# Subunit Interactions of Rabbit Muscle Aldolase. Dissociation of Aldolase in 1.2 M Magnesium Chloride†

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ABSTRACT: The dissociation of rabbit muscle aldolase in $MgCl_2$ solutions above 0.9 M has been studied by analytical ultracentrifugation. In 1.2 M $MgCl_2$–0.2 M Tris (pH 7.2) a single sedimentation peak with a decreasing $s_{20,w}$ with decreasing protein concentration (6–0.1 mg/ml) was observed. Under the same conditions, overlapping $M_w(r)$ *vs.* concentration curves were obtained by equilibrium sedimentation with different loading protein concentrations (0.2–1.3 mg/ml) and at different rotor speeds (14,000 and 26,000 rpm). Therefore, aldolase exists in a rapid, reversible association–dissociation system in 1.2 M $MgCl_2$. No preferential solvation occurs at this concentration of $MgCl_2$ since the apparent specific volume was determined to be the same as that of native enzyme by pycnometry and by evaluation of sedimentation coefficient–viscosity *vs.* solvent density data in different $MgCl_2$ concentrations. The molecular weight distribution of aldolase in 1.2 M $MgCl_2$–0.2 M Tris was analyzed by methods suggested for self-associating systems by Yphantis, Adams, and Steiner. The mode of dissociation was concluded to be tetramer–dimer–monomer. The calculated association constant for dimer formation from monomer was $1.4 \times 10^5$ l./mol ($\Delta G = -6.9$ kcal/mol) and for tetramer formation from dimer was $6.1 \times 10^5$ l./mol ($\Delta G = -7.8$ kcal/mol). Analysis of these data (Cornish-Bowden, A., and Koshland, D. E., Jr. (1971), *J. Biol. Chem. 246*, 3092) indicated that the bonding domains between subunits in aldolase are all isologous ($I_4$ or $I_6$). Activity measurements in 1.2 M $MgCl_2$ suggested that monomers and tetramers of aldolase have equal activity, but only about 0.2% of the $V_{max}$ of native enzyme.

The relationship between conformational changes and subunit interactions of enzymes can either be studied functionally in an oligomeric enzyme or studied in conformationally linked dissociation reactions of the enzyme. The former approach has suffered from a lack of quantitative methods whereas the latter approach has been limited by the extent of the conformational change that can be induced by specific perturbations. We have approached this problem by studying a well-characterized, nonallosteric enzyme, namely rabbit muscle aldolase, under conditions in which dissociation occurs with a relatively small, defined conformational change. These conditions were found to hold in concentrated $MgCl_2$ solutions, 0.9–3.2 M. The present paper establishes that a reversible association–dissociation of aldolase occurs in 1.2 M $MgCl_2$, that the mode of dissociation is tetramer–dimer–monomer, and that some conclusions can be made concerning the subunit interactions within the molecule. Future publications will discuss the concomitant conformational changes and the linkage between the two effects (Hsu and Neet, 1972).[1]

## Materials and Methods

Frozen rabbit muscle was purchased from Pel-Freeze Biological, Inc. Fructose 1,6-diphosphate tetrasodium salt (FDP)[2] and Tris base were obtained from Sigma Chemical Co. Magnesium chloride ($MgCl_2 \cdot 6H_2O$) was obtained from Fisher Scientific Co. Ammonium sulfate (ultrapure grade) was obtained from Mann Research Co. All chemicals were used without further purification.

Aldolase was purified from rabbit muscle by the method of Taylor (1955) except that chromatography on a DEAE column was performed prior to crystallization. The concentration of purified aldolase was measured spectrophotometrically using the determined value of 0.91 for $E_{1\,cm}^{0.1\%}$ at 280 nm (Taylor, 1955). The enzyme activity of aldolase was assayed by the hydrazine method (Jaganrathan *et al.*, 1956). One unit of specific activity by this assay method is defined as $(\Delta OD)_{1\,cm}^{240\,nm}$ per minute per milligram. $MgCl_2$ at concentrations used in this study did not interfere with the chemical reaction between the product, dihydroxyacetone phosphate, and the trapping reagent, hydrazine. The specific activity of purified aldolase was 16–24 units/mg. The purified aldolase was stored in 50% saturated ammonium sulfate at 4°.

### *Ultracentrifuge Experiments*

An aldolase solution, desalted by dialysis, was mixed with an appropriate amount of concentrated buffer and $MgCl_2$ solution to give the desired composition of solution. The solutions were then dialyzed against at least 200 volumes of solvent of the same buffer and $MgCl_2$ composition. The dialysis was carried out at room temperature with gentle stirring for a period of 18–26 hr. No visible turbidity or precipitate was seen after the dialysis and the solutions and dialyzate were directly used for analytical ultracentrifugation. All experiments were performed in a Spinco Model E analytical ultracentrifuge equipped with an RTIC unit, electronic speed control, photoelectric scanner, and multiplexer. A capillary type synthetic boundary cell with a 12-mm light path was used for sedimentation velocity studies. Schlieren photographs were recorded on Kodak metallographic plates. Ultraviolet optics

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[1] Hsu, L. S., and Neet, K. E. (1972), manuscript in preparation.

[2] Abbreviation used: FDP, fructose 1,6-diphosphate; DEAE, diethylaminocellulose.

were used for protein concentrations of less than 1 mg/ml. For studying the dependency of the $s_{20,w}$ of aldolase on protein concentration, an AnF rotor was used which incorporated three cells in a single experiment. Sedimentation velocity experiments were carried out at 20° with rotor speeds of 59,780 or 52,000 rpm. The observed sedimentation coefficients were corrected to values for a solvent with the viscosity and density of water at 20°. The relative viscosities of $MgCl_2$ solutions with respect to water at 20° were obtained by interpolating the viscosity data in the International Critical Tables. The densities of $MgCl_2$ solutions were taken from the Handbook of Physics and Chemistry.

High-speed sedimentation equilibrium was carried out according to the procedures of Yphantis (1964). All experiments were done using a charcoal-filled epon six-channel centerpiece assembled with sapphire windows. Each channel was filled with a liquid column height of about 2.2 mm. The menisci of solution and solvent sectors were carefully matched by using the same microsyringe. All experiments were carried out at 20° and equilibrium was judged to be attained at 20 hr since the molecular weight distribution calculated at 4-hr intervals after this time did not show significant differences. The experiments were routinely carried out for a total period of 25–28 hr. The experiments which employed white light to label the zero-order fringe were done according to the procedure of Goldberg and Edelstein (1969). The interference fringe patterns were analyzed with a Nikon microcomparator. The fringe displacements relative to that at the meniscus, $y - y_0$, were measured at a constant interval of 0.02 cm (comparator scale) along the $x$ coordinate. The fringe displacement corresponding to one fringe was measured to be 283 $\mu$. The specific refractive index increment of aldolase in $MgCl_2$ solution was not measured but was assumed to be the same as that of the native aldolase in dilute salt solution. Accordingly, 4.0 fringes were taken to correspond to a protein concentration of 1.0 mg/ml (Stellwagen and Schachman, 1962).

Pycnometric measurements were done using a 5.0-ml pycnometer with the temperature maintained in a water bath at 20 ± 0.02°. Protein concentration was measured by spectrophotometry at 280 nm; the $E_{280}^{0.1\%}$ for aldolase in 1.2 M $MgCl_2$ was corrected to a value of 0.90 by difference spectrophotometric measurement.

