The Molecular Characteristics of Yeast Aldolase*

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ABSTRACT: The preparation of a stable crystalline yeast fructose diphosphate aldolase has allowed the study of the molecular properties of this class II aldolase. Isoelectric fractionation shows the presence of three enzymatically active components each exhibiting similar molecular properties. Ultracentrifugal experiments demonstrate these molecules have a molecular weight of 80,000 ± 2,000 g/mole. In the presence of 6 M guanidine hydrochloride and 0.1 M 2-mercaptoethanol (or in acid solution), the molecule dissociates into two subunits which have molecular weights of 40,000 ± 1,000 g/mole. Yeast aldolase may be reconstituted from an acid solution of monomers to yield an enzymatically active preparation which is principally dimeric.

The metal ion is not necessary for the stabilization of the dimeric structure of yeast aldolase, but is essential for enzymatic activity. The similarities in subunit size and amino acid compositions of yeast aldolase and the rabbit aldolases suggest a structural relationship.

From comparative studies of FDP[1] aldolases obtained from phylogenetically divergent systems by Rutter and coworkers (Rutter, 1964), it has been proposed that there are two analogous classes of FDP aldolases each having distinct catalytic elements and molecular characteristics. Class I aldolases have molecular weights around 160,000, broad pH profiles, functional carboxyl-terminal tyrosine residues, and all appear to have a lysyl residue at the active site (Horecker *et al.*, 1963). Class II aldolases, on the other hand, have molecular weights of approximately 70,000 and sharp pH optima, and contain a divalent metal ion (Richards and Rutter, 1961). Recently, it has been demonstrated that the metal ion in yeast aldolase is required for catalytic activity (Kobes *et al.*, 1969).

In this paper we compare the basic molecular and structural characteristics of the yeast aldolase (the prototype of class II aldolases) with the well-studied enzyme from mammalian muscle (the prototype of the class I enzymes). The tetrameric subunit structure of rabbit muscle aldolase has now been rigorously established (Penhoet *et al.*, 1966, 1967; Kawahara and Tanford, 1966; Sia and Horecker, 1968). The molecular weight of rabbit muscle aldolase is approximately 160,000 g/mole (Kawahara and Tanford, 1966; Sia and Horecker, 1968). In the presence of 6 M guanidine hydrochloride (Kawahara and Tanford, 1966) or in acid (Sia and Horecker, 1968), the enzyme dissociates into four polypeptide chains of molecular weight of approximately 40,000 g/mole.

The experiments concerning the subunit structure of yeast aldolase have been hampered by the instability of the purified preparations. Preliminary evidence with these preparations indicated that the prototype of the class II aldolases had a molecular weight of 70,000 g/mole and two C-terminal leucine residues (Rutter *et al.*, 1966). Recently, we have developed a procedure which yields a stable preparation of yeast aldolase, thus permitting us to undertake studies on the molecular architecture of this enzyme. In this paper, we report that yeast aldolase has a molecular weight of 80,000 g/mole and in the presence of 6 M guanidine hydrochloride and 0.1 M 2-mercaptoethanol, the molecule dissociates into two subunits which have molecular weights of approximately 40,000 g/mole. Yeast aldolase may be reconstituted from an acid solution of monomers to yield an enzymatically active preparation which is principally dimeric. The metal ion is not necessary for the stabilization of the dimeric structure of yeast aldolase, but is essential for enzymatic activity. Even though the class I and class II enzymes have markedly divergent catalytic properties, the amino acid compositions of the yeast and rabbit enzymes are found to be quite similar.

Materials and Methods

Enzyme Preparation. Yeast aldolase was prepared according to the procedure of Rutter *et al.* (1966) with the following modifications. Fleischmann's bakers' yeast was extracted by homogenization with glass beads in a colloid mill at 5° and phenylmethylsulfonyl fluoride (2×10^{-4} M), a potent inhibitor of serine active-site proteases, was included in all steps of the purification procedure. The enzyme was recrystallized three to four times and yielded preparations which were essentially homogeneous on cellulose polyacetate electrophoresis at a number of pH values and on polyacrylamide disc gel electrophoresis at pH 8.3. The enzymatic activities of these preparations as assayed by the method of Richards and Rutter (1961) were 85–105 μmoles of fructose diphosphate cleaved per min per mg of protein.

The extinction coefficient at 280 mμ was taken as 1.02 optical density units/mg, based on the average of the results of three different methods: dry weight = 1.01 optical density units/mg, differential refractometry = 1.06 optical density units/mg, and fringe displacement in an artificial boundary cell in the ultracentrifuge = 0.99 optical density unit/mg.

Isoelectric focusing was carried out as described by Svensson (1962) and Vesterberg and Svensson (1966). An LKB 8101 electrofocusing column of 110-ml capacity was used and all solutions were prepared according to an instruction sheet provided by LKB Instruments, Stockholm, Sweden. The

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[1] Abbreviation used: FDP, fructose 1,6-diphosphate

density gradient was made up of sucrose (0–50%, w/v). The carrier ampholytes (pH 5–8) were purchased from LKB and made up to a final concentration of approximately 1% (w/v). For the runs in urea, the "dense" and "less dense" solutions were made up to a final concentration of 6 M urea, while 6 M urea replaced the water usually added to the anode and cathode solutions. Concentrated sulfuric acid (0.2 ml) was added at the anode (top of column) to prevent oxidation of the carrier ampholyte and 0.4 ml of ethylenediamine was added at the cathode (bottom of column) to prevent reduction. Crystalline yeast aldolase (15–30 mg) which had been centrifuged and dialyzed against 1% glycine for 24 hr replaced one of the less dense solutions when it was applied to the column. The column was run at 4° and focused using a final voltage of 900 V, for 40 hr. The column was then drained and 0.5-ml fractions were collected.

Amino Acid Analysis. For the amino acid analyses, enzyme was dialyzed against distilled water for 3 days with frequent changes of the dialysate. The samples were hydrolyzed in 6 N HCl in sealed evacuated tubes at 110° for 24 and 120 hr. Cysteine and cystine were determined as cysteic acid after performic acid oxidation of the protein for 4 hr at 20°, followed by acid hydrolysis. Amino acid analyses were carried out with a Spinco Model 120 automatic amino acid analyzer. The values for threonine and serine are extrapolated to zero time and isoleucine and leucine to infinite time; tryptophan was determined by the method of Edelhoch (1967).

Ultracentrifugation. A partial specific volume of 0.734 ml/g was determined from the amino acid analysis (McMeekin *et al.*, 1949). For all temperatures below 20°, a correction of $d\bar{v}/dt = 5.0 \times 10^{-4}$ ml per g was used (Svedberg and Pedersen, 1940).

Ultracentrifugation was performed in a Spinco Model E analytical ultracentrifuge equipped with electronic speed control and focused at the two-thirds plane of the cell (Svensson, 1954, 1956; Yphantis, 1964). Further, the lenses and other optical components were centered on the optic axis (E. G. Richards, R. H. Haschemeyer, V. D. Hoagland, D. C. Teller, and H. K. Schachman, unpublished data).

All molecular weight distributions are based on high-speed equilibrium experiments (Yphantis, 1964) using the six-channel Kel-F centerpiece designed by Yphantis (1964). Rayleigh patterns were recorded on Kodak II-G photographic plates, and read on a modified Nikon microcomparator (Teller, 1967). Computations were performed using computer programs developed in this laboratory (Teller *et al.*, 1969).

Sedimentation velocity experiments were carried out at 20° in a double-sector cell on samples varying in concentration from 2.14 to 21.40 g per l. Evaluation of the displacement of the schlieren pattern was made using a photographic grid of the ruled glass disk (Schachman, 1957). Solvent viscosities were determined using a Mechrolab viscometer. An apparent diffusion coefficient was determined at a single concentration using a synthetic boundary cell. Solvent was layered onto solute by rapidly increasing the rotor speed to 6000 rpm to form the boundary. The speed was then decreased to 2500 rpm to avoid sedimentation. The schlieren pattern was checked carefully for convection. Tracings of the photographs were made on graph paper using the Nikon microcomparator projection (20 ×) and D_{app} was evaluated by plotting the square of the inflection point against time, the slope of which is proportional to D_{app} (Svedberg and Pedersen, 1940).

For all high-speed sedimentation equilibrium experiments, the crystalline aldolase was equilibrated with 0.05 M glycylglycine (pH 7.5), 0.05 M 2-mercaptoethanol, and 0.05 M KCl by passage over a Sephadex G-25 column. For experiments on the native enzyme, the sample was further purified on a Sephadex G-100 column (1.1 × 59 cm) equilibrated with the same buffer. The peak fraction was diluted appropriately for the ultracentrifuge experiments.

