The Number of Polypeptide Chains in Rabbit Muscle Aldolase*

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ABSTRACT: The molecular weights of rabbit muscle aldolase and of its polypeptide chains have been measured by sedimentation equilibrium and by combination of the sedimentation velocity with values of the diffusion coefficient and intrinsic viscosity. The intact protein was found to have a molecular weight of 158,000. The mixture of polypeptide chains obtained by dissolving the protein in 6 M guanidine hydrochloride (with added β-mercaptoethanol) was found to be somewhat heterogeneous, giving $\bar{M}_n$ = 36,500–40,000, $\bar{M}_w$ = 37,000–41,000, and $\bar{M}_z$ = 39,500–43,000, the range of values reflecting uncertainty in the partial specific volume in the dissociating solvent. The number of chains in the intact molecule can be obtained unequivocally from the value of $\bar{M}_n$, and the result indicates that the molecule must contain four chains.

The studies of protein conformational change which have been carried out in this laboratory over the past several years have suggested that the most effective of all commonly employed denaturing media is a solution of concentrated guanidine hydrochloride, containing a moderate concentration of a sulfhydryl compound such as β-mercaptoethanol. Guanidine hydrochloride appears able to break all noncovalent interactions, and the sulfhydryl compound serves to rupture disulfide bonds when they are present and to prevent formation of new disulfide bonds by oxidation of sulfhydryl groups. Work currently in progress is demonstrating that most proteins, when dissolved in this medium, are dissociated to their constituent polypeptide chains, and that the chains themselves are probably completely unfolded to a state resembling the randomly coiled state of synthetic polymers. Except perhaps in strongly alkaline solutions, no complicating reactions occur.

As part of this project we are studying a number of physical properties of the unfolded polypeptide chains, as a function of their molecular weights. One of the proteins included in this study is rabbit muscle aldolase. As the molecular size of the polypeptide chains of this protein has not been definitely established, we have carried out a molecular weight determination both in dilute aqueous salt solution, where the protein is in its native state, and in our guanidine–mercaptoethanol mixture, where it is presumably completely dissociated to its polypeptide chains. The results are reported here. They indicate that aldolase contains four polypeptide chains, contrary to previous reports which suggest that it consists of three or six polypeptide chains (Kowalsky and Boyer, 1960; Stellwagen and Schachman, 1962; Deal *et al.*, 1963; Hass, 1964).

Materials and Methods

Materials. Rabbit muscle aldolase was a crystalline suspension, A grade, lot 52645, stated activity 11 units/mg, purchased from Calbiochem, Inc. The crystals were separated by centrifugation in the cold and dissolved in a minimal volume of *ca.* 1 M $(NH_4)_2SO_4$. A small amount of fibrous residue was removed by centrifugation. Excess $(NH_4)_2SO_4$ was added to the supernatant and recrystallization was allowed to take place slowly in the cold. The crystals were dissolved in 0.1 M NaCl and dialyzed against several changes of the same solvent for 30 hr. The final dialysis medium was used as reference solvent for subsequent dilutions and measurements. The concentration of protein in the protein solutions obtained in this way was determined both by dry weight and by measurement of optical density at 280 mμ, using absorbance data of Baranowski and Niederland (1949). Good agreement between the results was obtained. Solutions used for experimental measurements were prepared by quantitative dilution of such stock solutions.

The protein moved as a single peak in the ultracentrifuge (Figure 1), giving a sedimentation coefficient in good agreement with other determinations (see below). The enzymatic activity was determined by a modification of the hydrazine assay (Jagannathan *et al.*, 1956),[1] but the assay was performed only after all physical studies were made on a stock solution which had stood in contact with air for about 2 weeks. The activity was found to be rather low, 3 units/mg. In view of the homogeneity of the protein in the ultracentrifuge, the diminution of activity which had occurred on standing is not likely to be due to factors which would influence the molecular weight, and especially not the molecular weights of the polypeptide chains obtained after dissociation of the protein in the

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[1] The procedure used was that recommended by the manual of Worthington Biochemical Corp., Freehold, N. J.

guanidine–mercaptoethanol mixture which was employed.

