparation of samples. Significantly more heterogeneity is observed in 0.1 M acetic acid, suggesting incomplete dissociation. Conversely, aldolase in 10 M acetic acid appears to behave virtually as a single thermodynamic component. All these acetic acid concentrations were clearly effective in dissociating aldolase to subunits of about 40,000 daltons. The limiting nonideality observed in these acetic acid–water solutions has been analyzed to give estimates of the apparent effective charge per molecule. No unique models were found to describe higher terms in the nonideality of these systems. An analysis of the errors involved in assuming the native partial specific volume for the interpretation of experiments under such dissociating conditions showed that either these errors are individually small or they effectively cancel each other, quite closely in 0.1 and 1 M but not as closely in 10 M acetic acid solutions of aldolase.

Most large proteins appear to be aggregates of polypeptide chain subunits. Often these polypeptide chains are held together by noncovalent bonds (secondary forces) and can be dissociated by a variety of reagents. A common and elegant way of determining the quaternary structure of such a protein is to determine the apparent molecular weight of the molecule in its native state and to compare this value to that obtained under dissociating conditions. The ratio of these two values then gives an estimate of the number of subunits, provided the subunits are relatively uniform in size.

Systems under dissociating conditions are only seldom homogeneous in size. Perusal of the literature shows that some aggregated material is very often present under conditions commonly used for dissociation. The choice of technique for molecular weight estimation in such systems is critical. Any method that does not introduce fractionation during the experiment (*e.g.* low-speed sedimentation equilibrium, light scattering, osmometry), and that therefore gives a molecular weight average over the entire sample, may lead to apparent values for the subunit molecular weight that are grossly in error. High-speed sedimentation equilibrium offers the possibility of such a fractionation and therefore should be particularly useful for the study of dissociation into subunits (Yphantis, 1964).

The prime object of this work was the application of this high-speed sedimentation equilibrium technique to the study of proteins in dissociating media. We chose rabbit muscle aldolase for this study because of the controversial data in the literature at the time indicating three, four, and even six subunits (Stellwagen and Schachman, 1962; Deal *et al.*, 1963; Hass and Lewis, 1963; Hass, 1964; Schachman and Edelstein, 1966; Kawahara and Tanford, 1966; Chan *et al.*, 1967).

Studies of the amino- and carboxy-terminal amino acids indicated that rabbit muscle aldolase was a multichain enzyme. Thus, digestion of aldolase with carboxypeptidase gave 3 mol of tyrosine/mol of aldolase (Kowalsky and Boyer, 1960; Winstead and Wold, 1964). Stellwagen and Schachman (1962) dissociated aldolase into subunits. In accord with the chemical results, these authors found the molecular weight of the protein in acid (pH 2) or 4 M urea to be about one-third of the value for the native protein. Hence, they suggested a three-subunit structure for aldolase. Results supporting the above conclusion were also obtained by Deal *et al.* (1963).

However, Hass and Lewis (1963) and Hass (1964) working at alkaline pH obtained molecular weights closer to one-sixth of the accepted value for the native protein. Furthermore, quantitative determinations of the N-terminal amino acids gave four *N*-prolines/mol of aldolase; Hass (1964) interpreted this as representing minimal values and as further evidence for a six-subunit structure.

Further challenge to the trimeric structure came from the work of Kawahara and Tanford (1966). Using 6 M guanidine-HCl + 0.1 M β-mercaptoethanol as the dissociating agent, they obtained data for the molecular weight which suggested a tetrameric structure.

A four-subunit structure for rabbit muscle aldolase now appears to be well established. New physiocochemical and chemical data are all consistent with the above conclusion. Thus, hybridization experiments with different, naturally occurring aldolases (Penhoet *et al.*, 1966, 1967) and with chemically modified aldolases (Meighen and Schachman, 1970) are better explained on the basis of four subunits.

From studies in other laboratories (Sia and Horecker, 1968; Castellino and Barker, 1968; Reisler and Eisenberg,

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1969) and from our own work (Szuchet and Yphantis, 1968) it appears that the molecular weight of the subunit of rabbit muscle aldolase is in the vicinity of 40,000. This, in conjunction with the now accepted value for the native molecule (see Kawahara and Tanford, 1966; also see above references), points again, rather convincingly, to a tetrameric structure.

Finally, newer data on N- and C-terminal amino acids (Morse *et al.*, 1967; Kochman *et al.*, 1968) are also consistent with this structure.

The method we propose here for the study of protein subunits uses acetic acid without added electrolytes as the dissociating agent. This method should be potentially applicable to many dissociating systems. It has already been used for the study of the quaternary structure of myosin (Szuchet and Zobel, 1969).

Materials and Methods

Materials. Crystalline rabbit muscle aldolase [suspension in 2 M $(NH_4)_2SO_4$] grade A was purchased from Calbiochem Inc., Sigma Chemical Co., or Mann Research Laboratories, and was used without further purification. Prior to an experiment, the suspension of crystals was centrifuged at 3500 rpm and the precipitate dissolved in water. Originally, this solution was dialyzed against water for 24–48 hr and then against the desired solvent for another 24–48 hr. We soon abandoned this procedure in favor of the following more rapid method that required only 2–4 hr: the protein solution was desalted through a Dintzis (1952) column (*vide infra*). The protein contained in the effluent was then equilibrated with the desired solvent on a 100 cm × 1 cm column of Sephadex G-25. The effluent from this latter column was used directly for the experiments. Dialysis tubing from Union Carbide was used. Unless otherwise stated, all dialysis tubing was first boiled in 10% $NaHCO_3$, then washed with glass distilled water, and boiled in water, to remove the $NaHCO_3$. Finally, the tubing was stored in 0.01 M Na_2EDTA (pH ~7), in the cold. Treated dialysis tubing was never kept longer than 10 days and was thoroughly rinsed with glass distilled water before use. When using organic solvents, the dialysis tubing was soaked in the solvent for 2 hr. Ion exchangers were purchased from Bio-Rad Laboratories, Richmond, Calif.

Equipment and Procedures. The Spinco Model E analytical ultracentrifuge used was equipped with electronic speed control, a temperature-control unit calibrated against a National Bureau of Standards certified thermometer, and schlieren, Rayleigh interference, and ultraviolet (uv) scanner optics. The interference and schlieren optics were used for most of the experiments reported here. The alignment procedure has been described previously (Yphantis, 1960, 1964). Photographs were taken at both the midpoint and two-thirds focus of the camera lens and were recorded on Kodak spectroscopic plates, emulsion type II G, developed with H.R.P. developer (Kodak). Exposure times of about 15 min were necessary using a Polaroid filter on top of the 77-A filter with the limiting aperture of the interference optical system reduced to 1 mm and with the AH-6 lamp voltage reduced to 90% of its usual value (Ansevin *et al.*, 1970).

High-speed sedimentation equilibrium experiments were carried out as described (Yphantis, 1964) using externally loaded six-channel "Rexolite" centerpieces (Ansevin *et al.*, 1970) either 12 or 30 mm thick, with sapphire windows. This type of cell allows three solution–solvent pairs to be run simultaneously and enables blanks to be run before and after each experiment without disassembling the ultracentrifuge cell. Further operating details for these cells may be found in the last reference cited.

Equilibrium usually was attained in about 9 hr for the acetic acid solutions in the 3-mm high-solution columns used. Nevertheless, the runs were continued for a total time of at least 24 hr at any given speed. Photographs were taken at approximately 4-hr intervals to obtain a complete record of the experiment.

Photographic plates were measured by the usual method as described by Yphantis (1964) with the following modifications: blanks were measured every 200 μm on the *x* coordinate. Fringe displacements were determined every 200 μm in the meniscus region. The measurement spacing was changed to 100 μm when the apparent fringe displacement from the meniscus exceeded 50 μm. The alternative method of measuring the *x* coordinates corresponding to every one-half fringe displacement was used in regions with steep and extensive gradients, such as at high concentrations under highly nonideal conditions. In either procedure every point was an average of five measurements, with the mean value never differing from the middle point by more than 5 μm. Approximately 30–40 points were measured for each blank and 40–60 points for each experiment. The data were analyzed with the aid of a Fortran IV computer program developed in our laboratory (Roark, 1971; Roark and Yphantis, 1969; D. E. Roark and D. A. Yphantis, manuscript in preparation). This program calculates four of the standard apparent reduced molecular weight moments, $\sigma_n(r)$, $\sigma_w(r)$, $\sigma_z(r)$, and $\sigma_{z+1}(r)$, at each appropriate observation radius, *r*. These $\sigma_k(r)$ are defined as experimental observables and are proportional to the usual apparent molecular weight averages at each radius, $M_{k,a}(r)$ under certain assumptions (Yphantis, 1964). In addition, the program calculates seven "ideal" molecular weight moments that are identically independent of the second virial coefficient for systems composed of a single molecular species and for systems in which all the molecular species of the solute have effectively the same nonideality, such as reversibly associating systems wherein the mass action law is obeyed (see Yphantis and Roark, 1972, for details). For such systems one can write (Adams and Fujita, 1963; Roark and Yphantis, 1969)

$$1/M_{w,a} = 1/M_{w,ideal} + 2B_1c \qquad (1)$$

where B_1 is that part of the colligative second virial coefficient arising from the nonideality of the system above and beyond the association equilibrium. Equation 1 accounts for most of the nonideality usually observed with single species solutes: it expresses the major contribution from excluded volume effects (Tanford, 1961) and almost all of the nonideality from the presence of net charge on solute molecules (Donnan effect). Higher virial coefficients are only necessary to describe extreme nonideality: in the case of a symmetrical supporting electrolyte the third and all higher odd virial coefficients sensibly vanish leaving the second and fourth virial coefficients as the only practical contributors for even rather severe nonideality (Roark and Yphantis, 1971). These conclusions may be shown to hold for any system with all the solute species having a common charge to mass ratio. This is true to a first approximation for a single monomer–polymer system, whether reversibly associating or not. It is in general a poorer approximation if the subunits of the system have different effective charge to mass ratios than the polymer, although it may still be a good working hypothesis.

