Dissociation and Reassociation of Rabbit Muscle Enolase*

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ABSTRACT: When rabbit muscle enolase (mol wt 82,000) was exposed to 20% dioxane or 20% acetone in the presence of 0.2 M ammonium sulfate and 5×10^{-3} M Versene, a rapid dissociation to enzymatically inactive subunits of mol wt 39,000–44,000 was observed. In the absence of ammonium sulfate or Versene, or with magnesium added, the rate of dissociaton was greatly retarded. Upon dialysis, an enzymatically active dimer could be reformed in 45 and 87% yields, respectively, from the dioxane and the acetone system. High concentrations of ammonium sulfate alone also appeared to cause dissociation of rabbit muscle enolase. From the protein concentration dependence of the hydrodynamic parameters of the enzyme in 2 M ammonium sulfate, it is proposed that the process involves a dissociation, followed by unfolding or solvation, and finally a concentration-dependent association to higher molecular weight asymmetrical aggregates. It is also proposed that these aggregates of modified protein well may make up the crystalline form of the enzyme.

Subunit structure of proteins has recently received much attention both as a phenomenon with implications in biological control mechanisms and as an interesting problem in protein chemistry offering a convenient system for the study of protein–protein interactions. When it was recently found (Winstead and Wold, 1964) that rabbit muscle enolase of mol wt 82,000 contained two polypeptide chains, it was felt of interest to investigate possible means of dissociating the chains. This paper reports the reversible dissociation of enolase into 40,000 mol wt subunits in three different systems.

Materials and Methods

Recrystallized enolase was prepared from frozen mature rabbit muscle (obtained from Pel-Freez Biologicals, Inc.) by a modification of the procedure reported by Holt and Wold (1961) (Winstead and Wold, 1965). Protein determinations were made by the biuret–phenol method of Sutherland *et al.* (1949) or by direct spectrophotometric determination, using a value of 0.9 for the absorbancy at 280 mμ of a 1 mg/ml solution of recrystallized enolase (Holt and Wold, 1961). Enzyme activity was measured in imidazole buffer according to the published procedure (Holt and Wold, 1961). Most of the physical studies were also carried out in imidazole buffer. In some experiments, especially those involving prolonged dialysis, phosphate buffer was used, as it was found that dilute solutions of the enzyme were more stable in phosphate buffer than in imidazole buffer. Dioxane was freed of peroxides by passage through an alumina column. All reagents were of the highest chemical purity.

Sedimentation and Molecular Weight Studies. The sedimentation velocity and equilibrium experiments were carried out with the Spinco Model E ultracentrifuge, using the schlieren optical system. The photographic plates were measured with the aid of a Gaertner microcomparator.

Two different methods were used for the determinations of molecular weight. In one the diffusion coefficient was estimated from the schlieren curve formed in the valve-type synthetic-boundary cell, with the cup emptying at approximately 10,000 rpm. These experiments were conducted at constant temperatures in the range of 4–8°. The first photograph was taken immediately after the boundary had formed and at least four more pictures were then taken at 8-minute intervals for the diffusion calculation. The speed was then increased to 56,100 rpm for the determination of the sedimentation coefficient in the standard manner. The value of D_{app} was evaluated from the area, A, under the schlieren curve and the maximum ordinate, H_{max}, of the gradient curve (Schachman, 1957). The value of $M_{s/d}$ was calculated by substitution of the diffusion and sedimentation coefficients into the Svedberg equation (Svedberg and Pedersen, 1940).

Molecular weights were also determined by the Archibald (1947) approach to equilibrium method using a double-sector interference cell. A small volume (0.02 ml) of inert Kel-F fluorocarbon oil was added to the sample sector of the cell to facilitate the calculation of the molecular weight from the pattern at the bottom of the cell. (In the systems containing acetone or dioxane the Kel-F oil was omitted.) The experiments were conducted at 4–8° with the speed of 12,590–29,500 rpm. The initial concentration, c_0, was determined by in-

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creasing the speed to 42,040 after the photographs for the Archibald calculations were made, letting the schlieren pattern move out, and then calculating the area under the curve. Since no correction was made for sectorial dilution the molecular weights obtained in this manner should be too high by a factor of 4–8%. The data were obtained and the calculations were carried out according to Schachman (1957).