The guanidine hydrochloride used in this study was prepared as previously described (Kawahara *et al.*, 1965). All other reagents were the best available commercial products, used without further purification.

Sedimentation Equilibrium. Sedimentation equilibrium measurements were carried out essentially as described by Yphantis (1964), using a Spinco Model E analytical ultracentrifuge, Rayleigh interference optics, low protein concentrations, and a relatively high rotor speed, which assures that the concentration of sedimentating material at the upper meniscus of the solution column is essentially zero. We have used simple double-sector cells with sapphire windows and a relatively long solution column. The method has been previously employed in this laboratory to determine the molecular weights of the polypeptide chains of a number of other proteins (Marler, 1964; Kanarek *et al.*, 1964; Marler and Tanford, 1964).

Interference patterns were photographed on Kodak spectrographic plates, emulsion type II-G, and were measured with a Gaertner two-dimensional comparator. After alignment of the radial direction along the x coordinate of the comparator, any one fringe was selected for measurement of the y coordinate at suitable intervals of the x coordinate. A correction for minor effects of cell window distortion was made by running a water blank, as described by Yphantis (1964). The quantity used for calculation is the fringe displacement relative to the y coordinate at the upper meniscus (r_a). This is designated $f - f_a$, where f indicates fringe displacement referred to pure solvent. In all runs except the one shown in Figure 3, the fringes were parallel to the x axis in the upper portion of the cell, indicating the absence of sedimenting material, so that $f_a = 0$. All f values were taken as proportional to protein concentration, and the proportionality constant was established by observing the fringe displacement produced in a synthetic boundary cell with a known concentration of protein. This proportionality constant is not actually used in the calculation of molecular weight so that an error in its determination would have no effect on the results, but it is sometimes useful to be able to know actual concentrations instead of the f values read off the plates.

When $f_a = 0$, molecular weights are readily determined by a knowledge of f or $\ln f$ as a function of the position in the cell, designated by r. The local weight-average molecular weight $\bar{M}_{wr}$ at any position in the cell is determined from the slope of a plot of $\ln f$ *vs.* r^2,

$$\bar{M}_{wr} = \frac{2RT}{(1 - \bar{v}\rho)\omega^2} \frac{\mathrm{d} \ln f}{\mathrm{d}r^2} \qquad (1)$$

where $\bar{v}$ is the partial specific volume of the protein, ρ the density of the solvent, and ω the angular velocity of the rotor. If the plot is linear, the sample is homogeneous, and the molecular weight calculated by eq 1 is the unique molecular weight. If it is curved, the sample

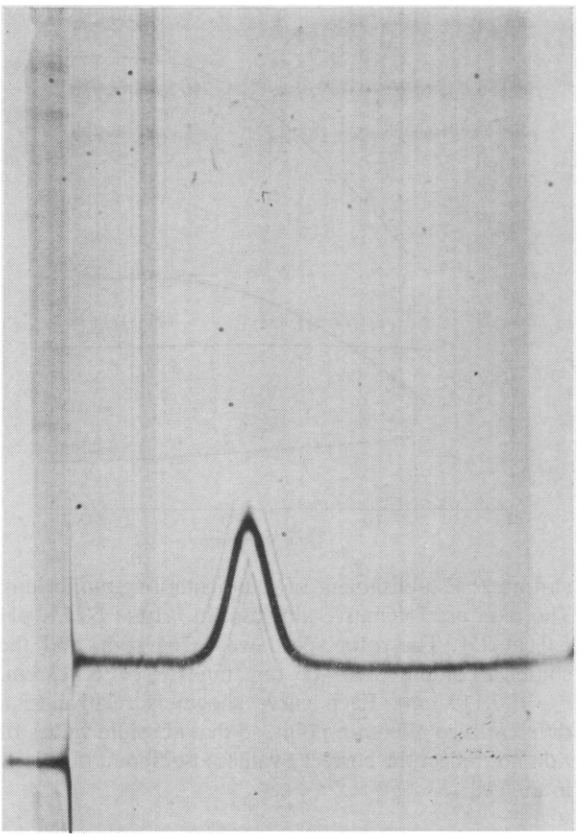

FIGURE 1: Sedimentation pattern for native aldolase, in 0.15 M NaCl, pH 6.1, and 25°. The rotor speed was 59,780 rpm.