Guanidine hydrochloride dissociation was carried out by prolonged dialysis of yeast aldolase (5–10 mg/ml) for 4 days against a solution of 6.0 M guanidine hydrochloride (Ultra Pure grade from Mann), 0.02 M glycylglycine (pH 7.5), and 0.1 M 2-mercaptoethanol. Before ultracentrifugation, the protein was diluted with dialysate to suitable concentrations.

Dissociation and Reconstitution. For the acid-dissociation studies, intact yeast aldolase was passed over Sephadex G-25 equilibrated with 0.05 M glycylglycine (pH 7.5)–0.05 M 2-mercaptoethanol. Citric acid (1 M) was added to the solution of desalted protein (1 ml) until the pH was 2.7. After standing at 5° for 30 min, the mixture was passed through a Sephadex G-25 column (2 × 10 cm) equilibrated with 0.05 M citric acid (pH 2.7) and 0.05 M 2-mercaptoethanol. Before ultracentrifugation, the proper protein dilutions were made.

Acidified yeast aldolase (pH 2.7) was reconstituted by a tenfold dilution with 0.3 M glycylglycine (pH 7.5) and 0.05 M 2-mercaptoethanol. Ultracentrifuge experiments and assays on the reconstituted enzyme were performed as usual.

Apoaldolase was prepared by incubation of 10 mg of native aldolase with 0.5 ml of 0.05 M EDTA (pH 8.0) for 1 hr at 4°. This mixture was then applied to a Bio-Gel P-6 column (0.9 × 15 cm) which had been freed of metals by washing with 0.01 M 8-hydroxyquinoline-5-sulfonic acid (pH 8.0) and then equilibrated with 0.05 M Tris-HCl buffer (pH 8.0). The protein was eluted with the latter buffer and diluted to the proper protein concentrations. This procedure has been shown to result in the removal of chelating agents and metals from the protein (Kobes *et al.*, 1969). Apoenzyme prepared in this fashion contained less than 1% of the original zinc content as determined by atomic absorption spectrometry.

The zinc used for reconstitution of the apoenzyme was a solution of the spectrographically pure sulfate salt (Johnson, Matthew Co., Ltd.) dissolved in metal-free water. All substrates and buffers were treated to remove trace metal contaminants by either extraction with dithizone in carbon tetrachloride or by passage over a Chelex-100 (Bio-Rad Corp.) column (Himmelhoch *et al.*, 1966). Other precautions to prevent contamination with adventitious metal ions were taken as previously described (Thiers, 1957). For the ultracentrifuge experiments with apoenzyme the ultracentrifuge cell parts were soaked in 50% nitric acid for 1 hr with frequent changes and rinsed carefully with glass-distilled water.

Assays of apoenzyme and zinc-reconstituted apoenzyme were performed before and after ultracentrifugation in the absence of potassium ion and at 15°. A suitable aliquot of aldolase was added to a mixture containing 75 μmoles of *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffer (pH 7.0), 3 μmoles of FDP, 0.9 μmole of DPNH, and 100 μg of a mixture of crystalline α-glycerol phosphate dehydrogenase and triose phosphate isomerase in a total volume of 3.0 ml. The decrease in absorbancy at 340 mμ was recorded.

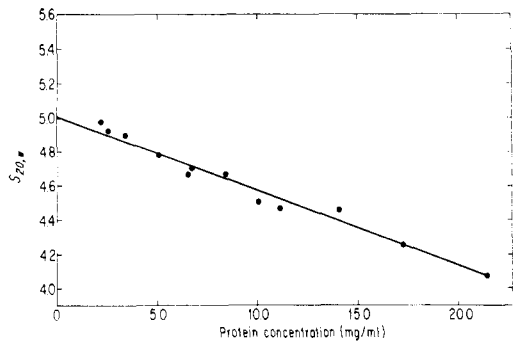

FIGURE 1: Concentration dependence of the sedimentation coefficient of yeast aldolase. Experiments were carried out at 20° in a buffer containing 0.05 M glycylglycine (pH 7.5), 0.05 M 2-mercaptoethanol, and 0.05 M KCl. The solid line was determined by least-squares calculation.

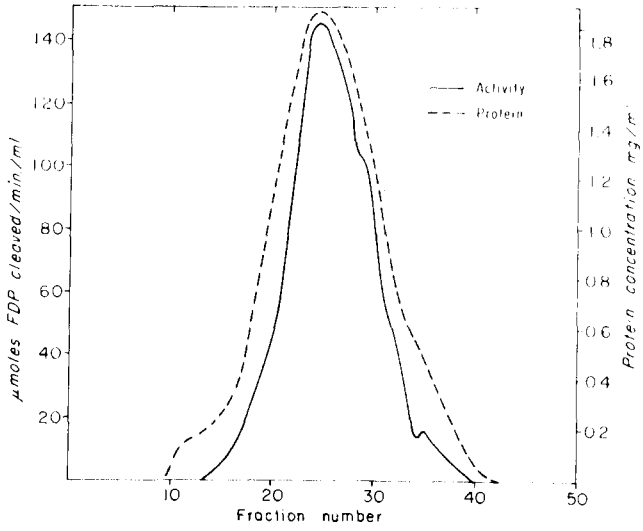

FIGURE 2: Elution patterns of protein and yeast aldolase activity from a column of Sephadex G-100. Crystalline enzyme (12 mg) was applied to a column (1.1 × 59 cm) previously equilibrated with 0.05 M glycylglycine (pH 7.5), 0.05 M 2-mercaptoethanol, and 0.05 M KCl. The column was eluted with the same solution. Fractions 1–10 contained 1.8 ml each and all other fractions were 0.55 ml. The flow rate was approximately 10 ml/hr at 6°. Protein and activity assays were carried out as described in the Materials and Methods.

Results

Sedimentation Velocity of Native Yeast Aldolase. Native yeast aldolase sedimented as a single boundary in 0.05 M glycylglycine (pH 7.5), 0.05 M 2-mercaptoethanol, and 0.05 M KCl. All schlieren patterns showed a slight leading edge indicating a small amount of heavy material. The concentration dependence of the sedimentation coefficient is shown in Figure 1. Least-squares analysis of these data gives a $s^0_{20,w}$ value of 5.02 ± 0.02 S. The concentration dependence, k, was evaluated from the equation $s = s°(1 - kC)$ (Schachman, 1959) and k was found to be $(8.9 \pm 0.2) \times 10^{-3}$ l./g. This value is similar to other globular proteins (Creeth and Knight, 1965).

Sedimentation Equilibrium Studies of Native Yeast Aldolase. Preliminary to the high-speed sedimentation equilibrium experiments of native yeast aldolase, an approximate molecular weight was determined using the Svedberg equation (Svedberg and Pedersen, 1940). A single diffusion experiment was performed at an initial concentration of 12.84 g/l. and gave $D_{20,w} = 5.79 \times 10^{-7}$ cm²/sec. $s_{20,w} = 4.54$ S at 6.42 g/l., so that $M_{s/d,\,app} = 76{,}000$ g/mole. This value was used to calculate the optimum conditions of rotor speed, initial concentration, and time to equilibrium for the sedimentation equilibrium experiments (Teller *et al.*, 1969).

Since the recrystallized enzyme showed a minor heavy contaminant in the sedimentation velocity experiments, the preparation was passed over a Sephadex G-100 column prior to sedimentation equilibrium experiments. A typical elution profile for yeast aldolase is shown in Figure 2. From this experiment it will be observed that both heavy and light materials are present in crystalline yeast aldolase preparations. Only the protein in the peak tube was analyzed in the equilibrium experiments. All molecular weights were determined in a solvent in which the protein was enzymatically active (0.05 M glycylglycine, pH 7.5, 0.05 M 2-mercaptoethanol, and 0.05 M KCl). Essentially the same results were obtained in three separate experiments, on two different preparations of enzyme; the results of one such experiment are presented. The initial concentration was 0.42 (centripital channel), 0.83 (middle channel), and 1.24 mg per ml (centrifugal channel). Number-average molecular weight values obtained from this experiment are presented in Figure 3A. Figure 3B presents the weight-average molecular weights. The solid lines in Figures 3A,B are theoretical lines as discussed below. From this figure it will be noted that molecular weight averages vary both as a function of initial concentration and the concentration point within the channel. In order to interpret these data, it is necessary to compare the molecular weight behavior of chemically equilibrated systems with nonequilibrium mixtures.