Protein concentrations were determined with a Zeiss spectrophotometer using an extinction coefficient $E_{1\,\mathrm{cm}}^{0.1\%} = 0.91$ at 280 nm (Baranowski and Niederland, 1949). No corrections were made for any change of the extinction coefficient in acid (Sia and Horecker, 1968) since knowledge of exact absolute concentrations was not required. The partial specific volume of aldolase was taken to be 0.742 ml/g (Taylor and Lowry, 1956; Sia and Horecker, 1968) except where stated. Solvent densities were estimated from entries in the International Critical Tables. No correction was applied for the density increment of the protein itself.

The Dintzis columns used for desalting were made from 10-ml pipets with the top cut off. A little glass wool in the tip supported three ion exchange resin layers: first there was a 4-cm layer of AG 50W-X12 in the H^+ form, then a 17-cm layer made of a mixture of equal parts of AG 50W-X12 (H^+) and AG 1-X10 (OH^-), and lastly a top layer (~7 cm) of a mixture of AG 1-X10 (acetate$^-$) and AG 50W-X12 (NH_4^+).[1] Such columns were freshly packed before use and were used only once.

Results

Native Aldolase. A series of high-speed sedimentation equilibrium experiments was performed on different preparations of the native protein. These experiments were planned to find out whether the commercial preparations appeared homogeneous, *i.e.* behaved as a single thermodynamic component, and to obtain a value for the molecular weight of native aldolase since conflicting results with values ranging from 142 × 10^3 (Stellwagen and Schachman, 1962) to 158 × 10^3 (Kawahara and Tanford, 1966) have been published in the literature.

Several preparations of native aldolase were examined near neutral pH to determine initial homogeneity and the "native" molecular weight. A "Sigma" sample examined in a solvent composed of 0.1 M NaCl and 1% sucrose at 20° and at 18,000 rpm in a 12 mm thick centerpiece gave the same constant values, within experimental error, for the apparent point weight average molecular weights, $M_w(r)$, observed as a function of radius for all three initial loading concentrations used (0.1, 0.3, and 1.0 g/l.); this provides a good indication that the sample is sensibly homogeneous (Squire and Li, 1961; Yphantis, 1964). Other samples also appeared homogeneous but showed some barely significant nonideality with $BM_1 = 27 \pm 10$ and 15 ± 16 ml/g under the same conditions. One sample in an experiment with three different loading concentrations gave barely significant apparent second virial coefficients that were negative. Even though the curves of apparent point weight average molecular weight as a function of observation concentration were within the (somewhat greater than usual) experimental error of each other for the different loading concentrations, we believe that this particular sample contained some irreversibly aggregated material: less than 5% dimer (or even less of a higher polymer) would have accounted for the data. Other aldolase preparations clearly showed significant amounts of aggregates and were not used for further studies, nor were any results from such samples included in the calculation of the average molecular weight.

The average value of the molecular weight extrapolated to zero concentration in 0.1 M NaCl + 1% sucrose was (161.0 ± 3.3) × 10^3 daltons using the usual value of 0.742 ml/g for the partial specific volume (Taylor and Lowry, 1956). The somewhat lower value ($\bar{v}$ = 0.737 ml/g) of Reisler and Eisenberg (1969) yields (157.6 ± 3.2) × 10^3 daltons for the mean estimate of the infinite dilution value of the molecular weight of native aldolase in dilute salt solutions. No evidence was found supporting the reported (Kawahara and Tanford, 1966) dissociation of aldolase into halves at low ionic strengths near neutrality. However, experiments at higher salt concentrations ($I > 0.5$ M) yielded progressively lower values for the apparent molecular weight of aldolase. These results appear most readily interpreted in terms of a partial dissociation by high salt concentrations and will be reported on elsewhere.

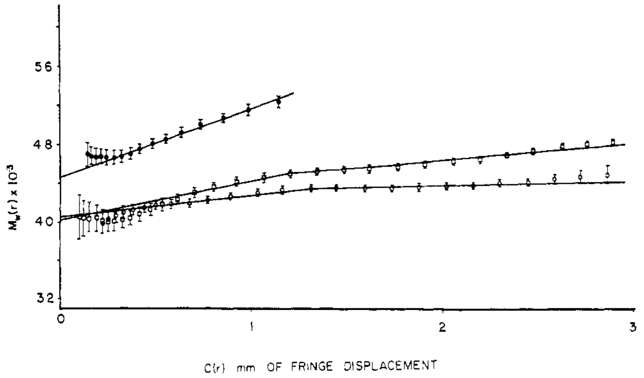

FIGURE 1: Apparent weight average molecular weights, $M_w(r)$, presented as a function of $c(r)$ for initial concentrations of (●) 0.08 g/l., (□) 0.24 g/l., and (○) 0.35 g/l. in 0.005 M HCl–0.1 M NaCl. The experiment was carried out at 20° and at 30,000 rpm in a 30 mm thick centerpiece. One-millimeter fringe displacement corresponds here to a concentration of about 0.4 g/l.

During the course of these experiments with the native aldolase it was noticed that an occasional preparation, after standing for several days in the cold, developed significantly increased heterogeneity and showed much lower values of molecular weight extrapolated to zero concentration. We believe that these observations represent progressive degradation of the aldolase with time, most likely from adventitious proteases or bacterial contamination. Not all of our samples showed such behavior, but to obviate complications which might arise for data obtained under dissociating conditions we reduced the time of handling of the initial material to a few hours (see Materials).

Dissociated Aldolase. Stellwagen and Schachman (1962) dissociated aldolase in 0.05–0.1 M NaCl at pH 2.0 and obtained an apparent weight average molecular weight of 46 × 10^3 by low-speed sedimentation equilibrium. We performed an experiment under similar conditions, but used high-speed sedimentation equilibrium instead. The results are shown in Figure 1. Here we plot $\sigma_w(r)$ *vs.* $c(r)$, the concentration at the points corresponding to $\sigma_w(r)$, for three different loading concentrations. The observation of three separate curves, one for each loading concentration, is a strong indication of heterogeneity. The data obtained at the lowest initial concentration give a curve lying well above the others. This would be expected if the system had high molecular weight material (aggregates) present since only in this channel with the lowest initial concentration could we observe the complete solute distribution including the base of the solution column.

Two types of extrapolations can be performed readily with such data from a high-speed sedimentation equilibrium experiment (Yphantis, 1964). Estimates of the values of σ for the smallest species present (to a significant extent) can be obtained by extrapolation of the $\sigma(r)$ *vs.* $c(r)$ to $c = 0$. In addition, a graph of $\sigma_n(r)$ *vs.* r, extrapolated to the base of the so-

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[1] The ammonium from the Dowex AG-50 was often omitted when the aldolase solutions contained only ammonium ions as cations.

lution column (to r_b), yields a measure of the apparent weight average molecular weight ($\bar{\sigma}_{w,a}$) of the solution observed and presumably of the original sample if adsorption of solute is assumed to be absent. Similarly, extrapolation of $\sigma_w(r)$ *vs.* r to the base gives an index of the apparent z-average molecular weight ($\bar{\sigma}_{z,a}$) of the solute. Such extrapolations to obtain average properties for the entire solute are meaningful only if the data to be extrapolated represent all of the solute in the cell. Since most of the solute lies near the base, reliable data must be available from this most centrifugal portion of the solution column. Observation of this base region is only possible at the lowest loading concentrations when using high speeds; the extreme gradients generated with the higher loading concentrations preclude any observations there. However, experiments with such high loading concentrations are useful in that they serve to emphasize the lower molecular weight species that predominate closer to the meniscus and often allow reliable estimation of their properties. This can be of significant advantage if, as seems to be the case here, the solute is contaminated by a few per cent of aggregated (or undissociated) solute.

Extrapolation of the data for the observation channel (A) with the lowest loading concentration to the base of the cell gave $M_n(b) = \bar{M}_{w,a} = (49.5 \pm 1.5) \times 10^3$ and $\bar{M}_w(b) = \bar{M}_{z,a} = (53.5 \pm 2) \times 10^3$ daltons.[2] These two values for the overall averages are almost within estimated error of each other and correspond to values observed by Deal *et al.* (1963) for their aldolase under somewhat similar conditions.

It is clear from Figure 1 that the apparent weight average molecular weights for the two channels with the highest loading concentrations extrapolate to values near 40,000 as the concentrations at which they were observed approach zero. Thus, there must be present some species with mol wt 40,000 or lower. The three curves of weight average molecular weight as a function of observation concentration for the different loading concentrations clearly are not superposable but rather are quite different. Thus, our sample of aldolase showed considerable heterogeneity in this solvent, indicating only partial dissociation and the presence of aggregates not in equilibrium with dissociated aldolase subunits. This heterogeneity would not have been detected by the usual criterion that the overall weight and z-average molecular weights differ for significant heterogeneity.

The presence of strong charge on any solute will cause nonideal sedimentation with apparent molecular weight averages decreased below their ideal values (see, for example, Williams *et al.*, 1958). The effect of this nonideality is greater on the z-average molecular weight than on the weight average. (Indeed, to first order, the decrease in the z average is twice that of the weight average: Van Holde and Baldwin, 1958; Roark and Yphantis, 1969). The lack of clear-cut heterogeneity discrimination by the cell average molecular weight moments observed here is most likely caused by partial compensation of the heterogeneity differences by the charge nonideality.