is heterogeneous and the limiting value of $\bar{M}_{wr}$ at the bottom of the solution column (r_b) is the z-average molecular weight of the original sample, $\bar{M}_z^0$. The weight-average molecular weight of the original sample, if it is heterogeneous, is determined as

$$\bar{M}_w^0 = \frac{RT}{(1 - \bar{v}\rho)\omega^2} \frac{f_b}{\int_a^b f r \mathrm{d}r} \qquad (2)$$

where f_b is the value of f at the lower meniscus. The local number-average molecular weight ($\bar{M}_{nr}$) is given by

$$\bar{M}_{nr} = \frac{RT}{(1 - \bar{v}\rho)\omega^2} \frac{f}{\int_a^r f r \mathrm{d}r} \qquad (3)$$

and that for the sample as a whole is

$$\bar{M}_n^0 = \frac{\int_a^b f r \mathrm{d}r}{\int_a^b (f r / \bar{M}_{nr}) \mathrm{d}r} \qquad (4)$$

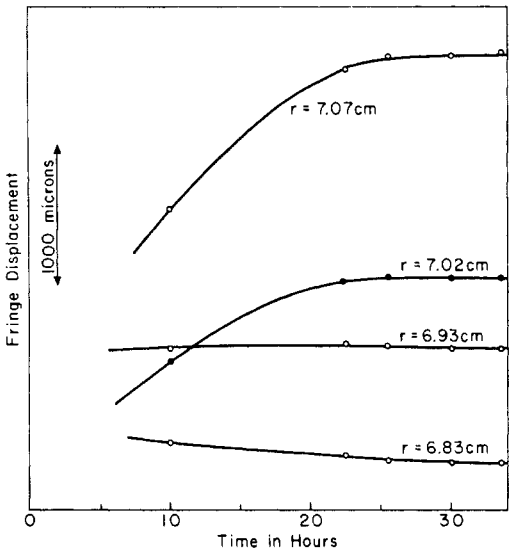

FIGURE 2: Establishment of sedimentation equilibrium. The data are for native aldolase, in 0.10 M NaCl, pH 6.0, at 25°. The rotor speed was 8766 rpm, and the solution column was 4.06 mm high (r_a = 6.713 cm, r_b = 7.119 cm). Each curve shown is relative to a different zero reference point, so that absolute values of f do not reflect the correct f values at different positions in the cell.

All the foregoing equations, except eq 1, apply only when $f_a = 0$, but the modifications applicable when $f_a = 0$ were not required; *i.e.*, only eq 1 was used for analysis of the single experiment in which f_a was not 0. The only additional problem in that run was, therefore, how to determine f_a so that experimental values of $f - f_a$ could be converted to f values to make a plot of ln f *vs.* r^2. This was done by estimating the ratio f_a/f_b to be expected for a reasonable assumed molecular weight, and combining with the known value of $f_b - f_a$. The value of f_a was checked by calculating the total protein content of the cell and comparing it with the protein content of the original solution; *i.e.*, using the conversion factor between f and true concentration (c),

$$\int_a^b cr\,dr = \frac{1}{2} c_0(r_b^2 - r_a^2) \qquad (5)$$

where c_0 is the initial concentration.