In a high-speed equilibrium experiment, the concentration of protein in the meniscus region of the cell approaches zero (Yphantis, 1964). Since the rotor speed for meniscus depletion in a mixture is calculated using the molecular weight of the species present in the greatest amount (Teller *et al.*, 1969), any protein in the mixture with a molecular weight less than that used to determine rotor speed will be preferentially observed in the meniscus region. The number-average and weight-average moments reflect this fact by being lower in the (low concentration) meniscus region than throughout the rest of the ultracentrifuge cell. The degree to which the presence of the lighter component affects the molecular weight averages at each point in the cell is a function of its concentration. Decreased molecular weights near the meniscus will occur for all dissociating systems, whether chemical equilibrium exists or not. A homogeneous nondissociating protein will not show such molecular weight dependence. In Figure 3, the number-average (A) and weight-average (B) molecular weights of yeast aldolase decrease sharply in the concentration region below two fringes. In this respect, the data are characteristic of a dissociating system (Hoagland and Teller, 1969).

For a protein in chemical equilibrium, the ultracentrifuge cell is characterized by a continuous sequence of thermodynamic phases (Goldberg, 1953). The various molecular weight moments at each point in the cell are functions only of the concentration at which they are calculated (Guggenheim, 1957; Adams, 1964). The values will not vary with the distance from the center of rotation, initial concentration, or

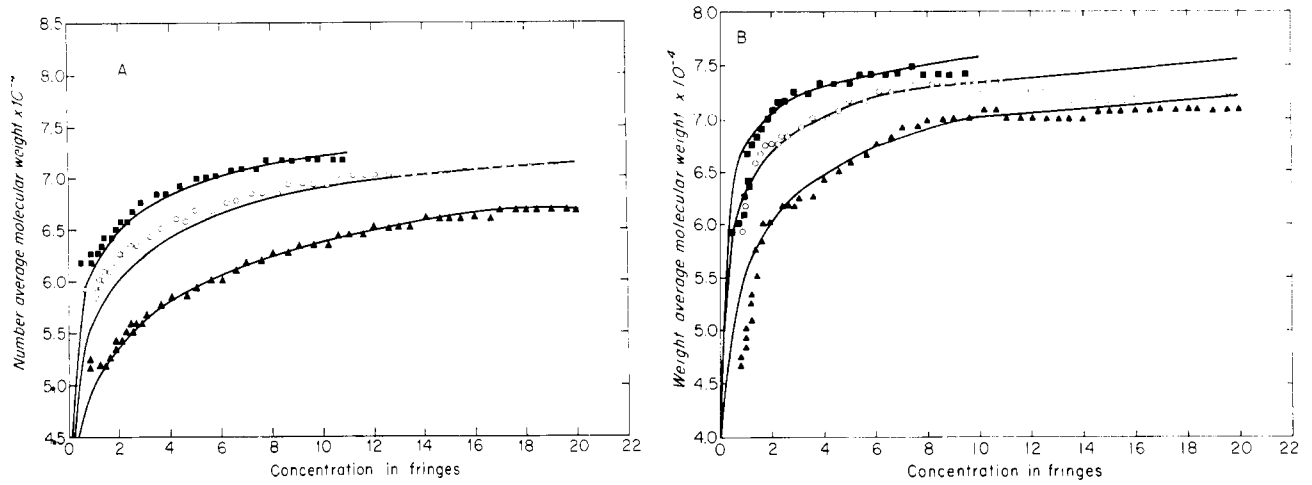

FIGURE 3: The molecular weight averages as a function of fringe concentration for native yeast aldolase. A is the number-average data; B, the weight average data. ■ represents an initial concentration of 0.42 mg/ml, ○, 0.83 mg/ml, and ▲, 1.24 mg/ml.

loss of mass due to "packing" at the cell base. Therefore, for a system in chemical equilibrium, graphs of the molecular weight *vs.* concentration should superimpose.

In contrast, a heterogeneous system not in chemical equilibrium will show a marked dependence of the molecular weight data upon the concentration. In order to illustrate this more thoroughly, the concentration distributions and molecular weight distributions of a simple two-component system have been simulated, assigning the experimental conditions used in the study of yeast aldolase. The model data were calculated using the Rinde equation (Svedberg and Pedersen, 1940).

Assuming that the individual components do not interact in solution, the distribution of mass will depend only upon the distance of the material from the center of the rotor. The Yphantis (1964) centerpiece contains three pairs of channels arranged approximately perpendicular to a radius centered on the axis of rotation; the over-all distance from the innermost sectors to the outermost is approximately 1.4 cm. At the same initial concentration for all three channels, a 15% increase in C_{bottom} would be predicted for a 1.4-cm change in the distance from the center of rotation. This does not represent a major shift in mass comparing the innermost and outermost sectors, since C_{bottom} is the point concentration most affected by the increased distance. The values of the weight-average molecular weight for the simulation calculations at a constant concentration near the sector base for the three different channels are shown in Table IA. These values vary only slightly, and such a difference normally would be difficult to detect. However, if the initial concentration, C_0, is different in each of the three channels, the molecular weight averages at a given concentration in a sector are dramatically altered. Table IB summarizes this change at the concentration value of 1.0 mg/ml. The effect occurs at all concentrations.

The disparity of molecular weights at a given concentration increases toward the base of the cell. The cause of this shift in molecular weights is illustrated in Figure 4. Concentration is expressed in milligrams per milliliter, and x reduced is defined by $(r - r_m)/(r_b - r_m)$, where r is the distance from the axis of rotation. Since the protein distribution depends only upon the speed, temperature, and distance from the axis of rotation, the composition and molecular weight moments at a given value of x reduced will be the same for all three values C_0. (The relatively small effect of increasing the distance from the center of rotation from channel to channel has been neglected.) The total concentration of protein at any point in a sector will increase with increasing initial concentration, as shown in Figure 4. Therefore, at a given sector concentration, the composition and average molecular weights will be a function of C_0, since the sector position at which this concentration occurs will change. For example, the ratio of heavy to light components at 1.0 mg/ml is illustrated in Figure 4. This ratio changes by a factor of two- for a threefold change in initial concentration. A graph of the various molecular weight moments as a function of protein concentration for different initial concentrations will clearly show whether a system can be described as chemically equilibrating.

The strong dependence of the molecular weights of yeast aldolase upon initial concentration, shown in Figure 3, has at least three possible interpretations: the first, that the system

TABLE I: Simulation Data for a Two-Component System.

A	C_0 (mg/ml)	Ratio 80,000/ 40,000 at 1.7 mg/ml	M_w at 1.7 mg/ml
Cell 1	0.50	1.32	63,846
Cell 2	0.50	1.28	62,932
Cell 3	0.50	1.14	62,874
B	**C_0 (mg/ml)**	**Ratio 80,000/ 40,000 at 1.0 mg/ml**	**M_w at 1.0 mg/ml**
Cell 1	0.25	1.642	64,844
Cell 2	0.50	1.042	59,919
Cell 3	0.75	0.732	56,828

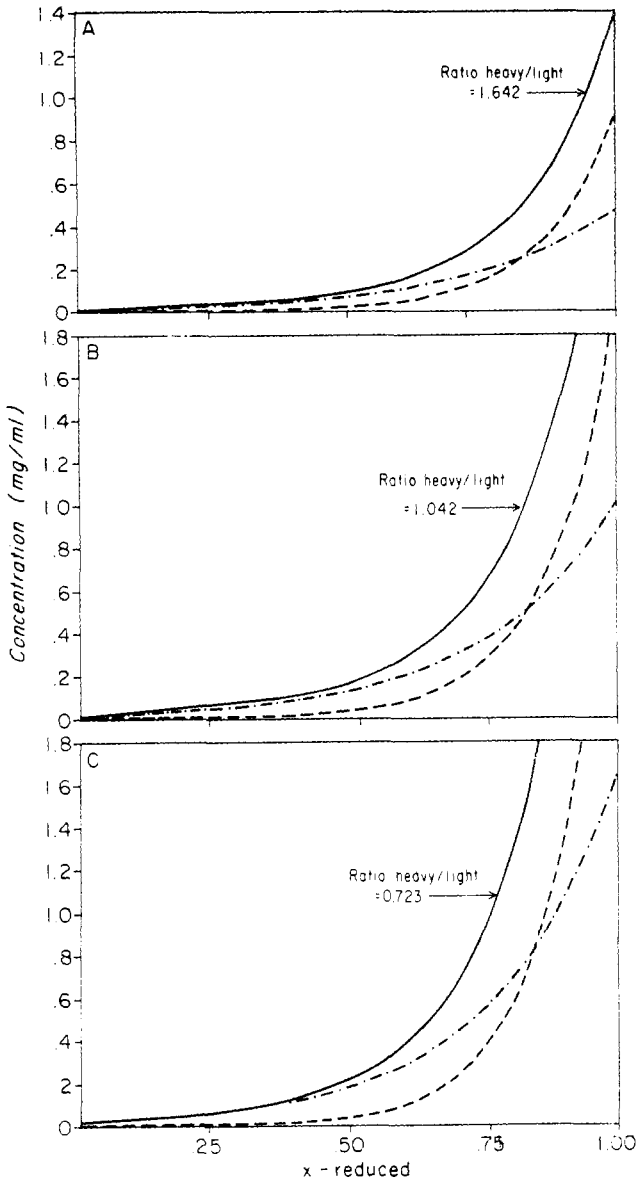

FIGURE 4: Theoretical distribution of a noninteracting light (40,000 g/mole) and heavy (80,000 g/mole) component mixture. (———) Total concentration, (– – –) heavy component, and (—·—·) light component. The simulation conditions are: 18,000 rpm; initial concentrations 0.25 mg/ml (A), 0.50 mg/ml (B), and 0.75 mg/ml (C); temperature 5°; equimolar mixtures of the two components. The arrows are drawn at a concentration of 1.0 mg/ml in all examples and the ratio of heavy to light component at that concentration is noted in the figure.