The observed sedimentation and diffusion data were corrected to standard conditions of water at 20° in the normal manner. The temperature-viscosity relationships for the organic solvent-water systems relative to water were determined and are given in Table I. The

TABLE 1: Viscosity of Buffer–Acetone and Buffer–Dioxane Mixtures.[a]

	Viscosity (cp)	
(°C)	Buffer–20% Acetone	Buffer–20% Dioxane
3.0	2.56	2.62
3.5	2.46	2.58
4.5	2.38	2.49
6.6	2.21	2.34
8.0	2.11	2.23
11.5	1.88	2.03
14.4	1.72	
15.0		1.82
18.9	1.50	1.61
22.2	1.38	1.49

[a] The data in this table were obtained for 20% acetone and 20% dioxane in 0.05 M potassium phosphate buffer, pH 7.1, each containing 0.2 M ammonium sulfate and 5×10^{-3} M Versene.

viscosities of the ammonium sulfate solutions were obtained from the International Critical Tables. The relative viscosity of 2 M ammonium sulfate was determined at 4° and found to be 1.42. Densities were determined in a 10-ml pycnometer. The densities (at 4°) of 2 M ammonium sulfate in 0.05 M imidazole buffer (pH 7.8), the dioxane system (20% dioxane in 0.05 M potassium phosphate buffer, pH 7.1, containing 0.2 M ammonium sulfate and 5×10^{-3} M Versene), and the acetone system (20% acetone in 0.05 M potassium phosphate buffer, pH 7.1, containing 0.2 M ammonium sulfate and 5×10^{-3} M Versene) were found to be 1.1367, 1.031, and 0.9952 g/ml, respectively. The partial specific volume ($\bar{v}$) of enolase in 2 M ammonium sulfate was also determined with the 10-ml pycnometer (Schachman, 1957). For other calculations $\bar{v}$ was assumed to be constant and equal to 0.728 (Holt and Wold, 1961).

Because of the nonideality of the systems used, the protein was always equilibrated with large volumes of the solvent system by dialysis for 4 hours prior to the experiments and the dialysate was used as the solvent phase in the synthetic-boundary cell and the double-sector cell. The partial specific volume of enolase in 2 M ammonium sulfate was also determined for a dialyzed sample relative to its dialysate.

Sucrose Density Gradient Centrifugation. The procedure described by Martin and Ames (1960) was used. The gradients were made from 2.3 ml each of 5 and 20% sucrose solutions made in 0.05 M imidazole buffer containing 10^{-3} M magnesium sulfate, pH 7.0. Beef liver catalase (Mann Research Laboratories), egg white lysozyme (Sigma Chemical Co.), and yeast enolase were chosen as standards. A solution (0.1 ml) containing catalase (0.2 mg), lysozyme (0.25 mg), and rabbit muscle enolase (6.8×10^{-3} and 3.4×10^{-2} mg) or yeast enolase (2×10^{-2} mg) was carefully layered on the gradients, which had been stored for 8 hours at 4°. The samples were centrifuged in the swinging-bucket rotor SW-39 in the Spinco Model L ultracentrifuge at 38,000 rpm for 14 hours at 4°. Immediately after centrifugation, the samples were fractionated by puncturing the bottom of the tubes and collecting samples of 10 drops each (approximately 0.15 ml). It was possible to collect 300–330 drops from each tube. The fractions were assayed for enzymatic activity. Catalase and lysozyme were assayed according to the procedure described by Martin and Ames (1960). The enolase assay was made on aliquots of 0.05 ml of the fractions from the sample containing 0.034 mg of rabbit muscle enolase. The entire samples (0.15 ml) from the dilute rabbit muscle enolase (6.8×10^{-3} mg) and yeast enolase runs were used for assay. In all cases the activities were expressed in absorbancy change per minute.

Viscosity Measurements. Viscosity measurements were conducted in a Cannon-Fenske viscometer No. 50. An average of three determinations were made for each sample. In addition to viscosity measurements of the various solvent systems, the viscosity of native enolase and enolase in 2 M ammonium sulfate in 0.05 M imidazole buffer, pH 7.8, was also determined. The viscosity measurements on enolase were conducted at 4°, and are reported as reduced viscosities η_{sp}/c, where η_{sp} is the specific viscosity and c is the protein concentration in grams per milliliter.

Fluorescence Polarization Studies. Rabbit muscle enolase was labeled with 1-dimethylamino-5-naphthalenesulfonyl chloride (DNS)[1] according to a method similar to that reported by Weber (1952a). Enolase (50–100 mg) was dissolved in 0.01 M potassium bicarbonate buffer, pH 8.25, to give a protein concentration of 8–10 mg/ml. DNS (California Corp. for Biochemical Research) was dissolved in acetone to give a concentration of 4–10 mg/ml. Both the enolase solution and the DNS solution were cooled to 0° in an ice bath and the DNS solution was added dropwise with con-

[1] Abbreviation used: DNS, 1-dimethylamino-5-naphthalenesulfonyl chloride.