Partial Specific Volume. The value of $\bar{v}$ for aldolase is required for all calculations of the molecular weight used in this paper. Taylor and Lowry (1956) obtained $\bar{v}$ = 0.742 cc/g at 20° for aldolase in aqueous salt solutions, and Hass (1964) obtained $\bar{v}$ = 0.745 under similar conditions. According to Taylor and Lowry (1956), the partial specific volume increases with temperature by about 0.001 cc/g per degree. We have assumed $\bar{v}$ = 0.747 cc/g for our measurements with the native protein at 25°, and $\bar{v}$ = 0.742 cc/g at 20°, but minor variation in this parameter would not affect the results significantly. On the other hand, the precise value used for $\bar{v}$ in the guanidine–mercaptoethanol mixture has a greater effect on the results because of the higher solvent density. There are conflicting results in the literature as to the effect of concentrated guanidine hydrochloride on $\bar{v}$. Kielley and Harrington (1960) found $\bar{v}$ of myosin in 5 M guanidine to be smaller by about 0.01 cc/g than the value for the native protein in dilute aqueous salt solution. We obtained a similar result for γ-globulin (Marler *et al.*, 1964). On the other hand, Reithel and Sakura (1963) have found little change in $\bar{v}$ for a number of proteins. We have considered both possibilities, and have made alternate calculations, based on $\bar{v}$ = 0.747 and $\bar{v}$ = 0.735 cc/g, respectively, in the guanidine–mercaptoethanol mixture at 25°.

Other Procedures. Sedimentation velocities were measured in the analytical ultracentrifuge, using schlieren optics. Synthetic boundary cells were used for measurements in the guanidine–mercaptoethanol mixture. Viscosity measurements were made in Cannon–Fenske capillary viscometers.

Solvent densities and viscosities of guanidine–mercaptoethanol mixtures of various compositions have been carefully measured, as reported elsewhere (K. Kawahara and C. Tanford, paper in preparation).

Results

Molecular Weight of Native Aldolase. The normal procedure for sedimentation equilibrium which is used in this laboratory is based on the method of Yphantis (1964). The initial protein concentration is extremely small (0.01–0.05 g/100 ml), and a relatively high rotor speed is used. We normally use a long solution column (3–10 mm), and the effect of the high rotor speed is to concentrate the protein to a concentration of the order of 0.1–0.2 g/100 ml at the bottom of the column, and to reduce the concentration in the upper part of the solution column essentially to 0.

When this procedure was applied to aldolase, dissociation of the protein was found to occur in the upper part of the column, beginning roughly at the point where the protein concentration had fallen to 0.02 g/100 ml. It is already well established that dissociation of aldolase occurs at higher protein concentrations, at pH 2 (Stellwagen and Schachman, 1962; Deal *et al.*, 1963). Evidently, at the low concentrations employed here, it takes place near neutral pH also.

An additional complication was that the dissociated protein appeared to have a tendency to aggregate slowly, perhaps by oxidation of thiol groups exposed by dissociation. The aggregated protein sedimented to the bottom of the cell, and the total amount of protein in the solution when equilibrium was ultimately attained (evaluated by eq 5) was substantially less than the amount originally introduced into the cell.

The results obtained in this way from a number of runs indicated that native aldolase has a molecular weight of 160,000, and that it dissociates in dilute solutions to subunits exactly half that size. In the experiment in which the lowest protein concentration was

used, the material present at the end of the experiment appeared to consist entirely of molecules with mol wt 80,000.

In view of the loss of material which occurred in these experiments, these data were deemed not entirely satisfactory for an unequivocal determination of the molecular weight. Moreover, as the chemistry of aldolase is not of primary interest to us, we did not wish to pursue the question of whether aggregation is in fact the result of oxidation of thiol groups. We have preferred to confine ourselves to the determination of molecular weight alone. The sedimentation equilibrium study was therefore repeated by carrying it out at higher protein concentration and lower rotor speed, the conditions being such that the final protein concentration was about 0.02 g/100 ml at the upper meniscus of the solution column, and about 0.8 g/100 ml at the bottom. Equilibrium was reached in about 24 hr as shown in Figure 2.

Under these conditions, as mentioned above in the experimental procedure, an estimate of the concentration (or fringe displacement) at the upper meniscus must be made before absolute values of f at each position in the cell can be determined. We based the ratio f_a/f_b on an assumed molecular weight of 160,000, because this was the most likely value which emerged from the low concentration runs.

The result of this determination is shown in Figure 3. It indicates that the protein is homogeneous, with a molecular weight of 158,000. When c_0 is evaluated by eq 5, we obtain 0.198 g/100 ml, which is identical within experimental error with the actual known concentration of 0.197 g/100 ml. There are no indications of loss of protein by aggregation.