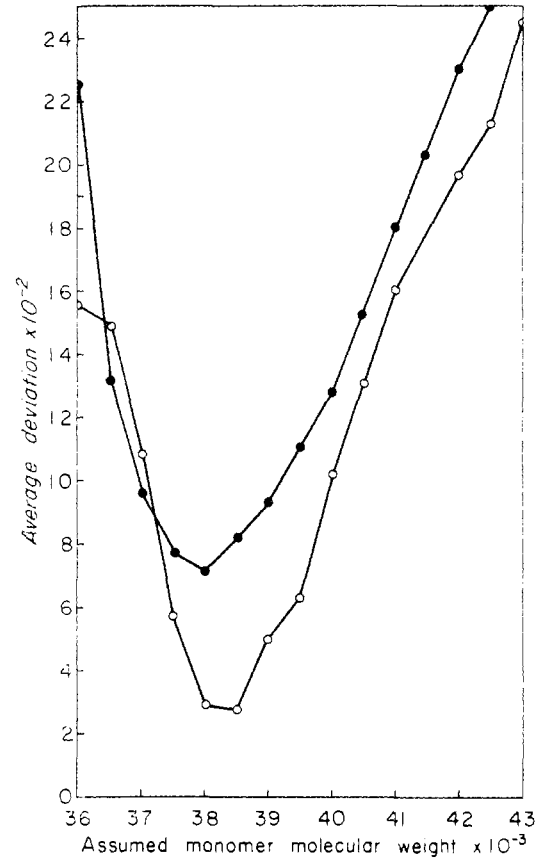

FIGURE 5: Determination of the molecular weight of the "monomer." Ordinate values are average deviations of predicted and observed data assuming a monomer–dimer equilibrium and a monomer molecular weight given on the abscissa. (—○—) Average deviation of M_n data and (—●—) average deviation of M_w data.

is not in chemical equilibrium; the second, that the system is in chemical equilibrium, but contains a finite amount of a non-equilibrating, low molecular weight material; and, finally, that the system is in chemical equilibrium but that the rate of equilibrium is slow in relation to the time required for the ultracentrifuge experiment. In any case, it is necessary to assume either that the enzyme redistributes itself following Sephadex G-100 fractionation or that the fractionation is incomplete. A protein fraction not in chemical equilibrium with the major species should be separated by gel filtration.

If one assumes that the data in Figure 3 can be described as a monomer–dimer mixture, it is possible to determine the subunit molecular weight that best fits all of the data. By assigning a monomer molecular weight, an apparent equilibrium constant can be calculated for a single channel, using the number-average and the weight-average molecular weight distributions as a function of concentration. If the average deviations of the predicted and observed molecular weight averages are plotted against the monomer molecular weight assigned, a minimum in the curve will be the molecular weight of the monomer that best predicts the experimentally obtained data (Hoagland and Teller, 1969). Figure 5 shows the results of such an analysis in the data from one cell. The assumption of an equilibrium is not restrictive, since the apparent equilibrium constant obtained for each cell simply represents a regrouping of the parameters describing a two-component system (Hoagland and Teller, 1969). If the protein contained different molecular weight subunits, the value obtained from M_n data probably should have been less than that from the M_w data, by the definition of these two moments. The average value of M_1, found from M_n data was 40,500 ± 1,000, and the average from M_w data was 40,280 ± 1,100.

It is possible to demonstrate that this system is adequately described as a monomer–dimer mixture as shown in Figure 6. This figure presents a summary of data for all three cells for the plots of M_w *vs.* $1/M_n$ and M_z *vs.* $1/M_w$ (Yphantis and Roark, 1969; Teller *et al.*, 1969) and $2M_w - M_z$ *vs.* $2/M_n - 1/M_w$ (Yphantis and Roark, 1969). The lines drawn are those cor-

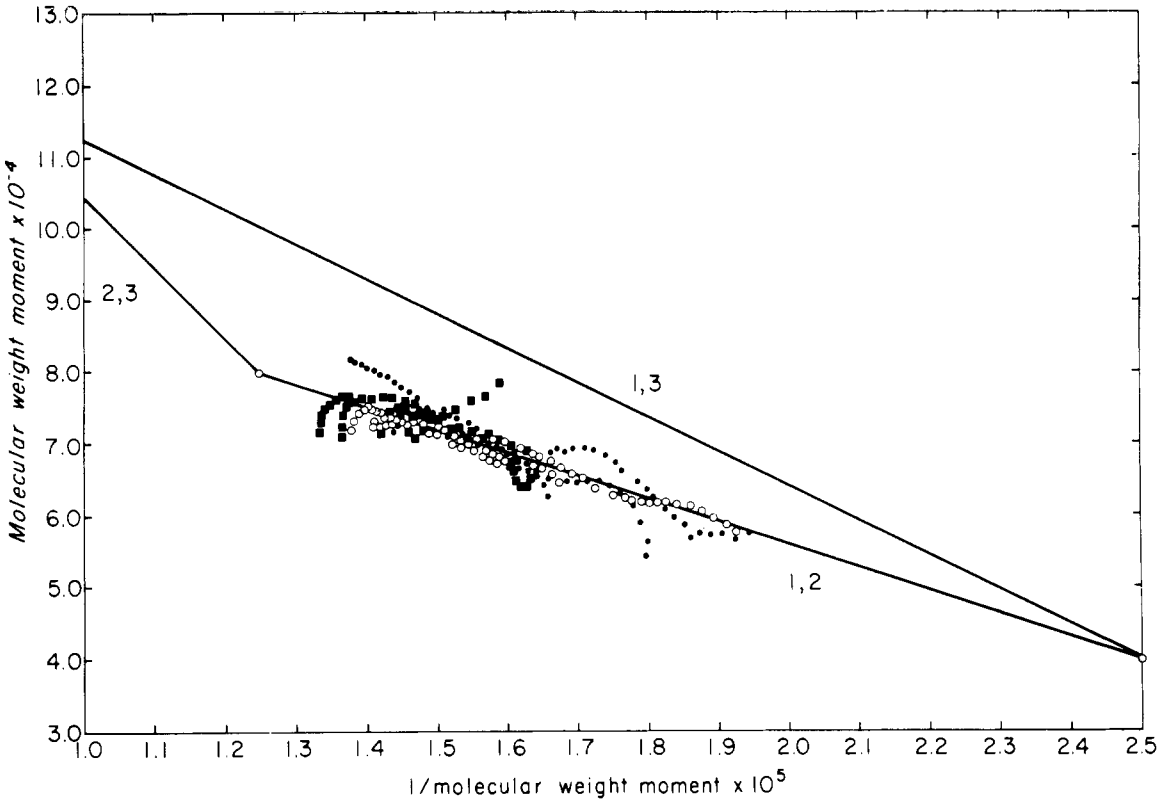

FIGURE 6: Sedimentation equilibrium data from Figure 3. Filled circles are $M_{z,r}$ *vs.* $1/M_{w,r}$ data; open circles are $M_{w,r}$ *vs.* $1/M_{n,r}$ data; and filled squares are $2M_{w,r} - M_{z,r}$ *vs.* $2/M_{n,r} - 1/M_{w,r}$ (Yphantis and Roark, 1969). The solid line labeled 1,2 is that calculated for a monomer–dimer system with mol wt 40,000 and 80,000 g per mole, respectively. The 2,3 line is for a dimer–trimer model and the 1,3 line for a monomer–trimer model.

responding to a subunit of molecular weight of 40,000 g/mole, a dimer of molecular weight of 80,000 g/mole, and a trimer of molecular weight of 120,000 g/mole. All of the points taken together are well described by the monomer–dimer line. These calculations reflect the composition of the mixture present and do not require the existence of chemical equilibrium. If the mixture were composed of more than two components (for example, different size subunits), then the points in this graph would describe a space rather than a line.