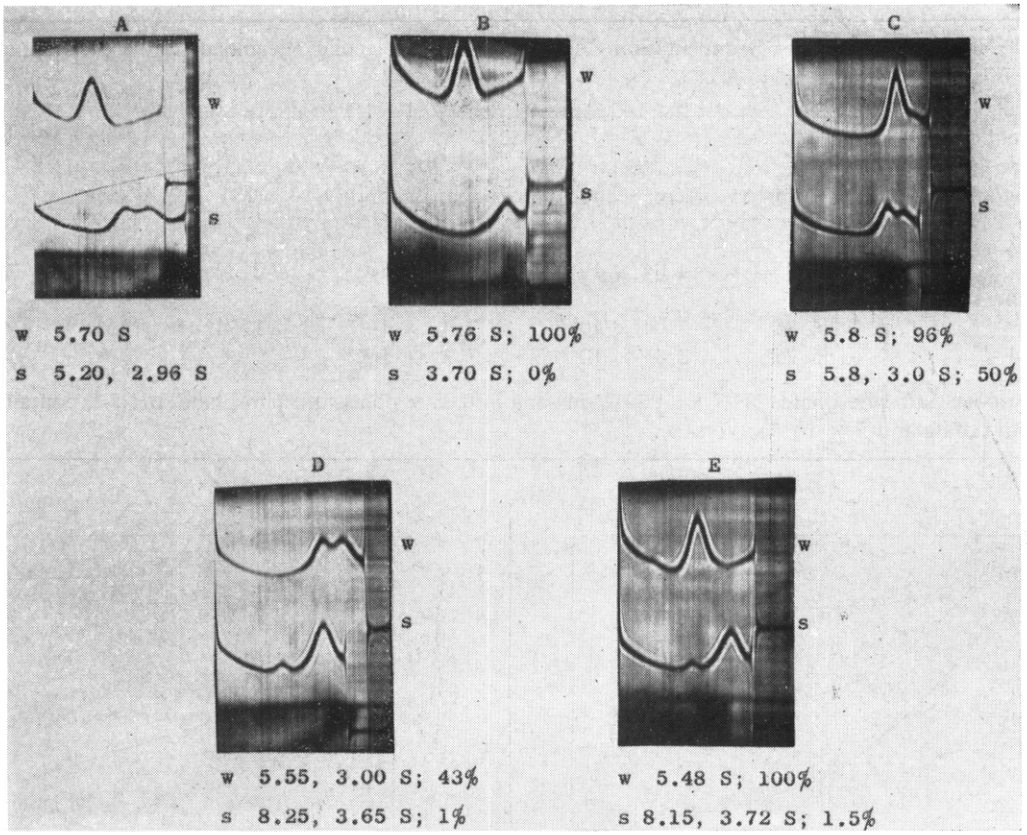


FIGURE 1: Sedimentation patterns of enolase. All samples contained 4 mg/ml of protein and 0.2 M ammonium sulfate and were made up in 0.05 M phosphate buffer, except C which was in 0.05 M imidazole buffer. Centrifugations were at 59,780 rpm at a constant temperature in the range 6–8°. Photographs were made 73–82 minutes after attaining speed (bar angle 50°). The $s_{20,w}$ and per cent activity remaining are given below the photographs. (A) w, enolase solution, pH 7.1; s, enolase solution, pH 10. (B) w, enolase solution, pH 7.1; s, enolase solution containing 20% dioxane, pH 7.1 (80 hours after mixing). (C) w, enolase solution containing 10^{-2} M magnesium sulfate and 20% dioxane, pH 7.1 (8 hours after mixing); s, enolase solution containing 20% dioxane, pH 7.1 (8 hours after mixing). (D) w, enolase solution containing 10^{-2} M magnesium sulfate and 20% dioxane, pH 7.1 (48 hours after mixing); s, enolase solution containing 5×10^{-3} M Versene and 20% dioxane, pH 7.1 (2 hours after mixing). (E) w, enolase solution containing 10^{-2} M Versene, pH 7.1 (2 hours after mixing); s, enolase solution containing 10^{-2} M Versene and 20% dioxane, pH 7.1 (2 hours after mixing).