*Data Calculation*

The apparent weight-average molecular weight ($M_w$) is defined by eq 1, where $R$ is the gas constant, $T$ is the absolute

$$M_w(r) = \frac{RT}{(1 - \bar{v}\rho)\omega^2}\left[\frac{\mathrm{d} \ln f}{\mathrm{d}(r^2/2)}\right]_r \tag{1}$$

temperature, $\bar{v}$ is the partial specific volume of aldolase, $\rho$ is the density of the solution, $\omega$ is the angular velocity, $f$ is the fringe displacement, and $r$ is the radial distance from the center of the rotor. The quantity [d ln $f$/d($r^2$/2)] was calculated by the sliding five-point least quadratic treatment (Yphantis, 1964). The apparent number-average molecular weight was evaluated by eq 2. The integral was evaluated by trapezoidal

$$M_n(r) = \frac{RT}{(1 - \bar{v}\rho)\omega^2}\frac{f(r)}{\int_{r=a}^{r} f(r)\mathrm{d}(r)^2/2)} \tag{2}$$

approximation. The apparent $z$-average molecular weight was evaluated from eq 3. The calculation of $M_z(r)$ was done by a least-squares treatment of five successive points on a ln $[M_w(r)(1 - \bar{v}\rho)\omega^2/RT]$ *vs.* $r^2/2$ plot.

$$M_z(r) = \frac{RT}{(1 - \bar{v}\rho)\omega^2}\left[\frac{\mathrm{d}^2 f}{\mathrm{d}(r^2/2)^2}\Big/\frac{\mathrm{d}f}{\mathrm{d}(r^2/2)}\right]_r \tag{3}$$

For extensive data manipulation, as in the Adams' method of analysis, the $M_w(r)$ *vs.* $c$ data were initially smoothed by obtaining the polynomial function which gave the best fit to the data. $M_n(r)$ was then calculated from the polynomial equation with eq 2a.

$$\int_0^c \frac{M_1}{M_w(r)} = \frac{cM_1}{M_n(r)} \tag{2a}$$

Two- and three-species plots (Roark and Yphantis, 1969) require the ideal quantities $M_w(c)$, $M_n(c)$, and $M_z(c)$, which were calculated from the apparent quantities $M_w(r)$ and $M_n(r)$, obtained from the smoothed data, with eq 1a, 2b, and 3a, where $B$ is the virial coefficient and $\psi$ is the quantity de-

$$\frac{1}{M_w(c)} = \frac{1}{M_w(r)} - Bc \tag{1a}$$

$$\frac{1}{M_n(c)} = \frac{1}{M_n(r)} - \frac{Bc}{2} \tag{2b}$$

$$M_z(c) = -\psi\frac{M_1^2}{cM_w(c)} \tag{3a}$$

fined in Table I. Both $B$ and $\psi$ were calculated from the results of the method of analysis (Adams, 1967a) presented later (see Table II). Since the computed value of $B$ was low, utilization of eq 1a, 2b, and 3a revealed only a small difference between the ideal and apparent molecular weights.

All data calculations were done by programming a Hewlett–Packard 9100A calculator with extended memory, printer, and plotter. The fitting of a polynominal function to experimental data and the solution of three or four simultaneous equations were based on existing calculator programs published by Hewlett–Packard. The solution of equations in Adams' method of analysis was done by a numerical method.

## Results

*Sedimentation Behavior of Aldolase in Concentrated $MgCl_2$ Solutions.* In concentrated $MgCl_2$ solutions, ranging from 0.3 to 3.2 M in concentration, only a single and essentially symmetrical Schlieren peak was seen in sedimentation velocity experiments. The observed sedimentation coefficient of aldolase decreased with increasing $MgCl_2$ concentration. To determine whether the observed decrease of sedimentation coefficient of aldolase in $MgCl_2$ solutions could be accounted for by solvent and/or preferential hydration effects, the data were plotted (Figure 1) as $s\eta$, *i.e.*, the product of the observed sedimentation coefficient and the relative viscosity of the solvent, *vs.* the density of the solvent (Aune and Timasheff, 1970). There is a linear relationship between $s\eta$ and $\rho$ up to a density of about 1.10. Within this limit of solvent density (which corresponds to 1.5 M $MgCl_2$), a least-squares line can be fitted through the data points and extrapolated to a density of $1.35_0$ on the abscissa. The reciprocal of this density, 0.740, represents the partial specific volume ($\phi'$) of the dialyzed

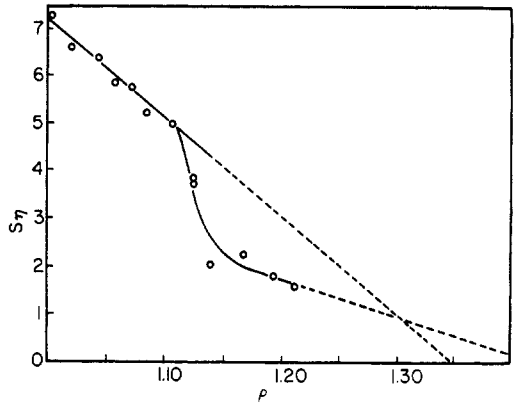


FIGURE 1: Viscosity-corrected sedimentation coefficient ($s\eta$) *vs.* solvent density, $\rho$, plot for aldolase in $MgCl_2$. Aldolase, dialyzed against the appropriate solvent, was centrifuged at 52,000 rpm in a synthetic boundary cell at 20° and a protein concentration of 2.5–3.0 mg/ml.

aldolase component in $MgCl_2$, as defined by Casassa and Eisenberg (1964). This estimated partial specific volume of aldolase in $MgCl_2$ is close to the reported $\bar{v}$ of 0.743 ml/g (Taylor and Lowry, 1956) and the value of 0.742 ± 0.003 ml/g we obtained for aldolase in dilute buffer. The estimated value is also consistent with the result of pycnometric measurements of aldolase in 1.2 M $MgCl_2$ at 20° which gave an apparent specific volume, $\phi'$, of 0.740 ± 0.008 ml/g. These data strongly suggest that no significant change in the partial specific volume of aldolase has occurred in $MgCl_2$ up to about 1.5 M in concentration. In fact, the $s_{20,w}$ of aldolase in $MgCl_2$ below 1.5 M calculated by applying the correction of the solvent properties (*i.e.*, viscosity and density) was within the uncertainty of $s_{20,w}$ of aldolase in dilute Tris buffer. Thus, aldolase was not preferentially solvated in $MgCl_2$, at least below 1.5 M.

No significant dissociation of the aldolase tetramer appears to occur below 1.5 M $MgCl_2$ under these conditions. However, the data of Figure 1 were obtained at a protein concentration between 2 and 3 mg/ml and, as will be seen in the following sections, aldolase does dissociate into subunits in 0.9 and 1.2 M $MgCl_2$ at lower protein concentrations. The value of $s\eta$ drastically decreases above 1.8 M $MgCl_2$ ($\rho$ = 1.13) as shown in Figure 1, indicating that either dissociation, a conformational change, or a change in preferential solvation has occurred. It can be estimated that if the observed $s_{20,w}$ of 4.2 at 1.8 M $MgCl_2$ is to be attributed to preferential hydration alone, an increase in preferentially bound water of 0.38 g of water/g of aldolase tetramer has to occur within the 0.3 M increment of $MgCl_2$ concentration from 1.5 to 1.8 M, assuming that there is no preferential hydration of aldolase in 1.5 M $MgCl_2$, as discussed above. Such an acute change of solution behavior is possible if a large conformational change has occurred in 1.8 M $MgCl_2$. Indeed, the major conformational transition of aldolase in $MgCl_2$ takes place at approximately 1.8 M (Hsu and Neet[1]). However, due to the steepness of the $s\eta$ transition, it is likely that dissociation has also occurred. The molecular weight of aldolase in 1.8 M $MgCl_2$ or above was not studied because of the difficulty involved in evaluating the partial specific volume from pycnometric measurements.