To test the alternative that the enzyme was in rapid chemical equilibrium but that the sample contained a finite amount of a nonequilibrating low molecular weight material, simulation calculations were made, employing the same procedures used to generate the values in Figure 4. The molecular weight of the light component was assigned as 20,000 g/mole on the supposition that this value would correspond to a significantly redistributed species under the normal experimental conditions. The dimer was given a molecular weight of 80,000 g/mole; the monomer, 40,000 g/mole. For a rotor speed of 18,000 rpm, a temperature of 5°, and an association constant of 800,000 l./mole, the light component would have to be present in the uncentrifuged sample in excess of 40% in order to see the effect of initial concentration which was noted in Figure 3. Although the simulation conditions do not exactly represent the experimental conditions, they do show the approximate amount of light component necessary to generate such data. The reciprocal graphs (Figure 6) and the average deviation calculations (Figure 5) for Sephadex G-100 fractionated enzyme show very little evidence for a polypeptide much less than mol wt 40,000 g/mole. It is therefore unlikely that the presence of a nonequilibrating light component can be used to explain the observed dependence upon initial concentration. The most likely model of yeast aldolase is a nonequilibrating 80,000–40,000 g/mole of dimer–monomer mixture. The solid lines drawn in Figure 3 are the theoretical distributions of molecular weights from the apparent equilibrium constant calculated for each cell, by assigning the monomer molecular weight of 40,000 g/mole. Table II gives the whole cell averages, the estimated per cent of monomer and dimer, and the apparent equilibrium constants of each cell. The amount of monomer–dimer and the apparent equilibrium constants were calculated by assigning a monomer molecular weight of 40,000 g/mole. The whole cell averages are lowered from the molecular weight of native dimer by the presence of the monomer. These values are generally lower than the molecular weight of 76,000 g/mole found from sedimentation velocity and diffusion. This increased value may be due to the presence of a minor heavy component observed in Sephadex G-100 fractionation.

Sedimentation Equilibrium in Guanidine Hydrochloride. To determine the molecular weight of the constituent polypeptide chains of yeast aldolase, the enzyme was dissolved in 6.0 M guanidine hydrochloride, 0.02 M glycylglycine (pH 7.5) and 0.1 M 2-mercaptoethanol. The reducing agent was included in the solution in order to reduce disulfide bonds and to prevent disulfide pairing of exposed sulfhydryl groups. Gua-

TABLE II: Native Yeast Aldolase Molecular Weight Moments.[a]

Initial Concn, C_0 (mg/ml)	Mol Wt × 10^{-3} (g/mole)				Estimated %[b]		App Equilibrium Constant and Root-Mean-Square Deviation[a]
	M_n	M_w	M_z	M_{z+1}	Monomer	Dimer	
1.24	62.4	68.1	70.9	71.8	21.0	79.0	86,983 ± 11,173
0.83	67.8	71.1	72.1	72.3	16.8	83.2	251,425 ± 67,892
0.42	69.2	72.4	71.8	67.9	12.2	87.8	573,257 ± 97,750

[a] Rotor speed = 18,000 rpm, temperature = 5°, time at speed = 16.5 hr. [b] Calculated assigning a monomer molecular weight of 40,000 g/mole as computed in the text.

nidine hydrochloride effectively disrupts most noncovalent interactions (Lapanje and Tanford, 1967).

The relationship between the concentration at the bottom of the cell and the whole cell number-, weight-, and z-average molecular weights is discussed in the Appendix. Figure 7 is a plot of the three averages against the concentration applicable in each case. If the enzyme were homogeneous in 6.0 M guanidine hydrochloride, the three lines should have superimposed giving identical intercepts. As shown in this figure, the lines are essentially parallel, indicating that the data are internally consistent in terms of the value obtained for the virial coefficient. Extrapolation by least-squares analysis to C = 0 gave the following values (Figure 7): M_n = 35,000 g/mole, M_w = 40,500 g/mole, and M_z = 45,100 g/mole. Since the values of the virial coefficient (Figure 7) are comparable with (but slightly larger than) values found by Lapanje and Tanford (1967), the heterogeneity seen in the molecular weights probably arises from a population of differing peptide chains rather than incomplete dissociation in the 6.0 M guanidine hydrochloride. The distribution of molecular weights may be due to (1) proteolysis occurring during isolation, even in the presence of phenylmethylsulfonyl fluoride, (2) a heterogeneity of the monomer molecular weights, or (3) the presence of protein contaminants not removed during isolation.

Characterization of the Reassociation Process for Yeast Aldolase. Preliminary experiments showed that when acid-treated yeast aldolase at pH 2.7 was directly added to an assay mixture at neutral pH at 25°, the initial activity was almost neg-

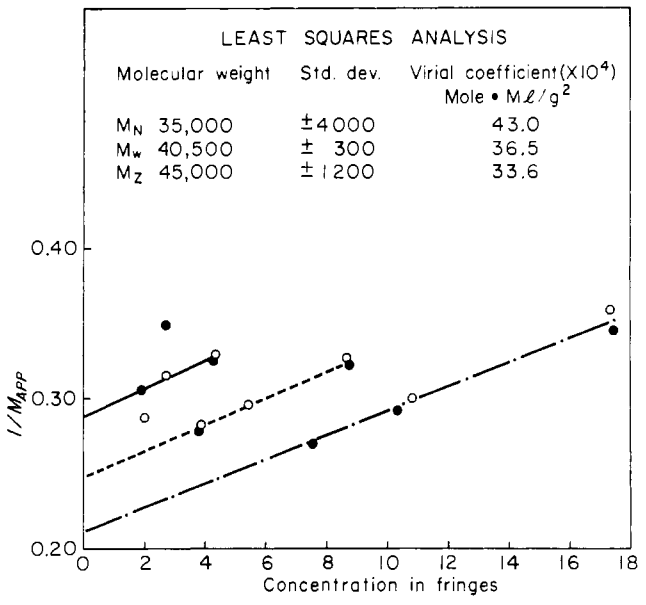

FIGURE 7: The reciprocal whole cell apparent molecular weight averages as a function of concentration in fringes for the high-speed sedimentation equilibrium experiments in 6.0 M guanidine hydrochloride. The concentrations have been normalized so that the lines would superimpose if the material were homogeneous, as discussed in the Theory section. (○) After 26.5 hr of ultracentrifugation; (●) after 36.0 hr of ultracentrifugation; (———) the M_n values *vs.* $C_{b/4}$; (– – – –) the M_w values *vs.* $C_{b/2}$; (–·–·–) the M_z values *vs.* C_b.

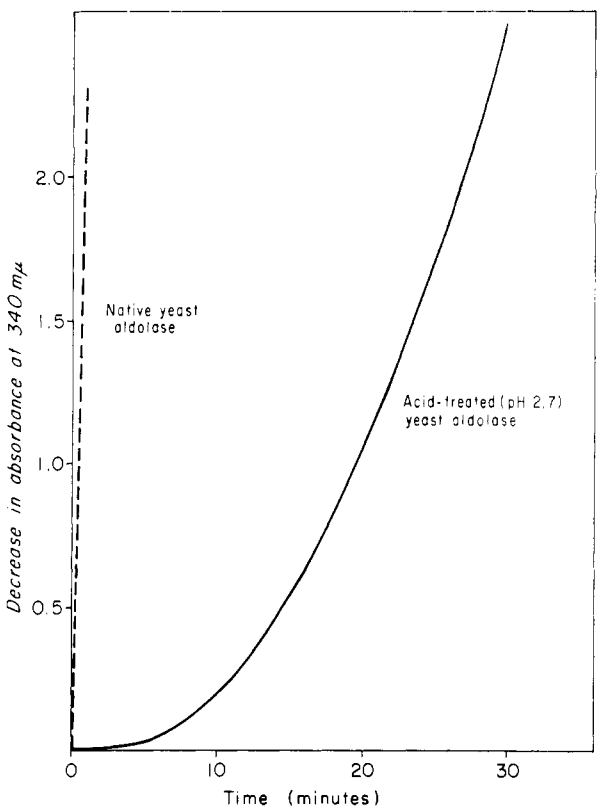

FIGURE 8: Time dependence of the recovery of catalytic activity upon neutralization of acid-dissociating solution. The line on the left is an assay of native aldolase and the curve on the right shows the regain of catalytic activity when the protein at low pH (2.7) is diluted into an assay medium of glycylglycine buffer at pH 7.5. In both cases, 2.3 μg of yeast aldolase was used for the assays.