stant stirring to the enolase solution until a 5–12:1 molar ratio of DNS to enolase was reached. The addition of DNS usually took 1 hour, and the stirring was continued until the reaction mixture became completely clear (3–5 hours). The DNS–enolase was dialyzed overnight at 4° against 100 volumes of 0.05 M potassium phosphate buffer, pH 7.1. The excess free DNS remaining was removed by applying the dialyzed reaction mixture to a 1.7×15 cm column of Dowex 2-X8 (200–400 mesh, ionic form Cl^-) and eluting the DNS–enolase with 0.05 M potassium phosphate buffer, pH 7.1, at 4°. The extent of DNS labeling was determined by measuring the optical densities of the DNS–enolase at 280 and 340 mμ. DNS has a molar absorbancy index of 4.4×10^3 at 340 mμ (Weber, 1952b). The absence of free dye in the DNS–enolase preparation was established by paper chromatography using ethanol–2 M acetate, pH 6 (60:40 v/v) solvent.

The measurements of fluorescence polarization of the DNS–enolase were carried out at 5° in the apparatus described by Weber (1956). The fluorescence was excited by using the 365-mμ line isolated from a high-pressure mercury arc with a Corning 7-60 filter. The fluorescent light was filtered through a 2-mm layer of 1 M sodium nitrite and a Corning 3071 filter. To increase the viscosity of the solutions, known amounts of sucrose were added. The viscosity of the sucrose solutions was determined from the data of Bingham and Jackson (1918).

Optical Rotatory Dispersion Measurements. The rotations were measured with a Rudolph automatic recording spectropolarimeter operating with a slit width of 0.15 mm and a full-scale scan equal to 2°. Rotations were measured at 5° in a 1-dm polarimeter cell with quartz windows using protein concentrations of 1.69–2.96 mg/ml. Values from −0.09 to −0.82°

TABLE II : Diffusion Coefficients, Sedimentation Coefficients, and Molecular Weights of Enolase Determined by the Synthetic Boundary Method.

Solvent and Enolase Concn (mg/ml)	t (°C)	D_{app} (cm²/second)	$D_{w,20}$ (cm²/second)	s_{obsd} (S)	$s_{20,w}$ (S)	Mol Wt
Buffer[a]						
3.0	6.95	4.04×10^{-7}	6.08×10^{-7}	3.87	5.7	80,000
Dioxane–buffer[b]						
3.0	6.85	3.04×10^{-7}	7.4×10^{-7}	1.34	3.4	41,000
4.24	5.25	2.85×10^{-7}	7.3×10^{-7}	1.36	3.6	44,200

[a] 0.05 M potassium phosphate, pH 7.1. [b] 20% dioxane in 0.05 M potassium phosphate, pH 7.1, containing 0.2 M ammonium sulfate and 5×10^{-3} M Versene.

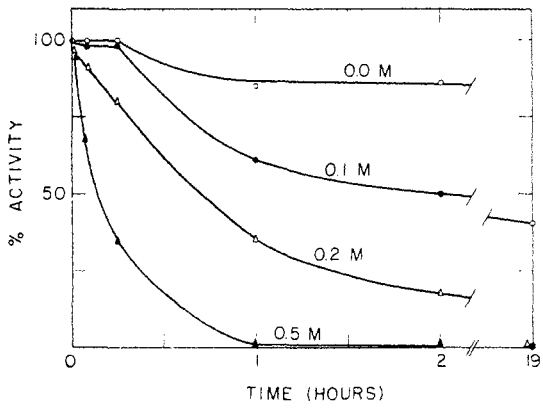


FIGURE 2: The effect of ammonium sulfate on the rate of inactivation of enolase in aqueous dioxane. Enolase (2 mg/ml) was incubated in 0.05 M imidazole buffer (pH 7.1), 5×10^{-3} M Versene, 20% dioxane, and with ammonium sulfate as indicated. Aliquots of the enzyme solutions were assayed under standard conditions.

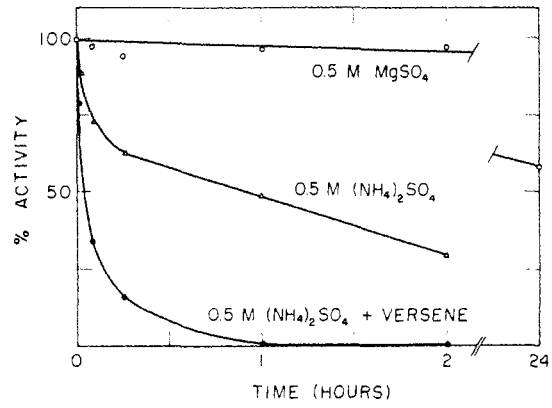


FIGURE 3: The effect of salt and Versene on the rate of inactivation of enolase in aqueous dioxane. Enolase (2 mg/ml) was incubated in 0.05 M imidazole (pH 7.1) and 20% dioxane and with the salt concentration indicated. The Versene concentration in the 0.5 M ammonium sulfate experiment was 5×10^{-3} M. Assays were made under standard conditions.

were recorded; thus the accuracy of the data is limited. The rotatory dispersion data were corrected for the blank instrument values from runs with solvent alone and plotted according to the simple Drude equation as $-1/[\alpha]_\lambda$ *vs.* λ^2. The Drude constants λ_0 were evaluated from the intercepts.