*Dependence of $s_{20,w}$ of Aldolase on Protein Concentration in 1.2 M $MgCl_2$.* Sedimentation velocity experiments were undertaken to characterize the behavior of aldolase in 1.2 M $MgCl_2$. There was no indication of boundary separation even at the lowest protein concentration studied although a broadening of the boundary was observed.[3] A decrease of $s_{20,w}$ with decreasing protein concentration was observed in the range of protein concentration from 1 mg/ml to 0.1 mg/ml (Figure 2). Such a positive dependence of the $s_{20,w}$ on protein concentra-

TABLE I: Result of Adams Analysis.

| Protein Concn (mg/ml) | Model | Eq[a] | Calcd[b] | True[c] | Difference[d] | $BM_1$ |
|---|---|---|---|---|---|---|
| 0.27 | Monomer– | I | 0.65 | 0.39 | 0.26 | −0.0041 |
| 0.55 | tetramer | | 0.84 | 0.54 | 0.30 | |
| 0.82 | | | 0.69 | 0.62 | 0.07 | |
| 1.18 | | | 0.93 | 0.71 | 0.21 | |
| 0.27 | Monomer– | II | −1.80 | −2.40 | 0.60 | −0.0041 |
| 0.55 | tetramer | | −5.38 | −6.09 | 0.71 | |
| 0.82 | | | −9.53 | −9.98 | 0.45 | |
| 1.18 | | | −12.46 | −13.80 | 0.34 | |
| 0.27 | Monomer– | IV | −0.30 | −0.30 | 0.00 | −0.0092 |
| 0.55 | dimer– | | −1.14 | −1.12 | 0.02 | |
| 0.82 | tetramer | | −2.07 | −2.02 | 0.05 | |
| 1.18 | | | −3.28 | −3.29 | 0.01 | |
| 0.27 | Monomer– | III | 2.19 | 2.16 | 0.03 | −0.0092 |
| 0.55 | dimer– | | 4.41 | 4.40 | 0.09 | |
| 0.82 | tetramer | | 6.43 | 6.56 | 0.13 | |
| 1.18 | | | 9.50 | 9.44 | 0.06 | |
| 0.27 | Monomer | V | −3.10 | −3.06 | 0.04 | −0.0110 |
| 0.55 | dimer– | | −7.84 | −7.75 | 0.09 | |
| 0.82 | trimer– | | −12.68 | −12.63 | 0.05 | |
| 1.18 | tetramer– | | −19.40 | −19.30 | 0.10 | |

[a] Equations used were as follows

$$\frac{4CM_1}{M_n(r)} - C = \psi + \frac{4}{[M_1/CM_w(r)] - BM_1} + 2BM_1C \quad \text{(I)}$$

$$\frac{20CM_1}{M_n(r)} - 21C = \psi + 10BM_1C^2 \quad \text{(II)}$$

$$8C = \frac{6}{[M_1/CM_w(r)] - BM_1} + \psi + 3\alpha \exp(-BM_1C) \quad \text{(III)}$$

$$\frac{8CM_1}{M_n(r)} - 6C = 3\alpha \exp(-BM_1C) + 4BM_1C^2 - \frac{1}{[M_1/CM_w(r)] - BM_1} \quad \text{(IV)}$$

$$\frac{24CM_1}{M_n(r)} - 26C = 6\alpha \exp(-BM_1C) + 12BM_1C^2 - \psi - \frac{9}{[M_1/CM_w(r)] - BM_1} \quad \text{(V)}$$

where $\psi = \{(d/dc)[M_1/CM_w(r)]\}/\{([M_1/CM_w(r)] - BM_1)^3\}$ and $\alpha = c \exp \int_0^c ([M_1/M_w(r)] - 1)(dC/C)$. [b] Right-hand side of appropriate equation. [c] Left-hand side of appropriate equation. [d] [Calculated − true].

[3] Occasionally, a small amount of slower moving component was observed. When commercial aldolase was used without further purification, two or three boundaries were evident in scanner tracings at low concentrations. The nature of this phenomenon is not known.

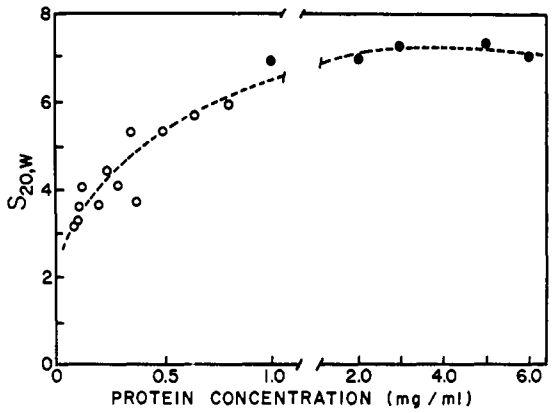


FIGURE 2: Dependence of $s_{20,w}$ of aldolase on protein concentration in 1.2 M $MgCl_2$–0.2 M Tris-Cl (pH 7.2). Filled circles are data obtained from experiments using the synthetic boundary cell and Schlieren optics. Open circles are data obtained by using the synthetic boundary cell and uv optics (280 nm) with photoelectric scanner and multiplexer. All solutions were previously dialyzed against the solvent for about 36 hr. Experimental conditions: rotor speed, 52,000 rpm; temperature, 20.0–22.2°.

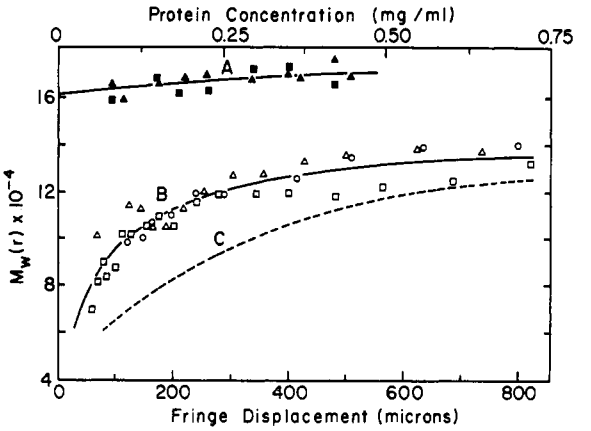


FIGURE 3: Dependence of point weight-average molecular weight on aldolase concentration in $MgCl_2$: (A) 0.6 M $MgCl_2$, loading concentration (▲) 0.85 mg/ml, (■) 0.21 mg/ml, 20.6°, 20,000 rpm; (B) 0.9 M $MgCl_2$, loading concentration (△) 0.22 mg/ml, (□) 0.43 mg/ml, (○) 0.80 mg/ml, 21.7°, 22,000 rpm; (C) 1.2 M $MgCl_2$ (see Figure 4), 20.8–21.6°, 26,000 rpm. All solutions were dialyzed against the corresponding solvents for about 36 hr.

tion is a distinct characteristic of a rapid dissociation–association system. In 0.1 M Tris alone such anomalous sedimentation behavior of aldolase was not observed.