TABLE III: Acid-Dissociated Yeast Aldolase Molecular Weight Moments.[a]

C_0 (mg/ml)	Reciprocal M_w *vs.* C	Reciprocal M_n *vs.* C	Extrapolated Reciprocal Moments >2.0 Fringe	
			M_w	M_n
0.50	38,600 ± 3,900	38,400 ± 2,300	40,000	40,000
0.83	30,800 ± 1,600	29,000 ± 600	37,200	33,300
1.16	29,300 ± 1,200	28,600 ± 500	34,800	33,300

[a] Rotor speed = 48,000 rpm, temperature = 5°, time at speed = 9.2 hr.

ligible, but increased with time (Figure 8). Assays with native enzyme were linear from zero time. After prolonged incubation of the neutralized acid-treated aldolase, 70–80% of the original activity was recovered. This behavior is reminiscent of that found for rabbit muscle aldolase A (Stellwagen and Schachman, 1962; Deal *et al.*, 1963; Sia and Horecker, 1968).

The recovery of activity was found to fit a first-order rate law as shown in Figure 9. Thus a unimolecular process is the rate-limiting step in the over-all recovery of the native state at these concentrations of enzyme. This process could be due to dissociation of the molecule into subunits or to loss and regain of the essential metal ion.

Sedimentation Equilibrium Studies of the Citric Acid Dissociation of Yeast Aldolase. Yeast aldolase was treated with acid in the absence of salt as described in the Materials and Methods section. Table III summarizes the data obtained from extrapolation of the reciprocal point-by-point molecular weights as a function of concentration to $C = 0$. The ultracentrifuge data obtained from the acid-treated yeast aldolase were variable. The graphs of molecular weight *vs.* concentration showed a strong effect of initial concentration on the determined molecular weight (6000 g/mole over-all molecular weight displacement for a threefold change in the initial concentration). The virial coefficients were quite significant at concentrations greater than 0.05 mg/ml, depressing the molecular weights calculated in some cases by more than 10,000 g/mole across the entire cell. Plots of reciprocal weight averages *vs.* concentrations were not linear, especially in the low concentration range. Finally, a duplicate experiment to that discussed here gave molecular weight averages varying from 22,000 to 28,000 g per mole, when the reciprocal apparent molecular weight averages were graphed as a function of concentration and extrapolated to $C = 0$.

In an attempt to eliminate the electrostatic charge effect, a number of experiments were carried out in 0.1 M KCl. Interpretation of the data was complicated by the presence of a significant amount of small molecular weight material and by extensive aggregation. The light material may be caused by the action of a yeast acid protease or by specific acid hydrolysis, as observed with rabbit muscle aldolase by Sia and Horecker (1968). On the assumption that light components were distributed mainly in the low concentration regions of the sectors, all data below two fringes concentration were discarded. The reciprocals of the remaining molecular weight averages were extrapolated to $C = 0$. The values obtained are given in Table III. With the qualification that data have been eliminated, these estimates are similar to the values found in 6.0 M guanidine hydrochloride.

Acid-Dissociation-Reconstituted Yeast Aldolase Molecular Weight Averages. Reconstitution of the acid-dissociated yeast aldolase by dilution with 0.3 M glycylglycine (pH 7.5)–0.05 M 2-mercaptoethanol produced a molecular species which behaved much like the native enzyme in sedimentation equilibrium. The whole cell molecular weight averages are summarized in Table IV.

By assuming a monomer molecular weight of 40,000 g/mole, it was possible to fit the concentration distribution in the cell to a monomer–dimer–trimer system (Teller *et al.*, 1965, 1969). The reconstituted enzyme showed the formation of a heavy component, corresponding to trimer and comprising 8–15% of the mixture. The trimer may be a nonspecific aggregate of variable molecular weight formed upon dilution of the en-

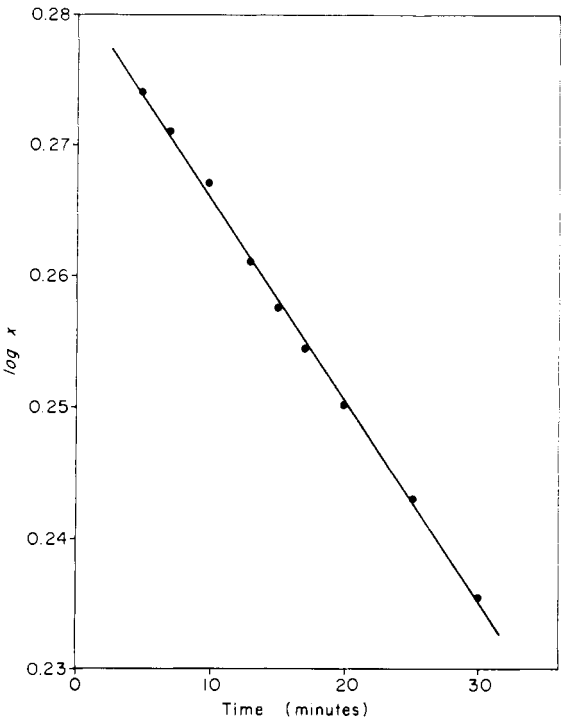

FIGURE 9: First-order kinetic analysis of recovery of enzymatic activity. The quantity, x, is the difference between the final activity and the activity at time, t, and is presumed to be proportional to the fractional recovery at a given time.

TABLE IV: Acid-Dissociated-Reconstituted Yeast Aldolase Molecular Weight Moments.[a]

Initial Concn, C_0 (mg/ml)	Mol Wt × 10^{-3} (g/mole)				Estimated %[b]		
	M_n	M_w	M_z	M_{z+1}	Monomer	Dimer	Trimer
1.16	71.6	77.4	83.9	94.9	18.3	74.5	7.2
0.83	65.4	74.6	84.5	102.8	21.7	69.5	8.8
0.50	70.0	76.1	82.7	92.1	19.7	75.2	6.1

[a] Rotor speed = 22,000 rpm, temperature = 5°, time at speed = 12.5 hr. [b] Calculated assuming a monomer molecular weight of 40,000 g/mole.

zyme and may be related to the wide range of low molecular weight species noted in the acid-dissociation data. In addition, since a significant amount of "packing" at the bottom of the centrifuge cell was noted during this experiment, the estimate of the percentage of heavy component may be low. The activity of the reconstituted protein was 75% of that of the original enzyme. The 25% decrease may be associated with the aggregate formed on reconstitution. The analysis of the concentration *vs.* distance data was made for the following systems: monomer–dimer, monomer–dimer–trimer, and monomer–dimer–trimer–tetramer. No evidence for tetramer was obtained. Materials of this size and larger may have been present in the solutions, but not observed because of the large amount of heavy material packed at the base of the cell.

Sedimentation Equilibrium of Apoenzyme. To investigate the possibility that zinc has a specific relationship to the molecular structure of the enzyme, apoenzyme was prepared (Materials and Methods) for high-speed sedimentation equilibrium. Table V is a summary of the data obtained. Plots of M_w *vs.* $1/M_n$, M_z *vs.* $1/M_w$, and $2M_w - M_z$ *vs.* $2/M_n - 1/M_w$ follow exactly the 40,000–80,000 g/mole of monomer–dimer model. The relative composition of the two species in the metal-free sample is also nearly that of the native yeast aldolase determined earlier (Table II). The activity of the apoenzyme taken directly from the ultracentrifuge cell following sedimentation equilibrium was less than 5% that of the original enzyme; addition of Zn^{2+} (1.0×10^{-4} M) completely reconstituted the activity. These results indicate that the metal dependence of yeast aldolase is not concerned with stabilization of the dimeric structure. Comparison of the data in Table V with those of Table II shows that the apoenzyme has slightly higher whole cell averages than holoenzyme. This is not considered significant, since the experiments on apoenzyme were performed on a different enzyme preparation and the metal-free enzyme was not fractionated on Sephadex G-100 prior to ultracentrifugation.

TABLE V: Yeast Apoaldolase Molecular Weight Moments.[a]

Initial Concn, C_0 (mg/ml)	Mol Wt × 10^{-3} (g/mole)				Estimated %[b]	
	M_n	M_w	M_z	M_{z+1}	Monomer	Dimer
1.24	73.0	73.9	73.2	69.9	12.8	87.2
0.83	69.8	71.6	70.0	64.6	12.0	88.0
0.42	71.3	74.3	74.5	73.0	11.9	88.1

[a] Rotor speed = 18,000 rpm, temperature = 5°, time at speed = 21.0 hr. [b] Based on a monomer molecular weight of 40,000 g/mole.