Results

Effect of Acid and Base. Enolase is very sensitive to both acid and base (Holt and Wold, 1961). At pH 4–5 the activity is lost very rapidly and irreversible denaturation and precipitation are observed. If the pH was lowered rapidly to 2.3, however, the enzyme remained in solution, but again all the activity was irreversibly lost. When this acid-treated sample at pH 2.3 was subjected to ultracentrifugation, it was found to contain highly aggregated material, and a sedimentation pattern could not be obtained. At alkaline pH, the activity loss was also rapid and irreversible, but the enzyme remained in solution and it was possible to get reasonable sedimentation patterns. An enolase sample titrated to pH 10 gave two peaks (Figure 1A) with $s_{20,w}$ of 5.20 and 2.96 as compared to a value of 5.7 for the control in buffer. The two peaks obtained in this experiment probably correspond to the two peaks obtained previously upon electrophoresis of enolase at pH 9 (Holt and Wold, 1961). Since these preliminary studies did not prove rewarding, other systems for dissociation of enolase were studied.

Effect of Dioxane. When enolase was exposed to 20% aqueous dioxane[2] and examined in the ultracentrifuge, a slow conversion of the normal 5.8S peak to a more slowly sedimenting peak was observed, indicating a

[2] Aqueous dioxane and aqueous acetone were both prepared on the assumption that there is no volume change on mixing. Thus, 20% dioxane or 20% acetone means that 20 ml of solvent was mixed with 80 ml of the appropriate buffer at 25°.