The $s^0_{20,w}$ of aldolase in 1.2 M $MgCl_2$ was difficult to evaluate since the precision of sedimentation velocity data tends to be lower at lower protein concentration. Nevertheless, as can be seen in Figure 2, it seems reasonable to estimate a value of $s^0_{20,w}$ somewhere between 2.0 and 2.5 S. The $s^0_{20,w}$ of aldolase in 1.2 M $MgCl_2$ is thus close to the reported $s^0_{20,w}$ of 1.9–2.1 S for the aldolase monomer in acid or urea (Deal *et al.*, 1963; Stellwagen and Schachman, 1962). The possibility of only monomers existing in rapid equilibrium with tetramers in 1.2 M $MgCl_2$ is not consistent with the observation of a single, symmetrical boundary at all protein concentrations. Based on the theoretical analysis of association models by Gilbert (1955) and Cox (1969) one would expect a markedly skewed boundary or the appearance of two boundaries for a monomer–tetramer interacting system. The observations made by sedimentation velocity studies, therefore, suggested that a significant amount of intermediate subunit species, *i.e.*, dimer and/or trimer, existed in reversible equilibrium with monomer and tetramer. Indeed, calculations by the asymptote method of Gilbert (1959) based upon the association constants obtained from sedimentation equilibrium studies (next section) predict a single, slightly skewed boundary for the monomer–dimer–tetramer case. The slight asymmetry of the curves was not detectable in the scanner tracings probably because of the noise and lower precision of the scanner at low protein concentrations. A quantitative analysis of subunit interactions was further undertaken using sedimentation equilibrium data as presented in the following sections.

*Sedimentation Equilibrium Studies in 1.2 M $MgCl_2$.* Sedimentation equilibrium studies of native aldolase in 0.1 M Tris-Cl–0.1 M NaCl at pH 7.2, using the high speed, meniscus depletion technique (Yphantis, 1964) revealed essentially a homogeneous preparation as evidenced by a linear $\ln f$ *vs.* $r^2$ plot and a linear dependence of apparent weight-average molecular weight on protein concentration. The extrapolated weight-average molecular weight of aldolase at infinitely dilute protein concentration is 162,000 ± 6,000, which is in good agreement with the reported molecular weight for the aldolase tetramer (Kawahara and Tanford, 1966; Sia and Horecker, 1968; Castellino and Barker, 1968). In agreement with the observations of Castellino and Barker (1968) and Eisenberg and Reisler (1969) there is no evidence for a spontaneous dissociation of aldolase tetramer with decreasing protein concentration to at least 0.1 mg/ml.

The effect of $MgCl_2$ concentration on the weight-average molecular weight distribution of aldolase is shown in Figure 3. In 0.6 M $MgCl_2$, it is evident that no significant dissociation of tetramer had occurred. In 0.9 M $MgCl_2$, however, a significant depression of molecular weight below that of the tetramer and a strong dependency of apparent molecular weight on protein concentration were observed. In 1.2 M $MgCl_2$ the apparent molecular weight of aldolase was further decreased and apparent heterogeneity was evident from the distinct distributions of apparent *z*-, weight-, and number-averaged molecular weights (Figure 4). The apparent hetero-

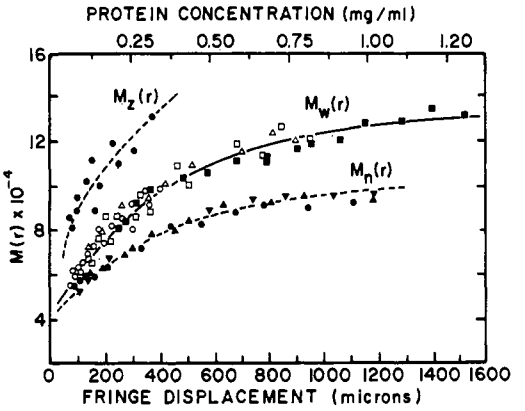


FIGURE 4: $M_w(r)$, $M_n(r)$, and $M_z(r)$ of aldolase in 1.2 M $MgCl_2$–0.2 M Tris-Cl, pH 7.2, as a function of protein concentrations. For clarity, only about half the data points obtained are shown. $M_w(r)$, loading protein concentration (mg/ml): (△) 0.54, 0.16; (□) 0.20, 0.64; (○) 0.74, 0.24; 26,000 rpm, 20.6–21.6°; (■) 1.27, 16,000 rpm, 21.1°. The curve was drawn based on the association constant calculated for a monomer–dimer–tetramer association (Table II). $M_n(r)$, loading protein concentration (mg/ml): (●) 0.58; (▲) 0.21; (▼) 0.78; 26,000 rpm, 20.6–21.6°. $M_z(r)$, loading protein concentration (mg/ml): (●) 0.26; 26,000 rpm, 21.6°.

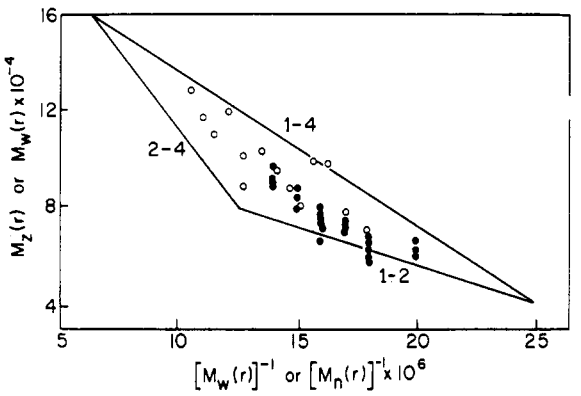


FIGURE 5: "Two-species plot" for analyzing the molecular weight data. The $M_n(r)$, $M_w(r)$, and $M_z(r)$ data were calculated from data obtained from a single sedimentation equilibrium experiment: (○) $M_z(r)$ *vs.* $1/M_w(r)$; (●) $M_w(r)$ *vs.* $1/M_n(r)$. The lines 1–4, 2–4, and 1–2 represent the theoretical line for a mixture of monomer–tetramer, dimer–tetramer, and monomer–dimer, respectively.

geneity in the molecular weight of aldolase in 1.2 M $MgCl_2$ is attributable to a reversible dissociation for the following reasons. First, there is no observable heterogeneity of molecular weight of aldolase preparations in the absence of $MgCl_2$ or in 0.6 M $MgCl_2$. Second, sedimentation velocity in 1.2 M $MgCl_2$ revealed no slow moving component which could be ascribed to a noninteracting monomer or a contaminating protein. Third, there seems to be a greater depression of molecular weight of aldolase with increasing $MgCl_2$ concentration. Fourth, the molecular weight data obtained from the same or different experiments at varying initial concentrations of the protein overlapped to form a continuous, characteristic distribution curve of apparent molecular weight with respect to protein concentration.

The molecular weight data shown in Figure 4 have been collected from four ultracentrifugation experiments, three using the conventional high speed method (Yphantis, 1964) and one using an intermediate speed with the fringe labeling technique (Goldberg and Edelstein, 1969). The scattering of the experimental results was judged to be due to random errors and the inherent uncertainty in the analysis of interference plates since there is a complete lack of systematic deviation of results with the radial position of samples or the initial loading concentration of samples. The data obtained from the experiments using an intermediate rotor speed overlapped with those obtained from the high-speed experiments. Thus, although a systematic study of the effect of pressure on the molecular weight distribution of aldolase has not been undertaken, it is likely that such an effect did not significantly alter the results.