The aldolase system appeared too complex in 0.1 M NaCl at acid pH to readily provide much further information on the subunits. In particular, these conditions did not seem well suited for answering questions as to whether or not the subunits are distinguishable in their sedimentation properties. We turned, therefore, to other dissociating systems, deliberately avoiding concentrated urea or guanidinium chloride solutions, largely because of the complications attendant to preferential solvent binding. (See Kirbe-Hade and Tanford (1967), Noelken and Timasheff (1967), and Reisler and Eisenberg (1969) for reports on preferential interaction of aldolase with guanidinium chloride and the effects of this binding on the interpretation of ultracentrifuge data.) We considered, instead, dissociating agents whose aqueous solutions show only small density increments over water. For these agents[3] $1 - \bar{v}_3\rho$ is small and, hence, preferential binding of such agents should affect observations of apparent molecular weight only slightly in comparison to agents with large density increments. The exact magnitude of the perturbation depends on exactly how the equilibrium experiment is observed. Williams *et al.* (1958) present equations appropriate to several observation techniques. They show that whenever one can observe the concentration of component 2 alone (and its concentration gradient) then the apparent molecular weight, extrapolated to infinite dilution, is given by

$$\lim_{c_2 \to 0} M_{2,a} = M_2[(1 + \Gamma'(1 - \bar{v}_3\rho)/(1 - \bar{v}_2\rho)] \qquad (2)$$

where Γ' is the preferential binding of component 3 on a per gram basis, $(M_3/M_1)(\partial m_3/\partial m_2)_{\mu_1,P,T}$, and where the symbols are assigned their usual meanings. This equation is directly applicable, for example, to data obtained using the absorbance of component 2 to estimate concentration distributions, as with the absorption scanning system. Equation 2 is also directly applicable to estimates of the z-average molecular weight by the usual refractometric procedures if Γ' is assumed to be constant and independent of the concentration of the macromolecule. In like vein, eq 2 can be shown to be applicable to all the moments observed (even refractometrically) in high-speed equilibrium experiments if the same assumption that Γ' is constant can be made. However, the usual use of refractometric optics to determine the weight average molecular weight in short column experiments (or even low-speed experiments as usually performed) leads to a different expression involving also the refractive index increments of components 2 and 3. (See Williams *et al.* (1958) for further details.)

Equation 2 predicts that when $(1 - \bar{v}_3\rho)$ vanishes there will be *no* perturbation from preferential interactions and the extrapolated values of the apparent molecular weight then will reflect the values of M_2 only. A choice of dissociating agents having small values of $(1 - \bar{v}_3\rho)$ (and therefore small density increments) could therefore sidestep the usual necessity for determination of ϕ' or equivalently Γ' (see Casassa and Eisenberg, 1961; Reisler and Eisenberg, 1969). There exist a large number of potential dissociating agents with small values of $(1 - \bar{v}_3\rho)$ in aqueous solutions; some of these have already been used as dissociating agents, *e.g.*, dioxane (Fredericq, 1957), acetic acid (Yphantis and Waugh, 1958), pyridine (Yphantis and Waugh, 1958), propionic acid (Fleischman *et al.*, 1962), and suitably substituted alkylammonium dodecyl sulfates (C. H. Paul and D. A. Yphantis, work in progress).

Aqueous pyridine (40% w/v)–0.1 M NaCl dissociated aldolase both at pH 7.0 and 9.7 giving apparent subunit molecular weights near 40,000. However, there was considerable

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[2] These values were calculated using the native value of $\bar{v} = 0.742$ ml/g and employing no corrections for "proper" definitions of solute components (*vide infra*).

[3] Following the usual convention the solvent component (water) will be identified as component 1, and the other solvent component (the dissociating agent) as component 3, while the solute of interest (the macromolecule) will be labeled component 2.

heterogeneity present from undissociated aggregates and this solvent system was therefore abandoned.

Dissociation in Aqueous Acetic Acid. We turned next to acetic acid without any other added electrolyte, reasoning that the low ionic strength should help minimize aggregation. Under these conditions most proteins appear to be highly nonideal, as predicted by considerations of the Donnan effect (Johnson *et al.*, 1954; Roark and Yphantis, 1971; also see Williams *et al.*, 1958, for an excellent description of the behavior of simple systems of ionized solutes). For a monodisperse solute of molecular weight M_2 and effective charge z, it can be shown in the limit as $C_2 \rightarrow 0$ that

$$\frac{1}{M_{\mathrm{w,a}}} = \frac{1}{M^*}\left(1 + \frac{z^2}{2}\frac{m_2}{m_3}\right) = \frac{1}{M^*}\left(1 + \frac{z^2}{2}\frac{C_2}{M_2 m_3}\right) \qquad (3)$$

where M^* is the molecular weight observed at infinite dilution,[4] M_3 is the molecular weight and m_3 the molality of the supporting electrolyte, M_2 is the molecular weight of the solute, and C_2 is the concentration of the solute in grams/kilogram of solvent.

Since care was taken to deionize the starting protein solution, the supporting electrolyte in these experiments is made up only of the hydrogen and acetate ions from the dissociation of the acetic acid. The dissociation constant of acetic acid serves to limit the ionic strength in these solutions to rather low values, which can be predicted in the limit $C_2 \rightarrow 0$ as $10^{-\mathrm{pH}}$ with the value of the pH well approximated by $(\mathrm{p}K_\mathrm{A} + \mathrm{p}m_{\mathrm{HA}})/2$, where $\mathrm{p}K_\mathrm{A}$ is the effective acid dissociation constant of the acetic acid and m_{HA} is the total molarity of acetic acid (see Edsall and Wyman, 1958, for an appropriate derivation procedure). This leads to estimates of the ionic strength (m_3) as $\sim(m_{\mathrm{HA}}K_\mathrm{A})^{1/2}$; the ionic strengths expected are only 0.014 M for 10 M acetic acid and 0.0014 M for 0.1 M acetic acid. Such low ionic strengths lead to large differences between $M_{2,\mathrm{a}}$ and M^* for only moderate values of C_2, and the resultant nonideality is, typically, great enough to be directly and distincly visible on the raw experimental records, as illustrated in Figure 2 for aldolase in 1 M acetic acid. Note that the Rayleigh interference fringes at the bases of the solution channels are almost straight and that the schlieren patterns are almost horizontal, in contrast to the exponentially increasing patterns that would be expected exactly for ideal or only approximately for moderately nonideal solutions.

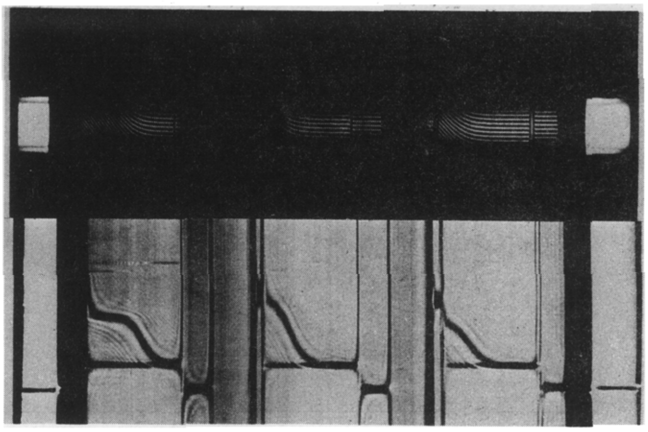

FIGURE 2: Optical records of an equilibrium sedimentation experiment on aldolase in 1 M acetic acid at 52,000 rpm showing the large nonideality of the system. Part a presents the Rayleigh interference pattern and part b the schlieren pattern observed at a 75° phase plate angle.

In addition to this strong nonideality, high-speed equilibrium ultracentrifugation in aqueous acetic acid without added salt suffers complications from the absence of a significant density gradient[5] from redistribution of solvent components in the centrifugal field that would act to provide stability against convective disturbances. Density gradients arising from compression of the solution in the centrifugal field do not provide stability (Pickels, 1942; Yphantis, 1963). Thus, such experiments are observed to be irreproducible, especially in the meniscus region where the redistribution of the solute itself also contributes virtually no density stabilization, unless precautions are taken; in these experiments we have added a little sucrose, typically 0.1%, to provide a stabilizing, self-generated density gradient (Yphantis, 1964).

Figure 3a presents the reciprocals of $\sigma_{\mathrm{w,a}}$, the apparent reduced weight average molecular weights calculated for the three solution channels of an experiment similar to that of Figure 2 but run at 40,000 and 52,000 rpm. A number of inferences may be drawn from these curves: (a) the system is obviously highly nonideal with the apparent reduced weight average molecular weight decreasing rapidly with increasing protein concentration; (b) extrapolations back to zero concentration give estimates of a mol wt near 40,000, about one-fourth the size of native aldolase, using the direct, but oversimplified approach of assuming the partial specific volume of aldolase in 1 M acetic acid to be 0.742 ml/g, the same value as for aldolase near neutral pH and under native conditions. It should be noted that such extrapolations to infinite dilution in high-speed equilibrium experiments are, at the same time, extrapolations toward the meniscus and thus should reflect, more or less effectively, the size of the smallest subunit present to any significant extent; (c) the curves for the three loading concentrations in each figure are almost, but not exactly, coincident. In addition, values of $\sigma_{\mathrm{w,a}}$ obtained at a given observation concentration, $c(r)$, but at the two different speeds are also quite close to coincidence. Thus, this system can *almost* be described, within the resolution of this technique, as a system composed of a single thermodynamic component. The lack of overlap among the three different loading concentration curves (Squire and Li, 1961; Yphantis, 1964) and between the two different speeds is somewhat greater than the expected experimental error and on this basis we believe that there is some residual heterogeneity, probably from undissociated or only partially dissociated aldolase that is *not* in chemical equilibrium with dissociated subunits. The amount of such heterogeneity in 1 M acetic acid depends on the particular sample of aldolase used and on the past history of the sample. For example, the aldolase preparations used in the experiment illustrated in Figure 2 showed significantly more heterogeneity

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[4] The value of M^* represents the effective molecular weight of the solute at infinite dilution; as mentioned before, it is influenced by the observation technique used but is independent of any definitions of components. For sedimentation equilibrium experiments, in general, that are observed with the absorption optics or for the high-speed equilibrium experiments utilizing any optical system the values of M^* may be predicted simply if the only interaction of the solute (component 2) with BX, the supporting electrolyte (component 3), is that imposed by the requirement of electroneutrality. Under these assumptions, M^* is given by eq 2 with $\Gamma' = -(z/2)(M_3/M_2)$ if component 2 is defined in the conventional way as PX_z, or with $\Gamma' = 0$ if we define component 2 according to Scatchard (1946) as $\mathrm{PX}_{z/2}\mathrm{B}_{-z/2}$. In both cases $M^* = M_{\mathrm{PX}_z}(1 - \{(z/2)[M_{\mathrm{BX}}(1 - \bar{v}_{\mathrm{BX}}\rho)]/[M_{\mathrm{PX}_z}(1 - \bar{v}_{\mathrm{PX}_z}\rho)]\})$.