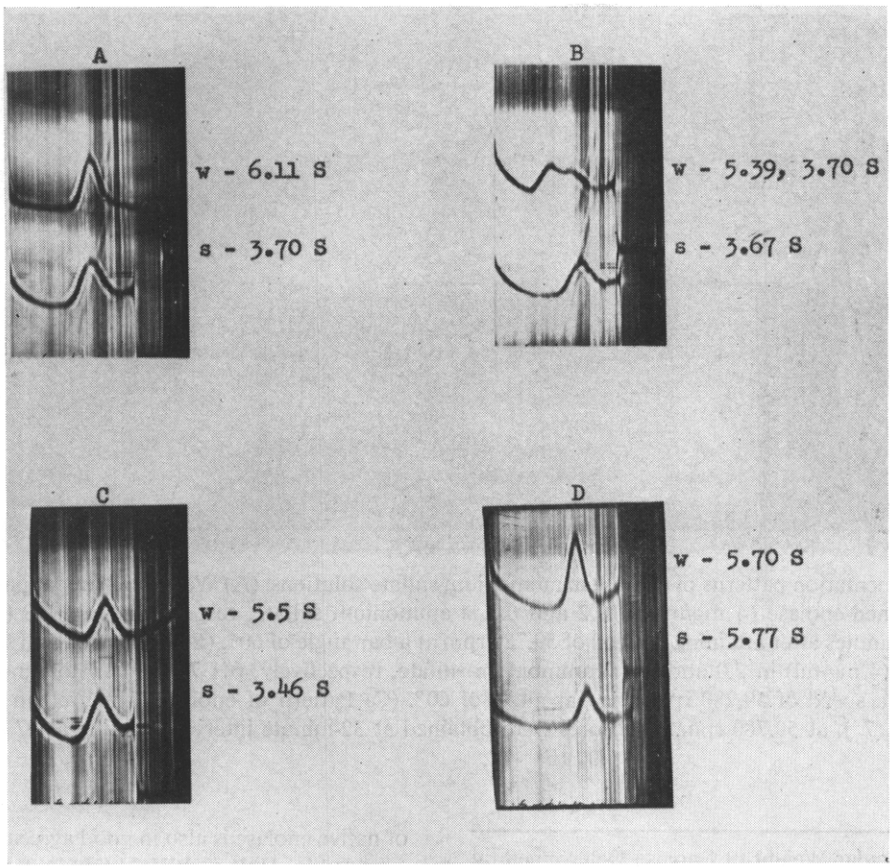


FIGURE 4: Sedimentation patterns of enolase. All experiments were conducted at 8° in 0.05 M phosphate buffer, pH 7.1, with an enolase concentration of 4 mg/ml except in the case of the reconstituted enolase, where the concentration was 1.65 mg/ml. The centrifuge speed was 59,780 rpm, and photographs were made at a bar angle of 50°. Time of photographs after attaining speed is given in parentheses. (A) (127 min) w, enolase in 20% acetone; s, enolase in complete acetone system (0.2 M ammonium sulfate, 5×10^{-3} M Versene, and 20% acetone). (B) (109 min) w, enolase in 0.2 M ammonium sulfate and 20% acetone; s, enolase in complete acetone system. (C) (100 min) w, enolase in 0.2 M ammonium sulfate and 5×10^{-3} M Versene; s, enolase in complete acetone system. (D) (56 min) w, native enolase in buffer; s, reconstituted enolase from C in buffer.

slow dissociation of the two chains (Figure 1B–E). In all cases the slow moving component must be inactive, as the activity of each sample was found to be directly proportional to the amount of the 5.7S component remaining.

It was found that the presence of Mg^{2+} ions greatly retarded the rate of dissociation, while the addition of Versene greatly increased the rate. Ultacentrifuge patterns for the 20% dioxane samples containing Mg^{2+} ions and Versene are shown in Figure 1. It should be emphasized that if ammonium sulfate were left out the rate of dissociation again was found to be extremely slow, and also that if the dioxane concentration were lowered to 10% no dissociation was observed.

Since the data in Figure 1 indicate that the activity is associated with the 5.7S component only, it was possible to evaluate the effect of ammonium sulfate, magnesium sulfate, and Versene on the rate of dissociation in 20% dioxane by measuring the rate of inactivation. The results of these studies are given in Figures 2 and 3.

Attempts to dissociate enolase with chelating agents alone failed, although a slight decrease in the sedimentation coefficients was observed. Sedimentation coefficients of 5.48 and 5.16 S were observed for solutions of enolase (4 mg/ml) containing 10^{-2} M Versene and 10^{-3} M *o*-phenanthroline, respectively, compared to 5.7 for native enolase. It was also found that the addition of 10^{-2} M Versene to enolase (4.75 mg/ml) in 0.50 M ammonium sulfate caused a decrease in $s_{20,w}$ from 5.0 to 4.8 S.

The molecular weight of the modified species in the dioxane system was determined by both the Archibald approach to sedimentation equilibrium method and from sedimentation and diffusion measurements made in the synthetic-boundary cell. The results are given in Tables II and III. There is good agreement between the results obtained by the two methods, and the diffusion