Figure 3 includes also the result of a calculation to show that the molecular weight determination does not depend appreciably on the choice of f_a made above. The dashed line in the figure shows the logarithmic plot obtained with a larger value of f_a (obtained from f_a/f_b values based on a molecular weight of *ca.* 145,000). The change has little effect over the lower half of the solution column (*i.e.*, where $f \gg f_a$) and one would still have to conclude that the major part of the protein has a molecular weight close to 158,000. The effect of the change in the upper half of the column would be to suggest the additional presence of some lighter component. A second consequence, however, is to increase the apparent total protein content, and the value of c_0 calculated by eq 5 from the dashed line is in fact 0.209 g/100 ml, sufficiently above the true value to allow us to discard the dashed curve as an impossible result. It should be noted that f_a values below that actually employed would also lead to an impossible result: a value of $\bar{M}_{wr}$ near the top of the column which is higher than that at the bottom of the column.

The molecular weight obtained by sedimentation equilibrium is confirmed by molecular weights calculated by use of sedimentation velocity. The sedimentation coefficient measured at a protein concentration of 0.46 g/100 ml, corrected to the viscosity and density of water at 20°, was found to be 7.82 S. With the regression

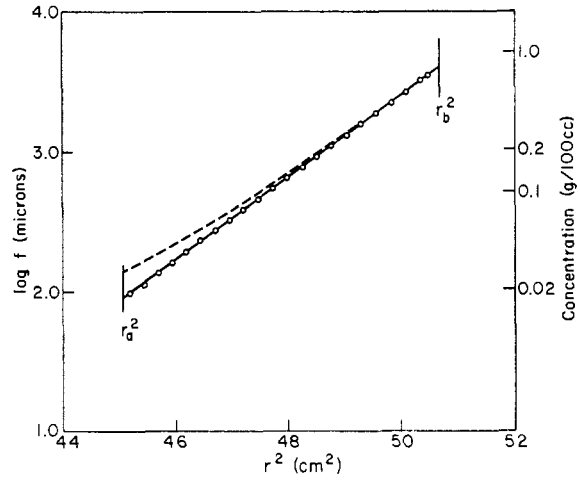

FIGURE 3: Sedimentation equilibrium for native aldolase, conditions as given in Figure 2. The points and solid line are based on a reasonable assumed value for f_a. The dashed line is based on an alternative value. As discussed in the text, this alternative value leads to an apparent total protein content of the sample which is too high.

formulas of Taylor and Lowry (1956) or Stellwagen and Schachman (1962), this corresponds to $s^0_{20,w} = 8.0$ S. This value is in good agreement with other published values. Stellwagen and Schachman (1962) obtained 7.9 S, and Deal *et al.* (1963) 7.8 S. Taylor and Lowry's value is 7.35 S, but this value was obtained before ultracentrifuges were equipped with temperature control units, and it is now known that experimental temperatures estimated in the manner then employed are too high by *ca.* 0.8°, because of adiabatic cooling during acceleration of the rotor (Waugh and Yphantis, 1952). Their sedimentation coefficient should therefore be corrected upwards by an amount corresponding to the viscosity difference of the solvent between actual and apparent temperatures. This correction raises their $s^0_{20,w}$ value from 7.35 to 7.6 S.

A molecular weight can be obtained by combining the sedimentation coefficient with the diffusion coefficient, an accurate value of which ($D^0_{20,w} = 4.63 \times 10^{-7}$ cm^2/sec) is available from the work of Taylor and Lowry (1956), or by combining it with the intrinsic viscosity, which is 4.0 cc/g (Stellwagen and Schachman, 1963; Hass, 1964). To get the molecular weight from $s^0_{20,w}$ and $[\eta]$ the equation of Scheraga and Mandelkern (1953) is used. The parameter β' which occurs in that equation has a value of 2.12×10^6 for spheres and for compact ellipsoidal molecules of moderate axial ratio. This is the value used here, since the intrinsic viscosity alone precludes any molecular conformation such as an elongated rod or a random coil (Tanford, 1961). As Table I shows, the results obtained by both these procedures are in excellent agreement with the result obtained by sedimentation equilibrium.