[5] The equilibrium density gradient from the redistribution of solvent component 3 is approximately proportional to $M_3(1 - \bar{v}_3\rho)^2/\nu$ where M_3/ν is the effective molecular weight of component 3, taking into account the dissociation of component 3 into ν ions.

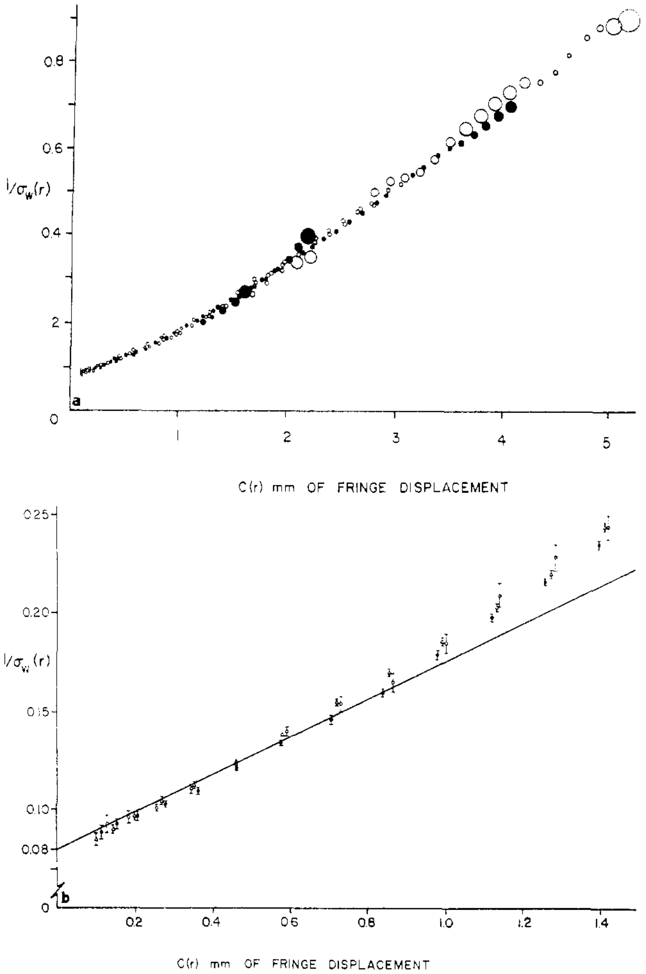

FIGURE 3: Aldolase in 1 M acetic acid + 0.1% sucrose. Reciprocals of $\sigma_w(r)$, the apparent reduced weight average molecular weight observed at each point r, are given as a function of $c(r)$, the concentration at each observation point. Part a depicts the complete concentration range observed for two speeds: the open circles were obtained at 52,000 rpm. The filled circles correspond to data from 40,000 rpm and the values presented were calculated by multiplying the observed values of $\sigma_w(r)$ by the factor $(52/40)^2$ to allow direct comparison with the data obtained at the higher speed. Assuming $\bar{v}$ = 0.742, a mol wt of 40,000 corresponds to σ = 12.3 cm^{-2}. Part b presents the lower concentration region for the experiment at 52,000 rpm. The solid line corresponds to the limiting slope and intercept used in the calculations (see the text for details). The initial concentrations loaded were 0.28 g/l. (○), 0.39 g/l. (●), and 0.98 g/l. (△). Note that the concentration units of millimeters of fringe displacement here and in Figure 4, 5, and 6 correspond approximately to units of 1 g/l.

than that evident in Figure 3a; the heterogeneity of that aldolase preparation appeared to be decreased markedly after fractionation on a Sephadex G-200 column in 1 M acetic acid. Similarly, aldolase aliquots aged in 1 M acetic acid for 1 week or more developed increased heterogeneity. The data shown in Figure 3a represent the most nearly homogeneous sample of aldolase seen in this solvent without fractionation after dissociation.

It is instructive to analyze this system further, in detail, without invoking the potentially questionable assumption of the native partial specific volume in a solvent such as 1 M acetic acid. The data of Figure 3a indicate moderately complex nonideality for aldolase in 1 M acetic acid. The overall shape of the curve appears somewhat sigmoidal; thus at least three virial coefficients are needed to describe the solution nonideality (*vide infra*). The near coincidence of the apparent reduced molecular weights is again evident for samples at different speeds and loading concentrations. The limiting behavior at low concentrations can be examined more closely in Figure 3b which illustrates the extrapolation to zero concentration and the estimation of the limiting slope of $1/\sigma_{w,a}$ *vs.* *c*. The linear region predicted for Donnan and simple nonideality in general (Johnson *et al.*, 1954; Williams *et al.*, 1958; Roark and Yphantis, 1971) is seen to be quite limited in extent here, with significant curvature apparent at concentrations of about 1 g/l. (see also later). The intercept, $1/\sigma_w{}^0$, gives the value of σ extrapolated to infinite dilution (and toward the meniscus) and should provide a measure of the molecular weight of the subunits, if the appropriate value of $\bar{v}_2$ to be used in eq 4 is known. Experimental determinations of such partial specific volumes—more specifically the ϕ' of Casassa and Eisenberg (1961, 1964)—require accurate density measurements under the conditions of the equilibrium experiments. Unfortunately, such determinations, although always preferable, may not be practical in any given case, in spite of the recent availability of sensitive equipment requiring only small volumes of solution—such as magnetically suspended densimeters (Ulrich *et al.*, 1964; Goodrich *et al.*, 1969) and the digital mechanical resonance densimeter (Kratky *et al.*, 1969).

Often the partial specific volume of a native protein is known or can be estimated from the amino acid composition (McMeekin and Marshall, 1952). In such cases it is possible to approximate a value for the effective partial specific volume of the protein in aqueous acetic acid solutions: component 2, the macroion, and its z counterions can be generated by titration of native isoionic protein with acetic acid. This titration requires q equivalents of acetic acid, where q, for any typical protein, is approximately equal to the sum of the effective net charge, z, on the protein and of the number of counterions (acetate) bound to the protein in the aqueous acetic acid solution.[6] This titration is primarily a transfer of protons from uncharged acetic acid to the protein carboxylate groups, a reaction whose net volume change appears to be essentially zero as shown by Rasper and Kauzmann (1962). Additionally, there are ν_c amino and histidyl residues whose titration by uncharged acetic acid is accompanied by the generation of an oppositely charged pair of ions and by a volume change ΔV_c, of about -20 cm^3/mol for each such residue (Kauzmann *et al.*, 1962). In addition to these direct ionic effects there is the likelihood of volume changes from the filling of some of the unfilled space in the "internal volume" (Waugh, 1954; Charlwood, 1957) during the denaturation attendant exposure to the aqueous acetic acid. This unfilled space in the internal volume is estimated to be about 3–4% of the volume of a typical globular protein. If some of this unfilled volume were made accessible to solvent, there would result a decrease, $\delta\bar{v}_p$, in the value of the partial specific volume of the protein ranging up to about 0.025 ml/g as a typical maximum value during the transition from native to a completely denatured state. Such an extreme disruption of the protein structure seems unlikely for aqueous acetic acid solutions of most proteins since aqueous acetic acid is found, for example, to cause no "substantial alteration in secondary structure" of ribonuclease (Cann, 1971) and since reversible association reactions of both insulin (Yphantis and Waugh, 1958) and tyrocidine B (Williams *et al.*, 1972) still occur in

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[6] This is true when the isoionic point lies below the titration region of the bulk of the tyrosyl groups in the molecule. Appropriate modifications to include volume changes on ionization of such titrable tyrosyl groups are otherwise necessary.

similar solutions. Some structure obviously still exists in the cases mentioned and in most systems only partial exposure of this unfilled volume is to be expected, especially with the lower concentrations of acetic acid. Values of perhaps one-tenth the maximum possible volume decrease are seen, for example, for ovalbumin (and even less for lysozyme) at a pH corresponding to 1 M acetic acid (Rasper and Kauzmann, 1962) but in 0.15 M NaCl as solvent. On the other hand significant volume changes, above and beyond the changes associated with the loss of electrostriction, are seen in bovine plasma albumin at such pH values (Kauzmann, 1958). These excess volume changes amount to decreases in the partial specific volume of ~0.0063 ml/g in 0.15 M NaCl and to 0.0113 ml/g with no added salt, using HCl as titrant. Thus a significant fraction of the unfilled internal volume can become exposed to solvent with some systems, especially at low ionic strength.