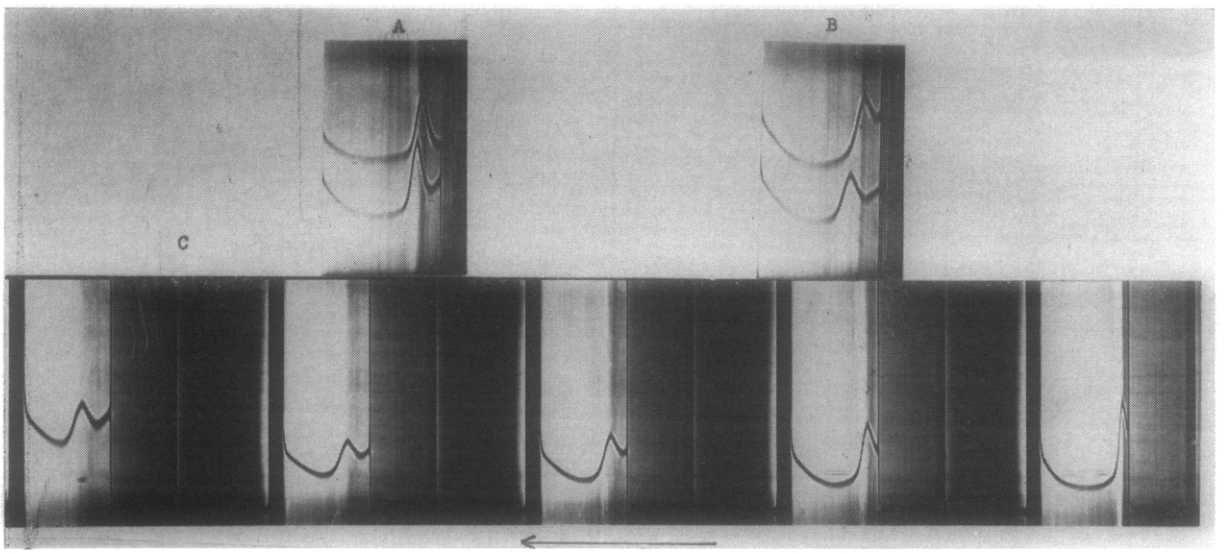


FIGURE 5: Sedimentation patterns of enolase in ammonium sulfate solutions. (A) Wedge cell (upper pattern) and standard cell contained enolase (4 mg/ml) in 0.2 and 0.5 M ammonium sulfate, respectively, pH 7.8 (5°). Photograph was taken 62 minutes after attaining a speed of 59,780 rpm at a bar angle of 60°. (B) Wedge cell and standard cell contained enolase (4 mg/ml) in 2.0 and 1.0 M ammonium sulfate, respectively, pH 7.8 (20°). Photographed 68 minutes after attaining a speed of 59,780 rpm at a bar angle of 60°. (C) Pattern of enolase (6 mg/ml) in 2 M ammonium sulfate, pH 7.8 (7°), at 59,780 rpm. Exposures were obtained at 32-minute intervals starting at 27 minutes after attaining speed.

TABLE III: Molecular Weight of Enolase Determined by the Archibald Approach to Sedimentation Equilibrium Method.

Solvent and Enolase Concn (mg/ml)	Speed (rpm × 10^{-3})	Time of Picture (min)	Mol Wt M_m[c]	Mol Wt M_b[c]
Buffer[a] 7	12.59	7	80,000	
		15	79,000	
		23	82,100	75,700
		31	82,400	
		39	82,800	
Dioxane–buffer[b] 4.24	20.41	17	44,300	
		49	46,700	

[a] 0.05 M imidazole, pH 7.1, containing 10^{-3} M $MgSO_4$. [b] 20% dioxane in 0.05 M potassium phosphate, pH 7.1, containing 0.2 M ammonium sulfate and 5 × 10^{-3} M Versene. [c] M_m and M_b signify the molecular weights calculated from the meniscus and the bottom of the cell, respectively.

constant determined for the native enolase in the synthetic-boundary cell agrees well with the reported value of 6.3 × 10^{-7} cm^2/second determined by free diffusion (Holt and Wold, 1961). The molecular weight of native enolase is also in good agreement with the one reported by Holt and Wold (1961). It is apparent from these data that enolase in the complete dioxane system dissociates into two monomers with a molecular weight of approximately 43,000.

As indicated in Figures 1–3, reactivation of the inactive, slowly sedimenting component did not occur spontaneously in the assay medium, and several attempts to reactivate enolase that had been subjected to dioxane, ammonium sulfate, and Versene for several hours (4 or more) gave very low yields of active enzyme (3–8%). However, when a sample incubated for only 30 minutes in the complete dioxane system (7% of the original activity remained) was dialyzed for 10 hours against 0.05 M potassium phosphate buffer, pH 7.1, which contained 5 × 10^{-3} M magnesium sulfate, and then stored at 4° for an additional 18 hours, 45% of the original activity was recovered. Only slight precipitation occurred in this sample, while the samples which had been subjected to the dioxane system for longer periods of time gave very heavy protein precipitation during the reactivation attempts. No attempts were made to characterize the reconstituted enolase from the dioxane system beyond activity measurements.

Effect of Acetone. Having established that dioxane in the presence of ammonium sulfate and Versene could cause dissociation of enolase, it was considered of interest to determine if other organic solvents would have a similar effect. Ethanol (20%) in the presence of ammonium sulfate and Versene had no observable effect on the sedimentation behavior of enolase, but a $s_{20,w}$ of 3.7 was obtained when 20% acetone was added to a solution containing 0.2 M ammonium sulfate and

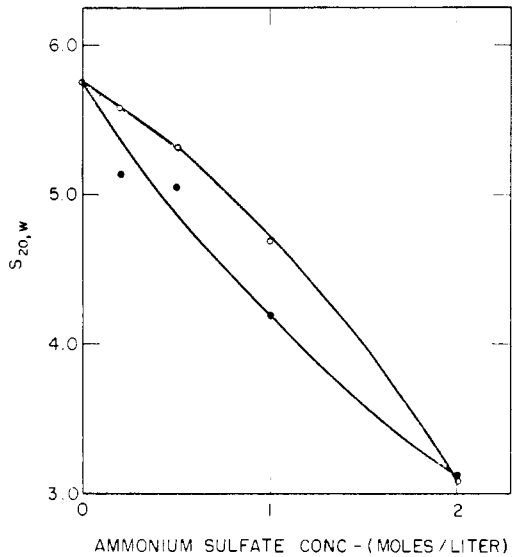


FIGURE 6: The effect of ammonium sulfate on the sedimentation coefficient of enolase. The sedimentation velocity experiments were conducted on enolase (4 mg/ml) in 0.05 M imidazole, pH 7.8. The closed circles refer to experiments at constant temperatures in the range 4–8°, and the open circles to experiments at constant temperatures in the range 18–22°. All runs were made at 59,780 rpm.