Polypeptide Chains in Guanidine Hydrochloride with

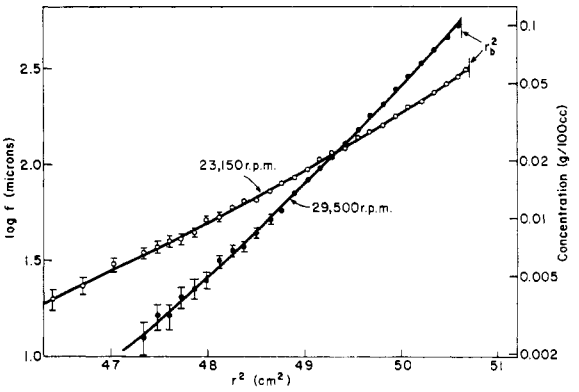

FIGURE 4: Sedimentation equilibrium results for aldolase in 6 M guanidine hydrochloride with 0.1 M β-mercaptoethanol, at 25°. The upper meniscus is near $r_a^2 = 42.7$ cm^2 in both runs.

TABLE I: Molecular Weights of Native Aldolase and Its Polypeptide Chains.

	Native Aldolase, 0.15 M NaCl, pH 6	
Sed equilibrum	158,000	
Sed and diffusion[a]	160,000	
Sed and viscosity[a]	161,000	
	Polypeptide Chains, in 6 M Guanidine, 0.1 M Mercaptoethanol	
	$\bar{v}_{25} = 0.747$ cc/g	$\bar{v}_{25} = 0.735$ cc/g
Sed equilibrium $\bar{M}_n^0$	40,000	36,500
$\bar{M}_w^0$	41,000	37,000
$\bar{M}_z^0$	43,000	39,500
Sed and viscosity	42,000	39,000

[a] These results are based on $s_{20,w}^0 = 7.9$ S, which is probably the best of the values cited in the text. With our own value of 8.0 S, the molecular weights would have been higher by *ca.* 2000.

Added Mercaptoethanol. The results of two independent sedimentation equilibrium runs for aldolase dissolved in 6 M guanidine hydrochloride, containing 0.1 M β-mercaptoethanol, are shown in Figure 4. The time required to reach equilibrium, by tests of the type shown for native aldolase in Figure 2, was about 3 days in both runs. The curvature of the plots of Figure 4 shows that the solute is not homogeneous; *i.e.*, the polypeptide chains of the protein are not all alike. Since the concentration of protein at the upper meniscus is 0, eq 1–4 can be used to evaluate the various molecular weight averages.

The results for the two runs are in reasonable agreement. $\bar{M}_z^0$ and $\bar{M}_w^0$ values differed by *ca.* 3%, and the $\bar{M}_n^0$ values by *ca.* 9% (38,000 and 41,400, respectively,

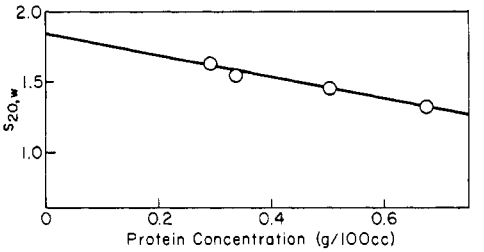

FIGURE 5: Sedimentation velocity in 6 M guanidine hydrochloride with 0.1 M β-mercaptoethanol, at 25°. A synthetic boundary cell was used. Rotor speed was 42,040 rpm. (These values are based on $\bar{v} = 0.747$ cc/g.)

with $\bar{v} = 0.747$ ml/g). The larger deviation in $\bar{M}_n^0$ values is not unexpected, since the integral which occurs in the denominator of eq 3 becomes quite uncertain when the value of f at the upper integration limit is small. At the same time, $\bar{M}_{nr}$ values obtained under these conditions do make a significant contribution to the integral in the denominator of eq 4. The results are summarized in Table I. As was pointed out earlier, the choice of a value for $\bar{v}$ has a significant effect on the results, and values are given for two values of $\bar{v}$, considered to be the extremes of the likely range in this parameter.