These considerations lead to an estimate for $\bar{V}_2$, the partial molal volume of component 2, as

$$\bar{V}_2 = M_2\bar{v}_2 = M_p(\bar{v}_p - \delta\bar{v}_p) + qM_{AH}\bar{v}_{AH} - \nu_c\Delta V_c \quad (4)$$

where $\bar{v}_2$ and $\bar{v}_p$ are the partial specific volumes of component 2 and of the native isoionic protein, respectively, M_2 and M_p are their molecular weights, M_{AH} is the molecular weight of acetic acid, and $\bar{v}_{AH}$ is the partial specific volume of (undissociated) acetic acid at the final concentration. The molecular weight of component 2 is $M_p + qM_{AH}$. Thus the partial specific volume of component 2 is given by eq 5.

$$\bar{v}_2 = \bar{V}_2/M_2 = \frac{M_p(\bar{v}_p - \delta\bar{v}_p) + qM_{AH}\bar{v}_{AH} - \nu_c\Delta V_c}{M_p + qM_{AH}} \quad (5)$$

The actual effective reduced molecular weight that is determined at infinite dilution by equilibrium ultracentrifugation depends on the details of the experimental procedure (Williams *et al.*, 1958). Complications can arise if the refractive increments of the initial solutions are obtained on undialyzed solutions and are used for weight average molecular weight determinations with refractometric optics assuming conservation of mass. Otherwise, and in particular here for "high-speed equilibrium" experiments with refractometric optics, the values obtained for σ_0, the value of σ at infinite dilution for a single thermodynamic component solute, correspond to the actual sedimenting component, defined essentially in the manner that components are defined naturally in osmotic experiments. This sedimenting component can most simply be considered to be composed of the component defined by Scatchard (for ionic components) plus whatever other molecular species are "bound" or associated with the macroion. Thus, for a single component solute in aqueous acetic solutions

$$\sigma^0 = \frac{\omega^2}{RT}[M_2(1 - \bar{v}_2\rho) - \frac{z}{2}M_{AH}(1 - \bar{v}_{A^-H^+}\rho) + \Gamma_{AH}M_{AH}(1 - \bar{v}_{AH}\rho)] \quad (6)$$

where $V_{A^-H^+}$ is the partial specific volume of *ionized* acetic acid, where Γ is the preferential binding coefficient of un-ionized acetic acid on a molar basis, *i.e.* $\Gamma_{AH} = (\partial m_{AH}/\partial m_2)_{\mu_1,\mu_3,P,T}$ and where we have also assumed that there is no volume change associated with the binding of any component (other than H$^+$) to the protein.

Let us consider now the magnitude of the error in the apparent molecular weight of the protein incurred by assuming the native value of $\bar{v}_p$ for the protein for use with the equilibrium experiments in aqueous acetic acid: the apparent molecular weight at infinite dilution that is calculated using $\bar{v}_p$ is $M_{p,a} = \sigma^0RT/(1 - \bar{v}_p\rho)\omega^2$ and the fractional error, ϵ, in M is

$$\epsilon \equiv (M_{p,a} - M_p)/M_p = \sigma^0RT/[M_p(1 - \bar{v}_p\rho)\omega^2] - 1 = [M_{AH}(q + \Gamma_{AH})(1 - \bar{v}_{AH}\rho) - (z/2)M_{AH}(1 - \bar{v}_{A^-H^+}\rho) + \nu_c\Delta V_c\rho + M_p\delta\bar{v}_p\rho]/[M_p(1 - \bar{v}_p\rho)] \quad (7)$$

where eq 6 and 5 have been inserted in turn. In the absence of titration data at low ionic strengths of these experiments we can only obtain estimates of the maximal value of q from the amino acid composition as the sum of all cationic groups in the molecule. The actual value of q is less than this maximal estimate even at the lowest pH attainable in aqueous acetic acid as solvent since there is a large repulsive electrostatic contribution to the free energy of protonation (see Edsall and Wyman, 1958; Tanford, 1961) under these conditions of low ionic strength, high charge, and somewhat lowered dielectric constant. On the other hand, z, the effective charge per molecule, provides a lower limit for q since $q = z + \nu_{A^-}$ and ν_{A^-} is the number of acetate ions bound to the protein, a positive number. In the absence of data about both proton and acetate ion binding we have estimated the values of z from the limiting concentration dependence, *i.e.* from the second virial coefficient, making the assumption that the observed virial coefficients in aqueous acetic acid solutions reflect only the Donnan nonideality and that all other sources of nonideality are unimportant. This appears to be a reasonable approximation in these cases since the magnitude of the observed nonideality is so much greater than any reasonable or even unreasonable contribution from sources such as excluded volume. There are about 360 amino acid residues per aldolase subunit of 40,000 daltons (Anderson *et al.*, 1969; Lai, 1968; Velick and Ronzoni, 1948). Making the improbable assumption that these residues are in an extended chain configuration and that aldolase forms a fully extended rod of length $L = 360 \times 3.615$ Å $\simeq 1.3 \times 10^{-5}$ cm in these solutions we may then calculate an upper limit for the magnitude of the contribution of the excluded volume to the second virial coefficient: the diameter of a rigid cylindrical rod of this length that is equal in volume to the aldolase subunit is $d = 2(M_p\bar{v}_p/N\pi L)^{1/2} = 6.95 \times 10^{-8}$ cm. The second virial coefficient contribution arising from the excluded volume of this rigid circular cylinder is (Tanford, 1961)

$$B_{ev} = \frac{L\bar{v}_p}{dM_p} = \frac{(1300 \times 10^{-8})(0.74)}{(6.95 \times 10^{-8})(4 \times 10^4)} \simeq 3.5 \times 10^{-3} \quad (8)$$

where B_{ev} is expressed as a usual colligative virial coefficient for concentrations in units of grams per milliliter. For a nonideal system containing a single molecular species we may write eq 3 in terms of the observable σ as

$$\sigma^0/\sigma_{w,a} = 1 + 2BM_1c \quad (9)$$

where σ^0 is the infinite dilution value of $\sigma_{w,a}$. The observed intercept and limiting slope of the curve presented in Figure 5b give the value $B = 1.39 \times 10^{-2}$ mol ml/g^2 if we assume that 1-mm fringe displacement corresponds to 1 g/l. and that

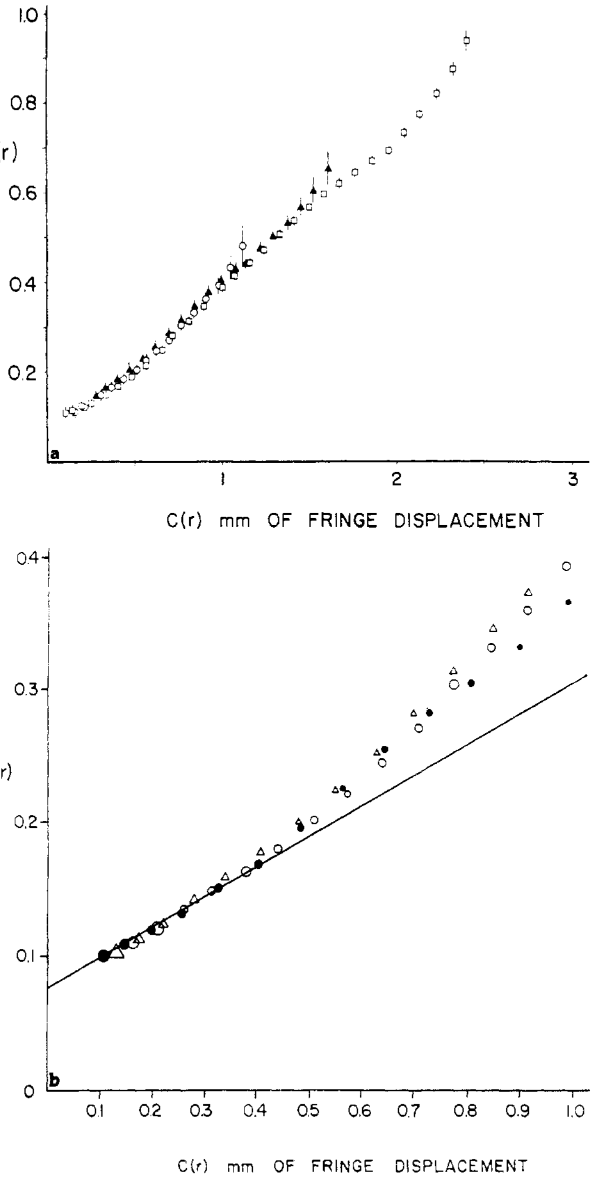

FIGURE 4: Aldolase in 0.1 M acetic acid + 0.1% sucrose run at 20° and 52,000 rpm in a 12-mm centerpiece. The reciprocals of $\sigma_w(r)$ are presented as a function of $c(r)$ in panel a, for all of the data with initial concentrations of (○) 0.13 g/l., (▲) 0.26 g/l., and (□) 0.43 g/l. The data from the initial region are given in panel b with the limiting line used for the calculations of the text indicated. In this panel the symbols for the loading concentrations are (●) 0.13 g/l., (○) 0.26 g/l., and (△) 0.43 g/l.

M = 40,000 daltons. Thus the excluded volume of the most extreme configuration imaginable for the aldolase subunit can account for only 28% of the observed second virial coefficient. More plausible configurations lead to much lower estimates for excluded volume contributions to the second virial coefficient.