TABLE VI: Amino Acid Analyses of Aldolases.

Residue	Residues/80,000 g			
	Yeast[a]	Rabbit A	Rabbit B	Rabbit C
Met	13.8 ± 0.1	6.0	10.0	6.0
Cys[b]	9.2 ± 0.6	4.0	15.5	14.0
Asp	78.7 ± 3.9	56.0	70.0	65.0
Thr[c]	37.0 ± 1.9	40.0	38.5	33.0
Ser[c]	38.6 ± 1.9	37.5	32.0	39.5
Glu	77.5 ± 2.3	80.0	86.0	81.5
Pro	32.6 ± 1.0	39.0	30.5	41.0
Gly	59.7 ± 1.8	56.0	56.0	61.5
Ala	67.0 ± 2.0	77.0	72.0	86.5
Val	44.6 ± 0.9	45.0	47.0	55.5
Ile[d]	48.4 ± 0.5	41.5	38.0	54.5
Leu[d]	45.2 ± 2.3	70.0	67.0	65.0
Tyr	24.2 ± 0.3	22.0	19.4	25.0
Phe	29.1 ± 0.3	14.5	21.5	16.5
Lys	50.6 ± 2.5	60.5	55.5	38.5
His	21.7 ± 0.4	22.5	20.5	14.5
Arg	19.0 ± 0.4	33.5	32.5	33.0
Trp[e]	9.1	6.0	7.0	7.0
Total	703.0	723.0	720.5	737.5

[a] Plus and minus values are root-mean-square deviations. [b] Obtained by performic acid oxidation. [c] Extrapolated to zero time. [d] Extrapolated to infinite time. [e] Determined by the method of Edelhoch (1967).

Amino Acid Composition of Yeast Aldolase. The amino acid composition was determined as discussed in Materials and Methods; four separate analyses on two different preparations were used to calculate the residues. The number of half-cystine residues determined as cysteic acid is 9.2/mole (80,000 g/mole); further analysis is required to establish whether these residues exist in disulfide linkages or as free sulfhydryl groups.

Table VI summarizes the result of this analysis together with the root-mean-square error estimates. The amino acid compositions of rabbit aldolase isozymes (Penhoet, 1968), normalized to 80,000 g, are included in the table for comparison. While there are obvious differences in the amino acid analyses, the over-all composition of these four enzymes is remarkably similar. For many of the amino acids, the differences among the rabbit aldolases A, B, and C are as large as the divergence of these enzymes from yeast aldolase.

In order to obtain a more objective comparison of the

TABLE VII: Divergence of Amino Acid Compositions of Rabbit and Yeast Aldolases.

	Muscle (A)	Liver (B)	Brain (C)	Yeast	Chymotrypsin[a]	Cytochrome C (Human)[b]
Muscle	0	0.0308	0.0449	0.0625	0.1210	0.1365
Liver	0.0308	0	0.0470	0.0493	0.1203	0.1390
Brain	0.0449	0.0470	0	0.0585	0.1213	0.1633
Yeast	0.0625	0.0493	0.0585	0	0.1143	0.1340
Chymotrypsin	0.1210	0.1203	0.1213	0.1143	0	0.1885
Cytochrome C (human)	0.1365	0.1390	0.1633	0.1340	0.1885	0

[a] Composition taken from Walsh and Neurath (1964). [b] Composition taken from Dayhoff and Eck (1967).

amino acid compositions of these enzymes, we have calculated a deviation function.

$$D = [\Sigma(X_{1,i} - X_{2,i})]^{1/2}$$

where $X_{1,i}$ represents the mole fraction of amino acid i in protein 1. $X_{2,i}$ represents the mole fraction of the same amino acid in the protein being compared. The sum of the squares of the deviations is made over all of the amino acids in Table VI. This value is a measure of the divergence of amino acid compositions. Table VII shows the results of this calculation for the aldolases of Table VI. Such calculations of divergence have been performed on many other proteins with the result that numbers as low as those in Table VII are usually found with proteins for which strong evidence of evolutionary homology exists (C. E. Harris and D. C. Teller, to be published). Chymotrypsin and cytochrome C (human) are included in Table VII as representative of proteins unrelated in composition. Independent evidence for homology between rabbit aldolases A, B, and C has been documented (Penhoet, 1968). It may be presumptuous to estimate the degree of sequence homology by simple amino acid compositions, but the values shown here are sufficiently low to suggest that yeast aldolase and rabbit aldolases A, B, and C are related.

Isoelectric Focusing of Yeast Aldolase. The results from cellulose polyacetate electrophoresis and polyacrylamide disc gel electrophoresis (Materials and Methods) suggested that the yeast aldolase preparation was a homogeneous preparation, migrating under the various conditions used as a single band.

Recent studies in this laboratory (W. Susor, M. Kochman, and W. J. Rutter, in preparation) have shown that rabbit muscle aldolase A and a number of other "homogeneous" enzymes can be resolved into multiple enzymatically active protein components by isoelectric fractionation. It was of interest to determine whether yeast aldolase was a single component using this technique. Figure 10 shows that yeast aldolase can be resolved into three peaks, all containing enzymatic activity. A second experiment on a different preparation gave results nearly identical with those depicted in Figure 10.

A sample of the original preparation was further submitted to an electrofocusing column in 6.0 M urea. The elution profile is shown in Figure 11. Three components were discern-

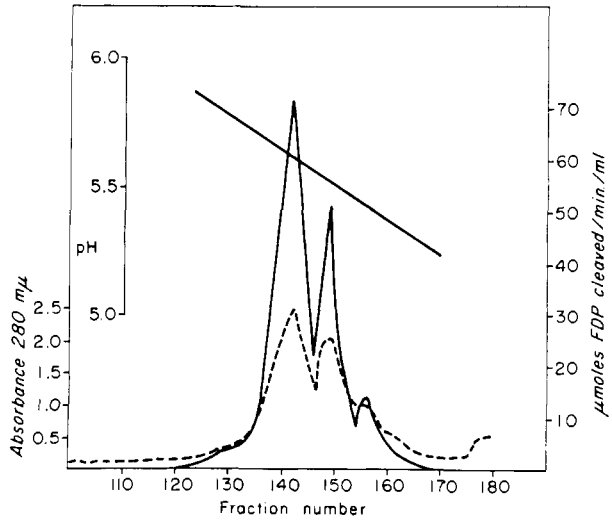

FIGURE 10: Isoelectric fractionation of crystalline yeast aldolase. The experiment was performed as described in the Materials and Methods. (——) Activity; (- - -) absorbance at 280 mμ.

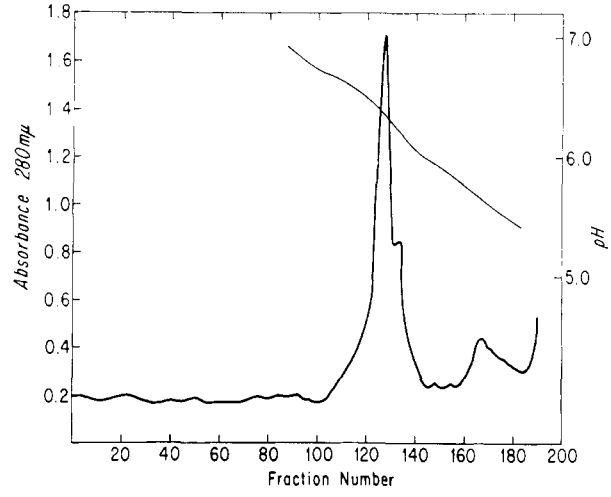

FIGURE 11: Isoelectric fractionation of yeast aldolase in 6 M urea. The experiment was performed as described in the Materials and Methods.

able: the major peak has a definite "shoulder" and the minor peak is not sharp. These results are compatible with (1) the presence of two differently charged subunits, which, when randomly combined at neutral pH, yield three possible molecular species; (2) modification of a specific protein molecule, perhaps during isolation; or (3) nonequivalent binding of small molecules to the protein. Preliminary evidence from high-speed sedimentation equilibrium analyses suggests that the three aldolase species separated by electrofocusing do not differ significantly in molecular weight. We believe the determined molecular weights for the monomer and dimer are valid for at least the two major active species. Some of the polydispersity detected in the preparations, for example, in guanidine hydrochloride, could, however, be related to the heterogeneity detected in the isoelectric focusing experiment.