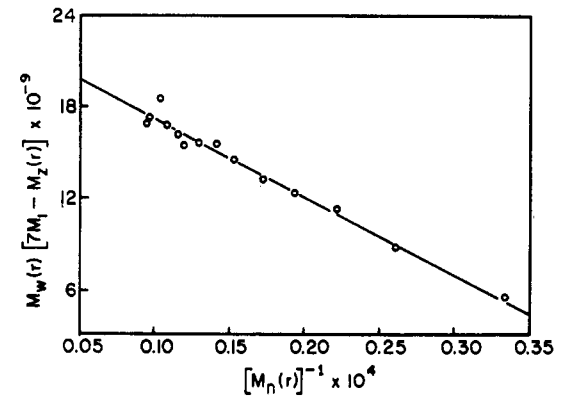


FIGURE 6: "Three-species plot" for analyzing the molecular weight data according to eq 10. The data were smoothed and corrected for nonideality as described under Methods. The line was drawn based on a nonweighted least-squares treatment of data.

Under dissociating conditions, it is desirable to know what subunit species are present in reversible equilibrium with the tetramer. The data were analyzed to determine if they were consistent with the presence of two species, *i.e.*, monomer–tetramer, or three species, *i.e.* monomer–dimer–tetramer. The simplest case involves only two species in the solution. To test this possibility, the "two-species plot" (Roark and Yphantis, 1969) was applied to analyze the molecular weight data. The equations used for such plots are

$$M_w(r) = -4M_1^2\left(\frac{1}{M_n(r)}\right) + 5M_1 \quad (4)$$

$$M_z(r) = -4M_1^2\left(\frac{1}{M_w(r)}\right) + 5M_1 \quad (5)$$

where $M_1$ represents the molecular weight of the smaller species. Figure 5 shows a typical two-species plot of a set of experimental data obtained from a single experiment. The experimental data do not follow any one of the three characteristic distribution lines which depict the theoretical relationship of $M_w(r)$, $M_z(r)$, and $M_n(r)$ (assuming a $M_1$ of 40,000) according to the models of a mixture of monomer–dimer, dimer–tetramer, and monomer–tetramer. The result of this analysis suggests that the data are not compatible with a model based on a mixture of only two species. Essentially the same conclusion was obtained when data from different experiments were combined for analysis. Smoothing of the $M_w(r)$ data with a polynomial function and utilization of eq 2a did not alter this conclusion.

The data were further analyzed in terms of a model consisting of monomer, dimer, and tetramer. Weight-, number-, *z*-average molecular weight data were used to fit a "three-species equation" which was derived from the following simultaneous equations

$$\frac{M_w(r)}{M_1} = f_1 + 2f_2 + 4f_4 \quad (6)$$

$$\frac{M_1}{M_n(r)} = f_1 + 0.5f_2 + 0.25f_4 \quad (7)$$

$$\frac{M_z(r)M_w(r)}{M_1^2} = f_1 + 4f_2 + 16f_4 \quad (8)$$

$$1 = f_1 + f_2 + f_4 \quad (9)$$

After eliminating the weight fractions, $f_1$, $f_2$, and $f_4$, of monomer, dimer, and tetramer, respectively, eq 10 is obtained.

$$M_w(r)[7M_1 - M_z(r)] = 14M_1^2 - 8M_1^3\left(\frac{1}{M_n(r)}\right) \quad (10)$$

Based on a known molecular weight of 40,000 for the monomer of aldolase, the molecular weight data were tested by plotting the left-hand side of eq 10 against $1/M_n(r)$ using the ideal quantities calculated from the smoothed, apparent molecular weight data (see Methods) (Figure 6). A least-squares

TABLE II: Apparent Equilibrium Constants and Free-Energy Change for the Association of Aldolase Subunits in 1.2 M $MgCl_2$–0.2 M Tris-HCl (pH 7.2).

| Method Calcn | $k_2$ (l./g) | $K_2$ (l./mol) | $\Delta G_2$ (kcal) | $k_4$ (l./g) | $K_4$ (l./mol) | $\Delta G_4$ (kcal) |
|---|---|---|---|---|---|---|
| 1. Steiner's method | 5.8 | $1.2 \times 10^5$ | −6.8 | 24.4 | $9.8 \pm 10^5$ | −8.0 |
| 2. Wt fraction method[a] | | | | | | |
| a. nonweighted av | $7.0 \pm 2.1$ | $1.4 \times 10^5$ | −6.9 | $20.1 \pm 7.9$ | $8.0 \times 10^5$ | −7.9 |
| b. weighted av | 6.9 | $1.4 \times 10^5$ | −6.9 | 15.3 | $6.1 \times 10^5$ | −7.8 |

[a] Adams' analysis.

analysis revealed a highly significant linear correlation coefficient and the $M_1$ calculated from the fitted line yielded values of 40,047 and 40,090 for $M_1$ from the slope and intercept, respectively. This suggested that the data are compatible with a three-species model provided that the molecular weight of monomer is assumed to be 40,000.

Further analyses of the dissociating system were carried out using methods developed by Adams (1967a) for analyzing self-associating systems. The method involves choosing an appropriate association model, deriving an equation in which the virial coefficient, $B$, is the unknown quantity, and solving the equation by a reiterative, numerical process. The analysis may then be checked and a decision made as to which model is best able to fit the data over a range of concentrations by comparing "calculated" values (employing $B$) of the appropriate equations to "true" values (from the primary data). A representative part of the result of the numerical analysis is shown in Table I. The degree of fit of any model can be compared by the difference between the calculated and true columns. The monomer–tetramer model (eq I or II) does not fit the data as well as the three- or four-species model (Table I). This supports the result of the analysis using the two-species plot. It is also evident that the behavior of the reacting system approaches ideal behavior since the calculated virial coefficients are all insignificantly small. The analysis, however, showed that a four-species model would fit the data as well as a three-species model. Thus, it is not possible to decide which model is correct based purely on the numerical fit. The difficulty may have arisen from the limitation of data in two respects. First, the data obtained may not be accurate enough for differentiating the two models. Second, the range of protein concentration may be too limited to permit a conclusive analysis. Similar difficulty has been reported in the analysis of association behavior of $\alpha$-chymotrypsin (Adams, 1967b) even though molecular weight data obtained from a wide range of protein concentrations were available for analysis. As pointed out, however (Adams, 1967b), a final examination of parameters should be made to determine whether a model chosen by mathematical analysis would indeed conform to physical reality. Such a final examination of parameters was incorporated in calculating the equilibrium constants by solving simultaneous equations, including the one expressing the conservation of mass, as will be shown in a following section.

Although Adams' method of analyzing an interacting system represents the most practical and sophisticated one so far, it demands data of high precision since a fairly extensive manipulation of primary data is involved. A method of successive analysis of a discrete associating system involving several intermediate species has been given by Steiner (1953). Since there is only one additional quantity, the weight fraction of monomer to be calculated from the primary data, it probably involves less risk of incorporating a propagated error in manipulating the data. The method also has the advantage of graphical evaluation of the fit of data to the models. It should be pointed out, however, that Steiner's method is limited to a system with ideal thermodynamic behavior. Since the molecular weight data on aldolase were obtained at low protein concentrations and the virial coefficient from the Adams' analysis was small, the assumption of ideality can be reasonably made to permit the analysis. Equation 11 describes a monomer–dimer–tetramer system with the reaction scheme

$$M_1 + M_1 \overset{k_2}{\rightleftharpoons} M_2$$

$$M_2 + M_2 \overset{k_4}{\rightleftharpoons} M_4$$

$$\left(\frac{M_w(r)}{M_1 f_1} - 1\right)/f_1 c = 2k_2 + 4k_4 k_2^2 (f_1 c)^2 \qquad (11)$$

where $k_2 = c_2/c^2$ and $k_4 = c_4/c_2^2$. Figure 7 shows a plot of the left-hand side of eq 11 against $(f_1 c)^2$. An excellent linearity of the plot was noted as predicted from the model. The association constants, $k_2$ and $k_4$, were calculated from the slope and intercept of a least-squares equation fitted through the data (Table II). Equation 12 describes a four-species model with the reaction scheme