The Donnan contribution to the second virial coefficient may be obtained from eq 3 as

$$B_D = 1000z^2/4M_1^2m_3 \qquad (10)$$

The observed value of $B = 1.39 \times 10^{-2}$ mol ml/g² corresponds to a charge of 19.9. The average of the slopes of $1/\sigma_w$ *vs.* c for the observation channel with the highest initial concentration (*i.e.* the channel in which effects of higher aggregates should be minimal) of eight other experiments gave 19.86 ± 0.53 charges per molecule. Subtraction of the calculated maximal excluded volume contribution to the second virial coefficient would lower this estimate of the effective charge to 17.2.

These numbers are much smaller than the estimates of $q_{\max} \simeq 52$ obtained by addition of all the cationic residues as given by all the amino acid analyses already cited. The difference arises from acetate binding, which is significant even at the isoelectric point (Velick, 1949) and is likely to be much more extensive at the low pH values of these experiments because of the large attractive electrostatic free-energy contribution that is expected even for macromolecules that are permeable to solvent under the present conditions (Tanford, 1961). In view of the uncertainty in the actual values of z it appears useful to recast eq 7 as

$$\begin{aligned}\epsilon = {} & \langle M_{AH}[(\Gamma_{AH} + \nu_{A^-} + z)(1 - \bar{v}_{AH}\rho) - \\ & (z/2)(1 - \bar{v}_{A^-H^+}\rho)] + \nu_c\Delta V_c\rho + M_p\delta\bar{v}_p\rho\rangle/\langle M_p(1 - \bar{v}_p\rho)\rangle = \\ & \rho\delta\bar{v}_p/(1 - v_p\rho) + \langle M_{AH}[(\Gamma^* + z/2)(1 - \bar{v}_{AH}\rho) + \\ & (z\rho/2)(\bar{v}_{A^-H^+} - \bar{v}_{AH})] + \nu_c\Delta V_c\rho\rangle/\langle M_p(1 - v_p\rho)\rangle \qquad (11)\end{aligned}$$

where we let $\Gamma^* = \Gamma_{AH} + \nu_{A^-}$ since we know neither binding coefficient. The remaining parameters are readily obtained from the literature: ν_c is the number of uncharged cationic residues at the isoionic point (IOP) that become charged under our observation conditions. The IOP of aldolase appears to be near pH 8 from measurements on aldolase solutions after passage through Dintzis columns and also by extrapolation to zero ionic strength (Velick, 1949). The only amide group estimates that we have found for aldolase are those of Velick and Ronzoni (1948): these analyses lead to 26 amides per subunit. These data, in combination with the two more recent amino acid analyses, can be used to infer 46 carboxylate ions per subunit. Since there are 52 cationic groups per subunit we can estimate that $\nu_c \simeq 6$. We calculate the partial specific volume of acetic acid in 1 M solutions to be 0.8604 ml/g at 20° and as 0.8694 ml/g at 25° from densities tabulated in the International Critical Tables (ICT). These values should correspond quite closely to $\bar{v}_{AH}$, the partial specific volumes of undissociated acetic acid, since less than 0.5% of the acetic acid is dissociated in a 1 M solution. The molal volume change at infinite dilution on ionization of acetic acid in water at 25° is reported to range from −9.2 to −11.47 cm³/mol (Owen and Brinkley, 1941; Redlick and Bigeleisen, 1942). The partial molal volume of *dissociated* acetic acid at 25° may be estimated to range from 40.7 to 43.0 ml/mol and the value of $\bar{v}_{A^-H^+}$ is thus estimated to be within the range 0.677–0.716 ml/g at 25° and about 0.009 ml/g lower at 20° in 1 M acetic acid. An additional estimate of this parameter can be calculated from infinite dilution values of available partial molal volumes for sodium acetate, sodium chloride, and hydrochloric acid as $\bar{v} = 0.702$ ml/g at 25°. We take the mean of these estimates for $\bar{v} = 0.70$ ml/g at 25° and, assuming the volume change on ionization to be independent of temperature, we obtain $\bar{v} = 0.691$ ml/g at 20°. Lastly, the density of the solvent in this experiment at 20° is calculated, using the ICT data, to be 1.0072 g/ml.

We can evaluate the fractional error of the apparent molecular weight that is calculated using the native value of $\bar{v}_p$ as

$$\epsilon = 4\delta\bar{v}_p + 7.9 \times 10^{-4}\Gamma^* - 0.014 \qquad (12a)$$

The average value of the molecular weight calculated using the native value of $\bar{v}$ from data of the channel with the highest

TABLE I: Apparent Molecular Weight, Colligative Second Virial Coefficients, and Net Effective Charge of Aldolase Subunits in Aqueous Acetic Acid with No Added Electrolyte.

	[Acetic Acid] (M)		
	0.1	1	10
M_{app},[a] daltons × 10^{-3}	41.9 ± 2.4	41.0 ± 0.8	41.6 ± 1.0
B, mol ml/g^2	0.038 ± 0.006	0.0137 ± 0.0007	0.0095 ± 0.0006
z_{app}	18.6 ± 1.4	20.0 ± 0.5	29.6 ± 1.3
$\bar{v}_{\text{AcH}}$,[b] ml/g	0.8561	0.8604	0.9180
Solvent density (ρ), g/ml	0.9994	1.0072	1.0625

[a] Apparent molecular weight calculated by assuming $\bar{v}_p$ = 0.742, the native value of the partial specific volume, and making no corrections other than for solvent density and temperature (see text for further details). [b] Partial specific volume of aqueous acetic acid calculated from data in the International Critical Tables.

loading concentration in each of eight experiments, five at 52,000 rpm and three at 40,000 rpm, in 1 M acetic acid is 41,000 ± 800 daltons. This result should be compared with the value (161,000 ± 3300)/4 = 40,350 ± 830 daltons calculated from the observed molecular weight in 0.1 M NaCl. The ratio of these two numbers is 1.016 ± 0.028, whereas eq 12a predicts 0.984. The difference (0.030) is not significant since the standard deviation of the observed ratio is 0.028. This suggests either of two possibilities: (a) both the value of the effective preferential interaction parameter $\Gamma^* = (\partial m_3/\partial m_2)_{P,T,\mu_1,\mu_3} + \nu_{A^-}$ and the value of $\delta\bar{v}_p$, the change in the partial specific volume from native conditions to 1 M acetic acid, are small or (b) their effects virtually cancel out (since Γ^* may become negative). We lean somewhat toward the former explanation by analogy with the limited data available for other proteins. Clearly, it would be best to actually measure the value of ϕ' appropriate to the solvent used.

Figure 4a presents values of $1/\sigma_{w,a}(r)$ as a function of the concentration $c(r)$, at which they were observed for the three loading concentrations of an experiment on aldolase in 0.1 M acetic acid. The nonideality here appears significantly greater, both in magnitude and complexity, than for aldolase in 1 M acetic acid, with the curves rising more steeply and showing considerable departure from the limiting straight line behavior. The lack of overlap of the three curves for the different loading concentration is an indication of significant heterogeneity in this solvent, again probably from the presence of undissociated or partially dissociated aldolase not in chemical equilibrium with the dissociated monomers. The limiting intercept and slope of these curves (Figure 4b) give estimates of the infinite dilution value of the monomer σ_0 (13.5 ± 0.7 cm^{-2}) and of the second virial coefficient, B. These estimates exhibit considerably greater uncertainty than before both because of the restricted range of linear behavior and the significant heterogeneity. The apparent molecular weight obtained by assuming the native partial specific volume, the second virial coefficient, and the magnitude of the net effective charge on the monomer (calculated on the assumption that all the nonideality arises from the Donnan effect) are presented in Table I. In addition, this table gives the values of all further variables needed to calculate estimates of ϵ as already has been done for aldolase in 1 M acetic acid. These data lead to the expression

$$\epsilon = 3.9\delta\bar{v}_p + (8.3 \times 10^{-4})\Gamma^* - 0.013 \qquad (12b)$$

for the fractional error of the apparent molecular weight as calculated with the assumption of the native value of $\bar{v}_p$ for aldolase in 0.1 M acetic acid. The ratio of the observed apparent monomer molecular weight to one-fourth the molecular weight of undissociated aldolase is 1.039 ± 0.061. Thus, as in 1 M acetic acid, either the values of $\delta\bar{v}_p$ and Γ^* are negligibly small or else they virtually compensate for each other.

Figure 5 presents curves for the reciprocal of the apparent weight average molecular weight as a function of observation concentration for aldolase in 10 M acetic acid. Here the nonideality clearly is smaller and the range of linear limiting behavior longer than in 1 M acetic acid. The close coincidence of the curves for the different loading concentrations implies that aldolase behaves as a single thermodynamic component in this solvent exhibiting rapid (with respect to centrifugation

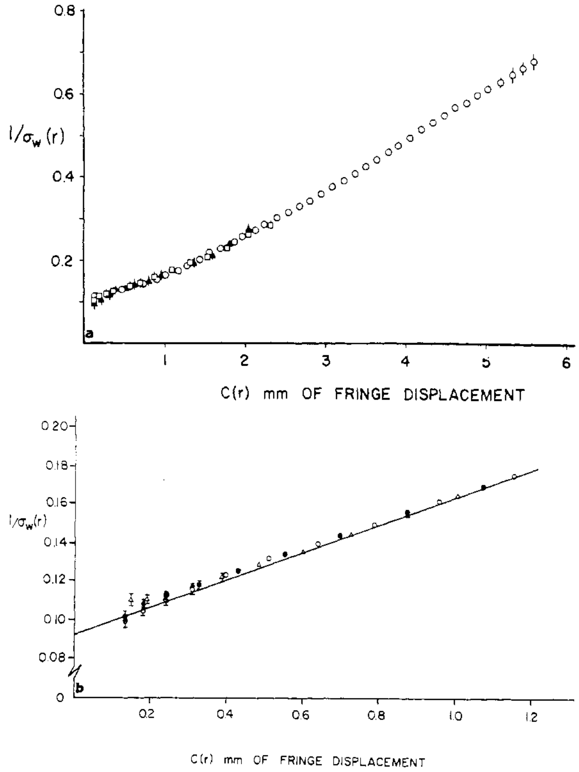

FIGURE 5: Aldolase in 10 M acetic acid + 0.1% sucrose run at 20° and 52,000 rpm in a 12-mm centerpiece. Values of $\sigma_w(r)^{-1}$ are presented as a function of c(r) for all the observations in panel a with c_0 = 1 g/l. (○), 0.34 g/l. (▲), and 0.18 g/l. (□). The lower concentration region is illustrated in panel b with the limiting line that was used in the calculations indicated.