The molecular weight obtained by sedimentation equilibrium has been confirmed by a determination based on sedimentation velocity and intrinsic viscosity. Values of the sedimentation coefficient were determined at several protein concentrations, and were corrected to the viscosity and density of water at 20°. The results are shown in Figure 5. The value of $s_{20,w}^0$ obtained by extrapolation to 0 protein concentration is 1.85 S. The measurements leading to the intrinsic viscosity are shown in Figure 6. The value obtained is 35.3 cc/g. To obtain a molecular weight from these data, one again uses the equation given by Scheraga and Mandelkern (1953), but the value of β' which must be used in this case must be altered to take into account the fact that the protein molecules can no longer be represented by compact spheres or ellipsoids. As was pointed out in the introduction, work in progress in this laboratory indicates that all polypeptide chains of proteins so far studied are essentially randomly coiled in our guanidine–mercaptoethanol mixture. The high intrinsic viscosity of 35 cc/g indicates that this is certainly so for aldolase.[2] The appropriate value of β' in that case is 2.5×10^6 (Tanford, 1961) and the molecular weight obtained, shown in Table I, clearly agrees very well with the results obtained from sedimentation equilibrium. The

[2] We have found that the intrinsic viscosity of a number of proteins in this solvent varies with molecular weight as $M^{0.68}$, a type of relation typical of random coils, but quite different from what would be expected for rod-shaped or globular molecules (Tanford, 1961). The intrinsic viscosity of aldolase chains falls on the same $[\eta]$ *vs.* M plot as do all the other proteins we have studied.

molecular weight average obtained in this way is a complex one, which should however be fairly close to the weight-average molecular weight.

Number of Chains in Native Aldolase. If native aldolase consists of n polypeptide chains, with molecular weights $M_1, M_2, \ldots, M_n$, then the molecular weight of the native protein is ΣM_i (i = 1 to n). The number-average molecular weight of the separated chains is $\Sigma M_i/n$, so that the number n can be obtained unequivocally from the ratio of the molecular weight of native protein to $\bar{M}_n^0$ for the polypeptide chains. Using $\bar{M}_n^0$ based on $\bar{v}$ = 0.747 cc/g (Table I) gives n = 3.95, and using $\bar{M}_n^0$ based on $\bar{v}$ = 0.735 cc/g gives n = 4.33. Since n must be an integer, these results indicate that the native molecule must consist of four polypeptide chains, rather than three (or six), as has been believed heretofore. The experimental uncertainty in $\bar{M}_n^0$ has little effect on this conclusion, for $\bar{M}_n^0$ cannot be higher than $\bar{M}_w^0$, which is subject to a much smaller error.

The exact molecular weights of the individual polypeptide chains cannot be determined with any precision from these data. $\bar{M}_z^0$, $\bar{M}_w^0$, and $\bar{M}_n^0$ do not differ greatly, so that the possible molecular weight differences are limited. If, *e.g.*, there are only two kinds of chains, they cannot differ from each other in molecular weight by as much as a factor of two. (Two chains of 26,000 and two of 52,000 would give $\bar{M}_n^0$ = 39,000, $\bar{M}_w^0$ = 43,300, and $\bar{M}_z^0$ = 46,800.) Chain weights of 47,000 and 32,000 would give $\bar{M}_n^0$ = 39,000, $\bar{M}_w^0$ = 40,900, and $\bar{M}_z^0$ = 42,200, roughly as observed, but the experimental error clearly allows considerable deviation from that result. Even the possibility that all four chains have the same molecular weight cannot be definitely discarded, as the curvature of the plots of Figure 4 comes close to being within the limits of experimental error.

Discussion

The molecular weights obtained in this study fall within the range of previous reports. For the native protein, Taylor and Lowry (1956) obtained 149,000, which, after correction of the sedimentation velocity, as described above, becomes 156,000. Stellwagen and Schachman (1962) obtained 142,000 for the native protein, but give values 159,000 and 160,000, respectively, for two samples which were reconstituted by dialysis after dissociation into subunits. An earlier value of 180,000 (Olikina and Firogenov, 1950) can probably be discarded because it is based on a sedimentation velocity which disagrees with all subsequent determinations.