$$M_1 + M_1 \overset{k_2}{\rightleftharpoons} M_2$$

$$M_2 + M_1 \overset{k_3}{\rightleftharpoons} M_3$$

$$M_3 + M_1 \overset{k_4}{\rightleftharpoons} M_4$$

$$\left[\frac{M_w(r)}{M_1 f_1} - 1\right](f_1 c)^{-1} = 2k_2 + 3k_2 k_3 (f_1 c) + 4(k_2 k_3 k_4)(f_1 c)^2 \qquad (12)$$

where $k_2 = (c_2/c_1^2)$, $k_3 = (c_3/c_2 c_1)$, and $k_4 = (c_4/c_3 c_1)$. A plot of the left-hand side of eq 12 *vs.* $(f_1 c)$ was nonlinear. The limiting intercept, $2k_2$, was evaluated from this plot, fed back into eq 12, and, after rearrangement, a plot of $[(M_w(r)/M_1 f_1) - 1 - 2k_2 f_1 c][f_1 c]^{-2}$ *vs.* $f_1 c$ was made (Figure 8). If a linear plot were to be imposed upon the data, as predicted from this model, a negative intercept on the ordinate would be obtained. Since the intercept on the ordinate represents a group of equilibrium constants, *i.e.*, $3(k_2 k_3)$, which cannot assume a negative value, the model failed in the sense that it yielded no physically meaningful parameter. Even considering an un-

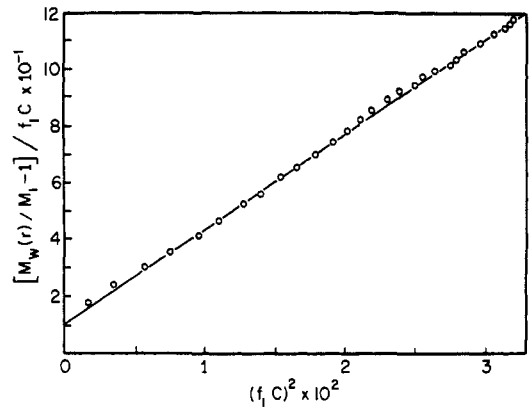


FIGURE 7: Test for a monomer–dimer–tetramer association by plotting the molecular weight data according to eq 11.

certainty in estimating the $2k_2$, negative intercepts on the ordinate were invariably obtained.

Similar difficulty occurred in calculating the distribution of weight fractions of subunits as a function of protein concentration based on a four-species model with the parameters derived from the Adams' analysis (Table I). The weight fractions of subunits at a given protein concentration were calculated directly by solving four simultaneous equations, 13–16,

$$\frac{M_w(r)}{M_1} = f_1(r) + 2f_2(r) + 3f_3(r) + 4f_4(r) \quad (13)$$

$$\frac{M_1}{M_n(r)} = f_1(r) + {}^1/_2 f_2(r) + {}^1/_3 f_3(r) + {}^1/_4 f_4(r) \quad (14)$$

$$\frac{-\psi(r)}{c(r)} = f_1(r) + 4f_2(r) + 9f_3(r) + 16f_4(r) \quad (15)$$

$$1 = f_1(r) + f_2(r) + f_3(r) + f_4(r) \quad (16)$$

where $\psi(r)$ is defined in Table I. Upon solving these equations, weight fractions $f_2$ or $f_3$ appeared as negative numbers, which are not physically meaningful. In contrast, a similar calculation based on a three-species model (*i.e.*, $f_3 = 0$) yielded positive values for weight fractions of monomer, dimer, and tetramer. The weight fractions from this analysis are plotted as a function of protein concentration (Figure 9). Thus, analyses by both Adams' method and Steiner's method agree that the monomer–dimer–tetramer model gives the best fit with meaningful parameters.

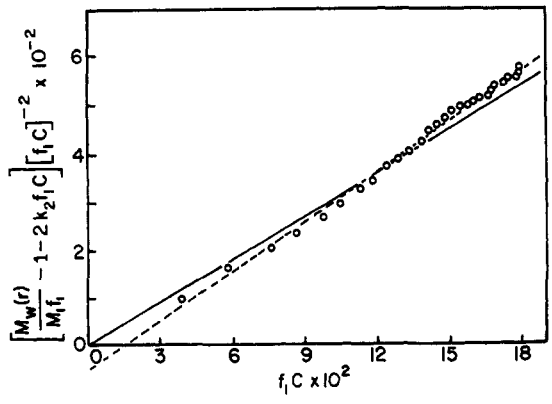


FIGURE 8: Test of monomer–dimer–trimer–tetramer association by plotting the molecular weight data according to eq 12, rearranged. The quantity $2k_2$ is 7.5 l./g, which was obtained from a plot of $[(M_w/M_1f_1) - 1]/f_1C$ *vs.* $fC$ (figure omitted). The dashed line represents the best fit line for the data. For comparison of fit, a solid line is drawn which yields a minimal positive intercept on the ordinate.

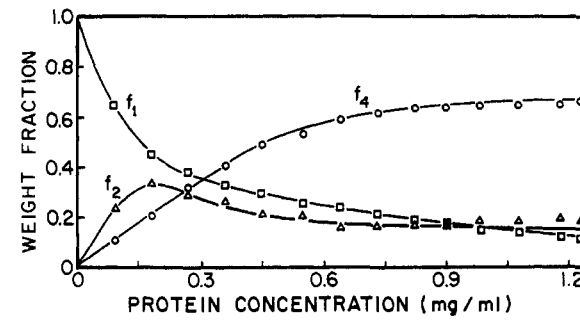


FIGURE 9: Weight fraction of monomer, dimer, and tetramer as a function of aldolase concentration in 1.2 M $MgCl_2$–0.2 M Tris-Cl. The values were calculated from eq 13–16.

The apparent equilibrium constants of subunit interactions were calculated from the values of the weight fractions (Adams analysis, Figure 9) at each radial position, $r$, according to the following relationships

$$k_2(r) = \frac{f_2(r)}{c(r)f_1^2(r)}$$
$$k_4(r) = \frac{f_4(r)}{c(r)f_2^2(r)} \quad (17)$$

To characterize the whole reaction system with a single set of apparent equilibrium constants, these point-average equilibrium constants were averaged (a) by simple arithmetic means without weighting factors, (b) by introducing a simple weighting factor based on the goodness-of-fit between experimental data and the model at a given point. The weighted-average equilibrium constant was calculated from eq 18. The weighting

$$k_2 = \frac{\Sigma w(r)k_2(r)}{\Sigma w(r)} \quad (18)$$

factor, $w(r)$, is $|f_1(r)e^{BM_1c(r)} - f_1'(r)|^{-1}$. The term $f_1'(r)$ is the experimentally observed data calculated from eq 19 (Steiner,

$$f_1'(r) = \exp\left[\int_0^c \left(\frac{M_1}{M_w(r)} - 1\right)\frac{dc}{c}\right] \quad (19)$$

1953). The quantity $f_1(r)$ is the calculated weight fraction of monomer based on the three-species model.[4] The equilibrium constants for the subunit interactions estimated by these methods, as well as the corresponding free-energy change, are in Table II.

*Enzymatic Activity of Aldolase in $MgCl_2$.* The catalytic activity of aldolase was markedly inhibited in concentrated $MgCl_2$ solutions at neutral pH. In 1.2 M $MgCl_2$, for example, only 0.2–0.5% of the original activity was observed. When $MgCl_2$ was removed by dilution, the activity was fully recovered except when aldolase was incubated in $MgCl_2$ above

[4] Since $BM_1c(r)$ is a very small quantity, $f_1(r)\exp(BM_1c(r))$ is approximately equal to $f_1(r)$.