times) equilibrium between any species that may be present. Values of the apparent molecular weight calculated on the assumption of the native $\bar{v}_p$ and of other parameters for these conditions are also given in Table I, along with equivalent data for both 0.1 and 1 M acetic acid solutions of aldolase. These data lead to the expression

$$\epsilon = 5.0\delta\bar{v}_p + (1.4 \times 10^{-4})\Gamma^* - 0.031 \tag{12c}$$

for the fractional error in the simply calculated apparent molecular weight. The ratio of the molecular weight calculated with the native $\bar{v}_p$ to one-quarter the undissociated aldolase molecular weight is 1.031 ± 0.032, significantly greater than the value of 0.969 predicted for $\delta\bar{v}_p$ and Γ^* vanishingly small. Thus, either $\delta\bar{v}_p$ or Γ^*, or more likely both, are significant for aldolase in 10 M acetic acid.

It is clear from the shapes of the curves of $\sigma_{w,a}^{-1}$ *vs.* c that expressions involving only second virial coefficients are grossly inadequate to represent the observations in all cases and that higher coefficients are essential. In the case of 0.1 M acetic acid these graphs (Figure 4a) appear fairly complex but initially there is positive curvature for all loading concentrations, implying that the most significant of the remaining virial coefficients are positive. Preliminary analyses of the data from the channel with the highest initial concentration (to minimize effects of possible irreversible higher aggregates) indicate that the data can be accounted for somewhat better under the assumption of a positive fourth virial and zero third virial coefficient than with a positive third and zero fourth virial coefficient. (It is not possible to account for experiment by assuming a positive fifth [or higher] virial coefficient and nonpositive third and fourth virial coefficients.) In addition, there are strong contributions from terms higher than the fourth in any such virial expansion of the data. It also appears quite likely that the best fit of even only the initial half of the data in this case to a virial expansion would require many such terms, including the third, fourth, and fifth (or higher) virial coefficients.

The positive third or fourth virial coefficients that appear to be essential here cannot be accounted for on the basis of charge effects since the contributions from the Donnan effect to the third virial coefficient vanish for a 1:1 supporting electrolyte and the fourth virial contribution is of negative sign (Roark and Yphantis, 1971). Similarly, we can rule out explanations based on contributions from reversible (or, within an individual solution column, irreversible) associations greater than dimer: for sufficiently small extents of association between monomer and N-mer[7] the apparent weight average molecular weights for an otherwise ideal system may be expressed as virial expansions (Stafford and Yphantis, 1972) of the form

$$\frac{1}{M_{w,1}} = \frac{1}{M_1}[1 - (N-1)Kc^{N-1} + \frac{1}{2}(2N-2)(2N-1)K^2c^{2(N-1)} - \ldots] \tag{13}$$

where M_1 is the monomer molecular weight, c the total concentration of solute in grams/milliliter, and K the association constant between monomer and N-mer in units of $(\text{ml/g})^{N-1}$. Thus, associations to species above tetramer contribute only to the fifth or higher virial coefficients. Monomer–trimer (or monomer–tetramer) associations can only supply negative contributions to the third (or fourth) virial coefficients. Positive contributions can be made to the third (simultaneously with smaller negative contributions to the fourth) virial coefficients only by associations involving monomer and dimer species. Thus, if association underlies the positive curvature observed at low aldolase concentrations firstly this association must necessarily involve monomers and dimers, and secondly it is the third virial coefficient that must be positive since association can never make a positive contribution to a fourth virial coefficient. Preliminary attempts to fit experimental observations to such a postulated monomer–dimer equilibrium in the presence of the extreme nonideality observed here were unsuccessful and indicated that the system "aldolase in 0.1 M acetic acid" was more complex than assumed, with the probable additional involvement of higher associations than to dimer and/or the participation of repulsive and attractive contributions expressed in the higher virial coefficients.

Essentially the same conclusions may be drawn from the experiments at the higher acetic acid concentrations: the Donnan nonideality is not responsible for the positive curvature in Figures 3a and 5a at low solute concentrations, but rather it is some other as yet unidentified solute–solute interaction—perhaps in part dimerization[8] (with higher simultaneous association stages) masked by the strong nonideality of these systems—that is responsible. We have undertaken studies of several proteins in other aqueous organic acids largely spurred on by the fact that the equivalent of the factor $(1 - \bar{v}_{AH}\rho)$ can be made to vanish sensibly at appropriate concentrations of some organic acids (propionic, butyric). Experiments in such solvents should then make available estimates of apparent molecular weight that are strictly independent of the extent of preferential interactions with solvent components. [A manuscript describing such experiments is in preparation (S. Szuchet and D. A. Yphantis).] These observations on other proteins in aqueous organic acids suggest, at present, that other interactions than associations may contribute to or indeed may be the sole cause of the excess nonideality observed above and beyond the expected Donnan contributions.

The nonideality expressed by means of the higher virial coefficients severely limits the range of utility of the nonideal molecular weight moments for the aldolase systems in aqueous acetic acid. Values of the "ideal" moments σ_{y1}, σ_{y2}, σ_{y4}, and σ_{y6} (see eq 2), along with the values of σ_w for comparison, are presented in Figure 6 for aldolase in 10 M acetic acid. At the lower observation concentrations (<800 μ fringe displacement) all of these moments provide estimates of the ideal (infinite dilution) value of σ $(=\sigma^0)$ for aldolase monomers as being between 10 and 11 cm^{-2}, with calculated standard deviations of about 10% in the plotted values of the σ_y. At higher aldolase concentrations these standard deviations decrease to about 4% of σ^0 but the nonideal moments now start to mirror the complexities of the system: for example, σ_{y2}, equal to $\sigma_{w,a}^2/\sigma_{z,a}$, follows the sign of the second derivative of the concentration. The value of $\sigma_{z,a}$, equal to

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[7] Virial expansions have been shown to be necessarily divergent above and beyond some critical extent of association that depends solely on the degree, N, of the association reactions. Thus all power series expansions for the reciprocal of any molecular weight average as a function of solute concentration are valid only below a critical weight fraction of polymer, a fraction that ranges down from a maximum value of 0.172 for dimer, through 0.077 for tetramer, and approaches zero with increasing N (Stafford and Yphantis, 1972).

[8] The possibility of dimerization is indeed real, as is suggested by the observation of apparent dimers by Hsu and Neet (1973) in solutions of Mg^{2+} salts.

$[\partial^2 c/\partial(r^2/2)^2]/[\partial c/(\partial r^2/2)]$, approaches zero as the slope of the schlieren pattern vanishes; thus the reciprocal of σ_{y2} must also vanish and σ_{y2} itself must necessarily exhibit a singularity at such points. Two such singularities are observed for σ_{y2} at aldolase concentrations of about 3300 and 4500 μm fringe displacement. The first of these coincides with a weak maximum and the second with a weak minimum in the concentration gradient. The maximum in the concentration gradient can be considered to arise from some positive virial coefficient beyond the second virial coefficient. Indeed Nichol *et al.* (1966) have observed such maxima in the equilibrium schlieren patterns from sodium polymethacrylate and from hyaluronic acid; they interpreted their observations in terms of apparent third virial coefficients for their solute systems, carefully recognizing the potential presence of significant contributions from higher virial coefficients.

It is not feasible to describe these aldolase systems in terms of only two apparent lower virial coefficients. This is illustrated by the behavior of σ_{y4} and σ_{y6}, both of which appear to decrease slightly below their low concentration values and then to rise again to values three or four times σ^0 near the base of the cell (off scale in Figure 6). This behavior cannot be accounted for simply by the contributions from a second and third or from a second and fourth virial coefficient since σ_{y6} and σ_{y4}, respectively, remove the effects of nonideality expressible in those coefficients. The behavior of these nonideal moments suggests, rather, the possible presence of molecular species larger than dimers: *i.e.*, comparison of the nonideal moments presented in Figure 6 with those calculated for simple monomer–polymer equilibria suggest that such species, if indeed present, are greater than hexamers in size.

The values of σ_{y4} and of σ_{y6} yield useful estimates for aldolase subunits over a wider concentration range than do the other nonideal moments, since σ_{y4} and σ_{y6} remove the effects of nonideality expressible in the fourth and third virial coefficients, respectively, as well as that arising from the second virial coefficient.

The behavior of the nonideal moments for aldolase in 1 and 0.1 M acetic acid is similar to their behavior for 10 M acetic acid except that the regions with constant σ_{yk} are progressively more abbreviated.