Discussion

The determination of the molecular weight of a native enzyme in a dissociating mixture is difficult. It is usually easier to determine the molecular weights of the subunits with a minimum of uncertainty and then define the molecular weight by the number of subunits in the intact molecule. The determination of subunit stoichiometry in the intact molecule and subunit molecular weights is difficult for complex mixtures, but for monomer–dimer systems, severe constraints apply and the sedimentation equilibrium data is interpretable (Hoagland and Teller, 1969). The sedimentation equilibrium data of the yeast aldolase preparation corresponds to a monomer–dimer mixture with molecular weights of 40,000 ± 1,000 and 80,000 ± 2,000 g/mole, respectively. The relation of 1:2 stoichiometry is confirmed by the observed "molecular space" of Figure 6, which discriminates between monomer–dimer and monomer–dimer–trimer models. Such results do not, in themselves, require the subunit of 40,000 g/mole to be the ultimate combinative unit; however, the studies in both guanidine hydrochloride and citric acid support this contention.

Some deviation of molecular weight averages was detected in both guanidine hydrochloride and in citric acid; this heterogeneity cannot be due to a simple mixture of two proteins of different molecular weights. Perhaps the most reasonable interpretation is that there are several differently sized molecules present, such as might have arisen by limited hydrolysis (proteolysis) in a small proportion of the molecules.

The molecular weights of the two subunits, if not identical, must be very similar since neither the data of Figure 5 nor Figure 6 deviate significantly from the 40,000 to 80,000 monomer–dimer model.

The results of electrofocusing experiments have a number of alternative explanations: (1) two discrete subunits which combine to give three molecular species; (2) three distinct yeast aldolases, each containing identical subunits; or (3) a single yeast aldolase which has been modified by degradation or by derivatization. At present we cannot resolve these possibilities, but we believe that the molecular properties described here are valid for the majority of molecular species present in the preparation. Our experience with isoelectric heterogeneity in other enzymes leads us to predict that there are only minor differences in amino acid composition and sequence in the multiple active forms detected.

Recently it has been demonstrated that divalent metal ions are directly involved in the activity of native yeast aldolase (Kobes *et al.*, 1969). Metal-free apoenzyme can be prepared with no enzymatic activity; the activity can be fully reconstituted by the addition of Zn^{2+}. A stoichiometry of 1 metal atom/40,000 g of protein was defined by (1) Zn^{2+} content of native enzyme; (2) recovery of activity by addition of Zn^{2+} to inactive apoenzyme; (3) the combination of Co^{2+} with the apoenzyme as measured spectroscopically (R. T. Simpson, R. D. Kobes, B. L. Vallee, and W. J. Rutter, in preparation); and (4) the ratio of bound Mn^{2+} to enzyme determined by electron paramagnetic resonance studies (A. S. Mildvan, R. D. Kobes, and W. J. Rutter, in preparation). The present results indicate that yeast aldolase binds 1 g-atom of metal/subunit or 2 g-atoms/enzyme molecule. These results should be compared with lower Zn contents of 1.2–1.6 g-atoms/80,000 g of protein (Richards and Rutter, 1961) and 0.6–0.9 g-atom/80,000 g of protein (Vanderheiden *et al.*, 1962) reported earlier. These lower metal contents (less than two) might arise from the partial loss of metal during the preparative procedure (R. T. Simpson, R. D. Kobes, B. L. Vallee, and W. J. Rutter, in preparation). The fact that metal-free apoenzyme has a nearly identical molecular weight distribution to the intact metalloenzyme shows that the metal ion is not involved in the maintenance of the dimeric structure.

The finding that the subunit size and amino acid composition of the yeast enzyme (prototype of the class II enzymes) are similar to those of rabbit aldolases A, B, and C (class I enzymes) was unexpected, but is of considerable significance. Class I and class II aldolases have different molecular and catalytic properties and it has been assumed that members of each class were structurally and evolutionarily distinct (Rutter, 1964). Although the similarities in subunit size and amino acid compositions could be fortuitous, they are sufficiently suggestive of a structural relationship to make a careful analysis of the primary sequences of these enzymes an important objective.

Acknowledgment

The authors thank Mr. Thomas Horbett for helpful discussions during the development of this research.

Appendix

In order to graph the data for whole cell molecular weight averages obtained in guanidine hydrochloride and 2-mercaptoethanol solutions, we wished to have the slopes proportional to the virial coefficient and lines superimposed if the solutions were homogeneous.

Van Holde and Baldwin (1958) and Fujita (1962) have shown that

$$\frac{1}{M_{w,a,cell}} = \frac{1}{M} + \frac{B^*}{2}(C_b + C_m) \qquad (1)$$

where $M_{w,a,cell}$ is the apparent weight-average molecular weight of the material (which is assumed to be homogeneous for the derivation) and $B^* = B_1 + \bar{v}/M$. Here B_1 is the second virial coefficient, defined by $\ln \gamma = MB_1C + 0(C^2)$, where γ is the activity coefficient based on the infinitely dilute standard state. $\bar{v}$ is the partial specific volume of the solute, C_b is the concentration at the base of the cell, and C_m is the meniscus concentration.

The relation for $M_{z,a,cell}$ has been derived by Richards *et al.* (1968). The result is

$$\frac{1}{M_{z,a,cell}} = \frac{1}{M} + B^*(C_b + C_m) + MB^{*2}C_bC_m \quad (2)$$

For the conditions of high-speed sedimentation in sectorial cells C_m is negligible so that eq 1 and 2 reduce to the approximations

$$\frac{1}{M_{w,a,cell}} = \frac{1}{M} + B^*\frac{C_b}{2} \quad (3)$$

and

$$\frac{1}{M_{z,a,cell}} = \frac{1}{M} + B^*C_b \quad (4)$$

Equations 3 and 4 suggest that if reciprocal $M_{w,a,cell}$ data are plotted *vs.* $C_b/2$ and $M^{-1}_{z,a,cell}$ data are plotted against C_b on the same graph, then a single straight line should describe all data if the solution is homogeneous.

Since high-speed sedimentation equilibrium experiments also provide number-average molecular weights of the solute, we wanted to find the concentration at which these data should be graphed to superimpose the $M^{-1}_{w,a}$ and $M^{-1}_{z,a}$ data for homogeneous materials. We shall show that the best point to use is $C_b/4$.

For a homogeneous, nonideal solute the number-average molecular weight observed is given by the relation (Teller *et al.*, 1969)

$$M^{-1}_{n,a,r} = M^{-1} + {}^1/_2B^*C \quad (5)$$

We also have the result of Lansing and Kraemer (1935) that,

$$M_{n,cell} = \int Cd(r^2) \Big/ \int \frac{C}{M_{n,r}} d(r^2) \quad (6)$$

where the integrals are taken over the entire cell. The apparent number-average molecular weight of the material becomes,

$$M^{-1}_{n,a,cell} = \int \frac{C}{M_{n,a,r}} d(r^2) \Big/ \int Cd(r^2) \quad (7)$$

Combination of eq 7 and 5 yields

$$M^{-1}_{n,a,cell} = M^{-1} + {}^1/_2B^* \int C^2 d(r^2) / \int Cd(r^2) \quad (8)$$

In order to evaluate the integral in the numerator of the second term of eq 8, it is necessary to have either C as a function of r^2 or r^2 as a function of C. From Goldberg (1953) and Fujita (1962) we have the ultracentrifuge relation

$$AMCd(r^2) = dC + MB^*CdC \quad (9)$$

where $A = (1 - \bar{v}\rho_0)\omega^2/2RT$. ρ_0 is the solvent density, ω is the angular velocity, R is the gas constant, and T is the absolute temperature. Substitution of eq 9 into the integrals of eq 8 and evaluation of the integrals yields the results

$$\int C^2 d(r^2) = \frac{1}{2AM}(C_b^2 - C_m^2) + \frac{MB^*}{3AM}(C_b^3 - C_m^3) \quad (10)$$

and

$$\int Cd(r^2) = \frac{1}{AM}(C_b - C_m) + \frac{MB^*}{2AM}(C_b^2 - C_m^2) \quad (11)$$

With the approximations that C_m is negligible and that $(1 + MB^*C_b/2)^{-1} = 1 - MB^*C_b/2$, we obtain for eq 8

$$1/M_{n,a,cell} = 1/M + B^*C_b/4 - MB^{*2}C_b^2/12 - M^2B^{*3}C_b^3/12 \quad (12)$$

so that the proper position to plot $M^{-1}_{n,a,cell}$ to obtain a slope of B^* is $C_b/4$ to a first approximation.

The relations 3, 4, and 12 were employed in Figure 6 to demonstrate that the virial coefficient is the same for all three molecular weight moments but the solution was heterogeneous since the three lines did not superimpose.

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