1.8 M. The extent of irreversible inactivation of enzyme activity increased with increasing $MgCl_2$ concentration and in 3.2 M $MgCl_2$, virtually no activity could be recovered by dilution. The inhibition or the recovery of enzyme activity by adding $MgCl_2$ or removing $MgCl_2$ by dilution in the assay occurred within seconds and no slow, time-dependent change in activity was observed during 15–20 min of assay. Urea at concentrations above 2.4 M at pH 7.2 causes a slow, time-dependent inactivation of aldolase as is evident from a kinetic assay curve with constantly decreasing slope (Deal *et al.*, 1963). Addition of $MgCl_2$ accelerated the rate of inactivation in urea (Hsu, 1972[5]).

Kinetic studies of aldolase in 1.2 M $MgCl_2$ were done in an attempt to correlate the enzymatic properties of aldolase with any changes in subunit interactions. It is evident from Figure 9 that the concentration of monomer (and dimer above 0.2 mg/ml) increased with decreasing protein concentration in 1.2 M $MgCl_2$. The specific activity of aldolase was studied with varying protein concentration and the result is shown in Figure 10. There is no tendency toward increasing or decreasing enzyme activity with varying protein concentration indicating a lack of correlation between the enzyme activity and the state of association.

Kinetic studies of aldolase in 1.2 M $MgCl_2$ also revealed that Michaelis–Menten kinetics were followed as evidenced by a linear double reciprocal plot. A Hill plot of these data yielded an interaction coefficient of 1.07. The $K_m$ and $V_m$ were calculated to be 0.11 M and 0.062 unit, respectively ($V_m$ of native enzyme = 18 units). If it is assumed that only $FDP^{4-}$ but not $MgFDP^{2-}$ is the true substrate for aldolase, the $K_m$ corresponded to $1.6 \times 10^{-4}$ M $FDP^{4-}$ in 1.2 M $MgCl_2$, using the reported equilibrium constant (McGilvery, 1965) for the reaction between $Mg^{2+}$ and $FDP^{4-}$ ($K_m$ of native enzyme = $2 \times 10^{-5}$ M). The linearity of the double reciprocal plot and the constant specific activity with protein concentration are consistent with the hypothesis that FDP binds and is cleaved equally well by monomer, dimer, and tetramer.

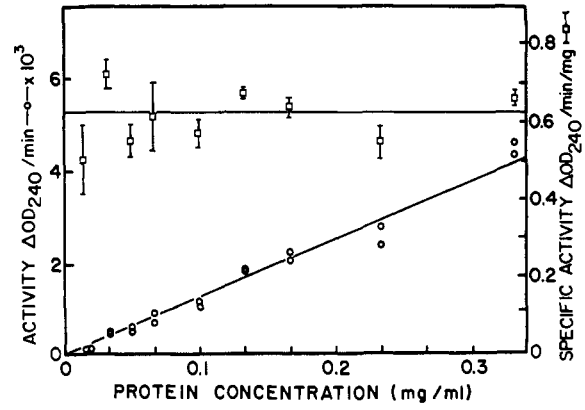


FIGURE 10: Dependence of aldolase activity and specific activity on protein concentration in 1.2 M $MgCl_2$. The substrate concentration used was 0.12 M FDP.

## Discussion

It is well known that the interpretation of ultracentrifugal data of a multicomponent system is complicated by the problem of preferential solvation. For example, recent studies on the dissociation of human carboxyhemoglobin in concentrated NaCl disclosed that the dissociation constants may be overestimated due to high degrees of preferential hydration of hemoglobin molecules (Edelstein *et al.*, 1971). Similarly, the dissociation of $\alpha$-glyceraldehydephosphate dehydrogenase in concentrated phosphate buffer was also found to be negligible if the preferential hydration effect was properly evaluated (Aune and Timasheff, 1970). To avoid this pitfall, we followed the thermodynamic treatment of a multicomponent system suggested by Casassa and Eisenberg (1964). The apparent specific volume of aldolase in 1.2 M $MgCl_2$ as defined by these authors was measured by pycnometry, and the protein concentration was determined by spectrophotometry. The close agreement of the apparent specific volume of aldolase measured in 1.2 M $MgCl_2$ with that measured in dilute Tris buffer and also that of reported values (Taylor and Lowry, 1956) suggested that aldolase molecules are not preferentially hydrated in 1.2 M $MgCl_2$. This conclusion is also supported by an analysis of sedimentation velocity data. Retardation of the elution of aldolase on a Sephadex G-100 column in 1.2 M $MgCl_2$ also indicates that dissociation has occurred rather than preferential solvation (Hsu, 1971). No attempt was made to evaluate the partial specific volume of aldolase in 1.2 M $MgCl_2$ by the isotope substitution method as devised by Edelstein and Schachman (1967) since it was known that water molecules in the hydration shell of $Mg^{2+}$ ion are not readily exchangeable with $D_2O$ in the bulk solvent (Hill and Cox, 1965).

The apparent equilibrium constants for subunit interactions obtained from ultracentrifugal analysis do not necessarily reflect the microscopic strength of interaction between interfaces of the subunits if a conformational isomerization reaction is occurring along with the association–dissociation reactions. A close examination of conformationally linked dimerization reactions has recently been made (Neet, 1972[6]). Theoretical analysis showed that even in relatively simple cases, the apparent equilibrium constants, as calculated from ultracentrifugal data, are actually made up of intrinsic association or dissociation constants and isomerization constants. In 1.2 M $MgCl_2$, a very small but definite conformational change of aldolase can be detected by sensitive techniques such as difference spectrophotometry and spectrophotofluorometry (Hsu and Neet[1]). Analysis of the conformational transition profiles revealed that, in 1.2 M $MgCl_2$, aldolase largely exists in a stable conformational state which is distinguishable from the native state. Thus, the apparent equilibrium constants of subunit interactions obtained in 1.2 M $MgCl_2$ can be viewed as a close approximation to the intrinsic equilibrium constants of the aldolase existing in the new conformational state. Even a very small perturbation of subunit interactions (either by a small displacement of interacting amino acids in the contact region) may have a large effect on the apparent molecular weight distribution (Klotz *et al.*, 1970) and cause the differences between aldolase in the native state and in $MgCl_2$. The analysis of the relationship of the conformational change of aldolase in 1.2 M $MgCl_2$ with the subunit interactions and the ramifications for the native molecule will be presented in subsequent communications (Neet and Hsu[1]).

Aldolase in 1.2 M $MgCl_2$ was deduced to be a rapidly interacting system of subunits. First, a plot of $s_{20,w}$ *vs.* protein concentration had a positive initial slope. Second, the apparent point weight-average molecular weight obtained from different experiments using different loading protein concentrations and

[5] Hsu, L. S., unpublished observation.

[6] Neet, K. E. (1972), manuscript in preparation.

rotor speeds overlapped with one another to form a unique distribution curve. The scattering of the data was judged to be random in nature since no systematic deviation with varying loading concentrations or radial positions could be detected. Although the precision of these data was somewhat limited they proved to be adequate for analyzing the association model based on existing theories advanced by Steiner (1953) and Adams (1967a,b).