For the dissociated protein, Stellwagen and Schachman (1962) observed a weight-average molecular weight of 37,000 in one experiment with urea as dissociating agent, and 42,000 in another. Both experiments were carried out at much higher protein concentrations than we have used, and are therefore influenced appreciably by thermodynamic nonideality. The true molecular weight which would have been obtained from measurements at several protein concentrations, and extrapolation to 0 concentration, would probably have been higher than either of the numbers quoted. On the other hand, these numbers are weight-averages, and the number-average, which is the important one here, is smaller.

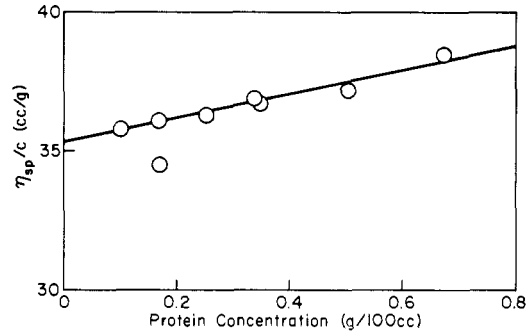

FIGURE 6: Determination of the intrinsic viscosity in 6 M guanidine hydrochloride with 0.1 M β-mercaptoethanol, at 25°. The single point which falls below the line has been neglected in determining the best straight line (by least squares) through the remaining points.

For acid-dissociated aldolase (pH 2), Stellwagen and Schachman obtained $\bar{M}_w$ = 42,000 and 46,000 in two experiments. Deal *et al.* (1963) obtained a weight average of 51,000 by sedimentation equilibrium and a value of 46,000 from sedimentation and diffusion for aldolase dissociated by exposure to pH 2.8. Hass (1964) has reported a wide range of values for dissociated succinyl aldolase, depending on the exact conditions. The lowest value, obtained after exposure to pH 12.5, was 27,000.

The fact that the presence of three polypeptide chains per molecule was deduced from earlier data both by Stellwagen and Schachman (1962) and by Deal *et al.* (1963) appears to result from use of the lowest of the reported molecular weights for the native protein and the highest possible value for the average molecular weight of the chains. The latter values were obtained simply by acidification of the native protein in aqueous solution, and it is quite possible that this is not sufficient for complete dissociation of aldolase to its constituent polypeptide chains. It may be noted that the chain molecular weights obtained by Stellwagen and Schachman in urea are similar to our results in guanidine hydrochloride. Allowing for uncertainty in the correction for nonideality, they are at least as compatible with four as with three chains per native molecule.

It should be emphasized that chemical estimates for the number of polypeptide chains, based on end-group analysis, favor a three-chain rather than a four-chain model (Drechsler *et al.*, 1959: Kowalsky and Boyer, 1960; Rutter *et al.*, 1963; Winstead and Wold, 1964). However, one study frequently cited to support a three-chain structure is that of Udenfriend and Velick (1951), who obtained 2.1 NH_2-terminal proline residues/aldolase molecule. In the same study, they reported only 1.9 NH_2-terminal residues for horse hemoglobin, which is exactly half the true number, so that their results

are certainly compatible with four chains per molecule, as well as three.

Finally, mention should be made of the finding by Lai *et al.* (1965) that aldolase contains two active sites per molecule. This result is at least suggestive of the possibility that the molecule may contain two identical halves, a possibility also suggested by our preliminary data on the dissociation of aldolase at low concentrations in aqueous solution, which indicated that subunits having half the weight of native aldolase are formed.

Though confirmation of our result by chemical means is of course essential before it can be fully accepted, we find it difficult to visualize any source of error which could have occurred in our procedure and which would alter the results sufficiently to lead to a ratio of 3:1 rather than 4:1 for the molecular weight of native aldolase to $\bar{M}_n^0$ for its chains.

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