Discussion

Aqueous acetic acid at various concentrations, in the absence of any added electrolyte, appears to be more effective in dissociating aldolase than does 0.05 M HCl with 0.1 M NaCl as supporting electrolyte. In the latter solvent there appears to be a significant amount of heterogeneity; indeed, so much that the expected nonideality appears to have been largely masked by the aggregated species present. By contrast, when care is taken in the selection and preparation of the sample, the apparent heterogeneity of aldolase dissociated in aqueous acetic acid appears considerably smaller. These dissociating systems of aqueous acetic acid are probably effective primarily through direct electrostatic effects; at the low ionic strengths used ($I = 1.4 \times 10^{-3}$ to 1.4×10^{-2} M) there is considerably less electrostatic shielding by the ion atmosphere than at the higher ionic strengths (~0.15 M) and thus electrostatic repulsion should be significantly stronger than usual. Other dissociating mechanisms are likely to be effective at the higher acetic acid concentrations, but it does not appear reasonable to account for significant dissociation in 0.1 M acetic acid by such mechanisms as competition by acetic acid molecules for intersubunit hydrogen bonds, through augmentation of electrostatic repulsion by decreases in solvent dielectric constant or by invoking a perturbation of water structure by acetic acid. These later mechanisms probably all operate, to some extent or another, in 10 M acetic acid, but simple electrostatic repulsion appears to be the only common mechanism that is likely to be significant at all the concentrations of acetic acid used in these experiments. The higher acetic acid concentrations appear to be significantly more effective in dissociating aldolase, as judged by the decreased heterogeneity of the dissociated aldolase subunits. It should be noted, however, that from the data available, we cannot rule out possible reversible equilibria between subunits and higher associated species in any of the dissociating solvents used.

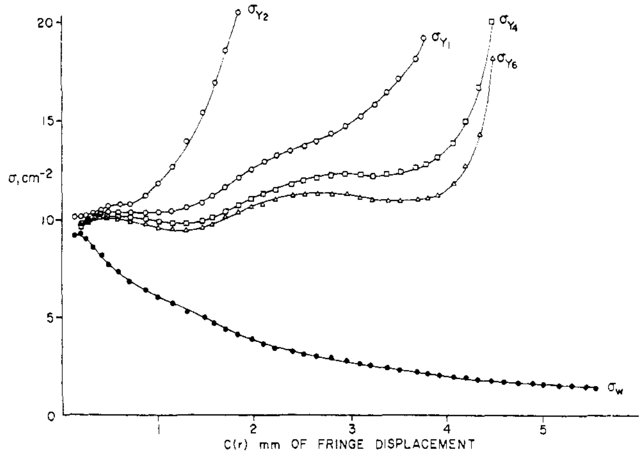

FIGURE 6: Values of "ideal" moments observed for aldolase at an original concentration of 1 g/l. in 10 M acetic acid plus 0.1% sucrose at 20° and at 52,000 rpm. The "ideal" moments $\sigma_{y1}(r)$, $\sigma_{y2}(r)$, $\sigma_{y4}(r)$, and $\sigma_{y6}(r)$ are presented, along with $\sigma_w(r)$, as a function of $c(r)$. The uncertainty (calculated standard deviation) of these "ideal" moments is about $0.1\sigma^0$ for the lowest observation concentrations and decreases to about $0.04\sigma^0$ at the higher observation concentration.

It appears effective to use the native value of the partial specific volume to estimate the molecular weight of aldolase in these experiments, largely because the corrections from preferential interaction of the solute with the solvent components and the corrections from volume changes on transfer of the protein from "normal conditions" (where $\bar{v}$ is known) into the aqueous acetic acid solutions either are both small or else almost completely cancel each other, at least for aldolase in 0.1 and 1 M acetic acid. In 10 M acetic acid, on the other hand, these effects appear to contribute sufficiently to make the apparent molecular weight calculated assuming the native $\bar{v}$ to be about 6% too high. These observations and data from other proteins in aqueous organic acids (S. Szuchet and D. A. Yphantis, manuscript in preparation) suggest that the error to be incurred by assuming the native value of the partial specific volume for a simple direct calculation of apparent molecular weight is likely to give approximate molecular weights—within 10–15%, or so—for many systems. However, such estimates should be treated as provisional and with considerable caution; these estimates should be replaced, whenever possible, by proper calculations using observed values of ϕ' obtained through density measurements on solutions that have been well equilibrated with all solvent components (Casassa and Eisenberg, 1961).

The departures from ideality observed for aldolase in aqueous acetic acid solutions appear moderately complex. In all cases, the dominant nonideality at the lowest aldolase concentrations appears to arise from the Donnan effect, *i.e.*, from the requirement that counterions sediment with macroions so as to maintain electroneutrality. Excluded volume effects are likely to make only a small contribution in the 0.1 and 1 M acetic acid solutions of aldolase; we cannot rule out some significant contribution from excluded volume effects in the 10 M acetic acid, but we believe that such contributions could account for only a small fraction of the observed second virial coefficient. The magnitude of the effective charge, z, that we have estimated from the limiting nonideality is only a fraction (ranging from about 40% in 0.1 M acetic acid to about 64% in 10 M acetic acid) of the total number of cationic groups of the aldolase subunits. Most of this difference is likely to be accounted for by the binding of acetate ions to the positively charged protein; such behavior is usually observed with macroions—see Tanford (1961) for a discussion. The actual values of z that have been estimated entail significant uncertainties since the counterion concentrations were simply calculated from the dissociation constant of acetic acid, without making any corrections for dielectric constant changes, ionic strength effects, etc., and since all the nonideality was imputed to the Donnan effect. These numbers could also be underestimates (as well as potentially overestimates) especially since we cannot exclude, on the basis of the data available, some reversible association to dimers (as well as to higher aggregated species). Such dimerization could mask some of the nonideality present (*i.e.*, make the apparent second virial coefficient more negative) at the same time that this association would be masked by the nonideality itself.

Ordinarily one might expect to use the deviations from nonideality ascribed to higher virial coefficients to delineate the extent of dimerization actually present. However, as mentioned, there appear to be large and significant third, fourth, and/or higher virial coefficients—with indications of possible associations involving several subunits—so that we cannot even be sure if such dimerization is even present, let alone determine its magnitude. We have been unable to explain our experimental observations through any sets of simple unique models. However, we have fit the raw experimental data, in the form of fringe displacements, using a nonlinear least-squares fitting program (Johnson and Yphantis, 1971; M. L. Johnson and D. A. Yphantis, manuscript in preparation) in some experiments by assuming a single nonideal component with several different sets of virial coefficients up to and including a fifth virial coefficient. Such fits, although describing the experimental data well,[9] are not unique: the differences in the sums of the squares of the residuals for distinctly different sets of virial coefficients corresponded to only rather small fractions of the expected experimental error. At present our conclusions are that the second virial coefficients are fairly well determined but that the higher nonidealities are uncertain, largely because we lack a satisfactory model for fitting the experimental data. Clearly more work on such nonideal systems is needed.

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[9] In an experiment on aldolase in 10 M acetic acid, for example, the standard deviation of the experimental data from the fitted curve corresponded to less than 3-μm fringe displacement for the data from a single loading concentration, and to less than 5-μm fringe displacement when fitting all the data from three loading concentrations simultaneously to the same model.

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Plant Carbonic Anhydrase. Properties and Carbon Dioxide Hydration Kinetics†

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ABSTRACT: Carbonic anhydrase from spinach is shown to be an hexameric enzyme of mol wt 180,000 and to contain six Zn atoms per molecule. In the presence of sodium dodecyl sulfate it can be dissociated into a mixture of pentamers, tetramers, trimers, dimers, and monomers as identified by electrophoresis on polyacrylamide gel. The monomer subunits can be reassociated into active enzyme if the latter is dissociated in guanidine hydrochloride rather than in sodium dodecyl sulfate. In contrast to all previous reports, a sulfhydryl reducing agent is not needed for stabilizing the enzyme. 5,5′-Dithiobis(2-nitrobenzoate) does not react with the intact enzyme; however, in the presence of guanidine hydrochloride the dissociated enzyme is shown to contain 12 sulfhydryl groups. The enzyme strongly catalyzes the hydration of CO_2. The enzyme–substrate interaction appears to follow a Michaelis–Menten mechanism with a Hill constant of 1.02. Hydrase activity rises continuously but nonuniformly with increasing pH between 6.0 and 9.0. The pH profile of k_{cat} ($\equiv k_2$) exhibits a simple sigmoidal curve with an inflection at pH 7.7. Phosphate, and to a lesser extent other buffers, have significant effects on both kinetic parameters but especially on K_m. When extrapolated to zero buffer concentration, values of K_m are pH independent. It appears that the acidic form, $H_2PO_4^-$, lowers the CO_2 affinity of the plant enzyme by increasing the value of K_m while the basic form, HPO_4^{2-}, behaves as a mild activator in that it increases the value of k_{cat}. The possible role of the enzyme in promoting photosynthesis is discussed.

Plant carbonic anhydrase was first obtained from leaf cytoplasm by Neish (1939). This carbonate hydrolyase (EC 4.2.1.1) is present in chloroplasts of species which carry out photosynthesis *via* the Calvin cycle (Everson and Slack, 1968), often in close association with ribulose diphosphate carboxylase, an enzyme which uses CO_2 as the C_1 substrate for the carboxylase reaction (Poincelot, 1972a). Recently, the plant enzyme was isolated from spinach (Rossi *et al.*, 1968), parsley (Tobin, 1968, 1970), and pea leaves (Kisiel and Graf, 1972) as well as from *Tradescantia albiflora* Kunth (Atkins *et al.*, 1972) and is reported to exhibit fundamentally different properties from the one isolated from erythrocytes. Both the pea and parsley enzymes were found to be hexameric, mol wt

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† From the Department of Chemistry, University of Washington, Seattle, Washington 98195. *Received June 25, 1973.* Support of this work by Grant AM 09221 from the National Institutes of Health of the U. S. Public Health Service is gratefully acknowledged.

‡ Taken in part from the dissertation of Ms. Joan S. Y. Ng to be submitted to the University of Washington in partial fulfillment of the Ph.D. degree.