The interacting system of aldolase subunits in $MgCl_2$ was analyzed by the method of two- and three-species plots, which does not invoke any assumption about the interactions among the molecular species, by the curve-fitting method of Steiner (1953) and by the numerical method of Adams (1967a,b). The latter two methods also yield information on the strength of the interaction. The results from these different types of analyses all consistently indicated that monomer, dimer, and tetramer are the interacting species existing in 1.2 M $MgCl_2$. The four-species model was rejected due to the failure of such a model to give physically meaningful parameters, such as weight fractions or equilibrium constants. It seems unlikely that the convergence on the monomer–dimer–tetramer model is coincidental due to inaccurate data, since there is little chance that by coincidence different types of analyses will all give the three-species model as the best fit. An indefinite association of monomer in an independent (*i.e.*, not at thermodynamic equilibrium), parallel reaction was not considered possible since the $M_w(r)$ *vs.* $c$ curves did not exhibit a systematic variation with loading concentration of protein.

There is considerable evidence indicating that the subunits of rabbit muscle aldolase are nonidentical (Edelstein and Schachman, 1966). Chan *et al.* (1967) have actually succeeded in separating the two types of subunits by chromatography of carboxymethylaldolase on DEAE-cellulose in 8 M urea. It was found, however, that there is an overall similarity of chemical structure between the two types of subunits. Despite the chemical differences existing in the two types of subunit, recent X-ray diffraction studies of aldolase crystals by Heidner *et al.* (1971) revealed that the four subunits of aldolase appear identical at a resolution of 4 Å. In light of this evidence suggesting similarity in the chemical structure and the conformation of aldolase subunits, it is appropriate to analyze the thermodynamic data obtained in this study in terms of a model of aldolase as a tetramer composed of identical subunits. This approximation will allow us to analyze the data by making use of the analytical methods recently developed by Klotz *et al.* (1970) and by Cornish-Bowden and Koshland (1971) to deduce more detailed information concerning the subunit interactions of aldolase. Specifically, one would like to know whether or not the subunit interactions in aldolase involve isologous or heterologous binding sites and the distribution of energy of interaction among these sites.

Based on a statistical analysis, Cornish-Bowden and Koshland (1971) deduced that for a tetramer with tetrahedral symmetry, only $I_6$ (*i.e.* all subunit interactions involve isologous sites) and $IH_5$ (*i.e.* subunit interactions involve one isologous and five heterologous sites) types would occur in preponderance. Similarly, for square-planar symmetry $I_4$ and $H_4$ would most likely occur. Furthermore, differentiation between all isologous models and models including heterologous sites can be made if thermodynamic data, such as equilibrium constants, are known. While $K_2'$ and $K_4'$, *i.e.* the dimerization and tetramerization constants (from monomer), are readily calculated from ultracentrifugal data, $K_3'$, the trimerization constant (from monomer), is not directly available since the reaction mixture contained no measurable concentration of trimer. The quantity $K_3'$, however, may be evaluated from knowledge of the arrangement of four subunits and the known free-energy change involved in the formation of dimer and tetramer. Following the treatment of energetic data suggested by Klotz *et al.* (1970), the free-energy change associated with the formation of a dimer can be equally divided between the two monomeric units and the resultant quantity represents the average free-energy difference between a mole of "free monomer" and the monomer existing as a subunit in a dimer. The free-energy difference per mole of monomer, designated as $(\Delta G_m)_{M-D}$, is $\Delta G_2/2$, namely $-3.45$ kcal. Similarly, a quantity designated as $(\Delta G_m)_{M-T}$, representing the average free-energy difference between a mole of "free monomer" and a mole of monomer existing as a subunit in a tetramer, would be calculated to be $-5.4$ kcal $[(2\Delta G_2 + \Delta G_4)/4]$. The $(\Delta G_m)_{M-T}$ includes $(\Delta G_m)_{M-D}$ and also the contribution from one or two additional site(s) on a monomer which are engaged in the interactions with the corresponding sites in the neighboring subunits to form a tetramer. For either tetrahedral or square-planar symmetry the average free-energy change for the formation of a trimer can be estimated to be $-10.8$ kcal, *i.e.*, $2(\Delta G_m)_{M-D} + 2[(\Delta G_m)_{M-T} - (\Delta G_m)_{M-D}] = \Delta G_2 + \Delta G_4/2$, and the corresponding trimerization constant, $K_3'$, would be $1.2 \times 10^8$ $l.^2/mol^2$. The tetramerization constant $K_4'$, corresponding to $4(\Delta G_m)_{M-T}$, would be $1.32 \times 10^{16}$ $l.^3/mol^3$.

When these values were examined with the contour map as reported by Cornish-Bowden and Koshland (1971) it was found that the set of equilibrium constants allowed an $I_6$ or $I_4$ but not an $IH_5$ or $H_4$ type of tetramer. Thus, it is suggested that the four subunits of aldolase interact with one another through isologous binding sites. The validity of this conclusion depends on two assumptions: (1) aldolase can be approximately viewed as a tetramer composed of four identical subunits; (2) the original assumptions by Cornish-Bowden and Koshland in deriving the contour map for subunit models are correct. As a check of the internal consistency of this approach, the theoretically predicted maximal concentration of intermediate species was calculated, based on these equilibrium constants (Cornish-Bowden and Koshland, 1971). The maximal weight fractions of dimer and trimer calculated from the set of equilibrium constants are 28 and 0.07%, respectively. The total protein concentration at which maximal concentration of dimer occurs is calculated to be 0.082 mg/ml. These values are in reasonable agreement with the values calculated from the primary data (Figure 9).

There have been some controversial results reported in regard to the observation of a stable dimer of aldolase under various conditions. Blatti (1968) concluded that a 3.4-S sedimenting species in sucrose-gradient ultracentrifugation at pH 3.7 was a stable dimer. Deal *et al.* (1963), however, suggested that the 3.4-S species observed in sedimentation velocity experiments at pH 3.7 was an unfolded aldolase tetramer since the molecular weight of this 3.4-S species was close to that of the tetramer. Kawahara and Tanford (1966) observed that the molecular weight of aldolase in 0.15 M NaCl (pH 6) decreased to about 80,000 (dimer) below 0.2 mg/ml. Woodfin (1967) observed that aldolase dissociated into active dimers when incubated with high concentration of substrate FDP. These interesting results were, however, not confirmed (Castellino and Barker, 1968). More recently Steinman and Richards (1970) also reported that aldolase dissociated into monomers in concentrations of guanidine-HCl above 1.1 M. It is likely, however, that the stability of the aldolase dimer depends on the dissociating conditions and also on the nature of reagents used to perturb the subunit interactions. The existence of an

appreciable amount of dimer in $MgCl_2$ is likely to be a consequence of the presence of two types of subunit interactions in a tetramer. These two types of subunit interactions are presumably distinguishable with respect to their strength and/or their nature of interactions. Using the terminology of Klotz *et al.* (1970), these two types of interactions may be called the dimer–dimer and monomer–monomer bond. It is clear that $(\Delta G_m)_{M-D'}$ (−3.45 kcal/mol) is significantly greater than $(\Delta G_m)_{M-T} - (\Delta G_m)_{M-D}$ (−1.95 kcal/mol) which involves one (or two) sites of a monomer to form a tetramer. In other words, the free-energy change which results from one site on a monomer engaging in an interaction is significantly greater than that of the other one (or two) sites. If all six interactions among the monomers in a tetramer are present so that there are three sites on each monomer, the difference in the strength of various site–site interactions may be even greater. The difference between the types of interaction sites may be that one type of subunit contact is more extensive and stronger than the other two, and/or that one type of subunit bonding is more stable than the other two in $MgCl_2$. The reversibly interacting system defined in this study affords an opportunity for further investigation of the molecular nature of subunit interactions in aldolase.

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