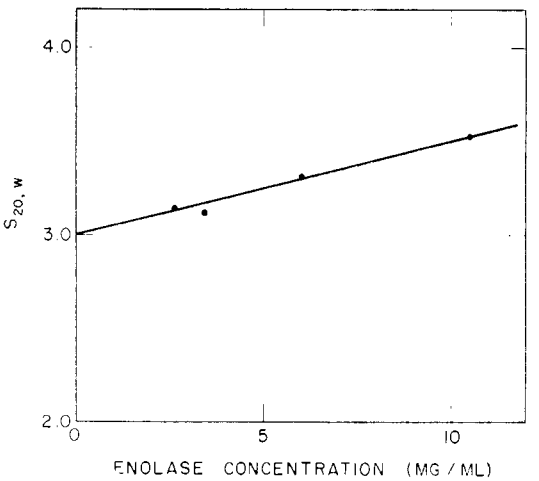


FIGURE 7: Concentration dependence of the sedimentation coefficient of enolase in 2 M ammonium sulfate. Enolase was in 0.05 M imidazole, pH 7.8, containing 2 M ammonium sulfate. Centrifuge runs were conducted at constant temperatures in the range of 4–8° at 59,780 rpm.

5×10^{-3} M Versene. The requirement for ammonium sulfate and Versene was found to exist in this system also as illustrated by the sedimentation patterns of the complete acetone system and the patterns obtained in the absence of salt and Versene (Figure 4). If Versene were omitted, the rate of dissociation was again slow enough to give two separate peaks in the sedimentation pattern (Figure 4B). Acetone (10%) even in the presence of salt and Versene gave no dissociation.

The loss of enolase activity in the acetone system was very similar to that observed in the dioxane system, but it was found to be more readily reversible. If the acetone were diluted or dialyzed away in the presence of 10^{-3}–10^{-2} M magnesium sulfate, an immediate reactivation was observed. One sample containing 3 mg/ml of enolase incubated in the acetone system until all of its activity was lost was dialyzed overnight at 4° against 0.05 M potassium phosphate buffer, pH 7.1, containing 5×10^{-3} M magnesium sulfate, and then centrifuged to remove some precipitated protein before subjecting it to ultracentrifugation. The sedimentation pattern of this reconstituted enolase is shown in Figure 4D, and the sedimentation coefficient was calculated to be 5.77 S for a concentration of 1.65 mg/ml. This is in good agreement with the value 5.70 S for native enolase (4 mg/ml). The specific activity of this reconstituted enolase was found to be 355, which compares favorably with the value 375 for native enolase. The yield of the reconstituted enzyme was 46%. Activity measurements on other reconstituted samples in which precipitation was essentially completely prevented by working with more dilute protein solutions gave activity recoveries of 83–87%.

The molecular weight of enolase (3.98 mg/ml) in the acetone system was determined by the Archibald approach to equilibrium method at 5° using a rotor speed of 20,410 rpm. Molecular weights of 40,100 and 38,900 were calculated at the meniscus from photographs made at 16 and 42 minutes, respectively.

The properties of the acetone system closely paralleled the dioxane system in the fact that three components (solvent, ammonium sulfate, and Versene) were required for dissociation. Protein precipitation, presumably due to aggregation, occurred in both systems as the concentration of enolase increased and as the duration of the treatment was extended. Based on the above observations, it is suggested that the molecular species of enolase in the dioxane and acetone systems are similar.

Effect of Ammonium Sulfate. Having observed the requirement for ammonium sulfate in both the organic solvent systems, it was of interest to investigate the effect of ammonium sulfate alone on the molecular properties of enolase. Since the enzyme crystallizes from approximately 2.2–2.4 M ammonium sulfate solutions, concentrations up to 2 M were used. Representative sedimentation patterns from the ammonium sulfate system are given in Figure 5. The data in Figure 6 show that the sedimentation coefficient of enolase decreases with increasing ammonium sulfate concentrations. It is also evident that the change in sedimentation coefficient was slower at room temperature than at 4–8°, but that the same value was reached at both temperatures in 2 M ammonium sulfate. The sedimentation coefficient of enolase in 2 M ammonium sulfate was also

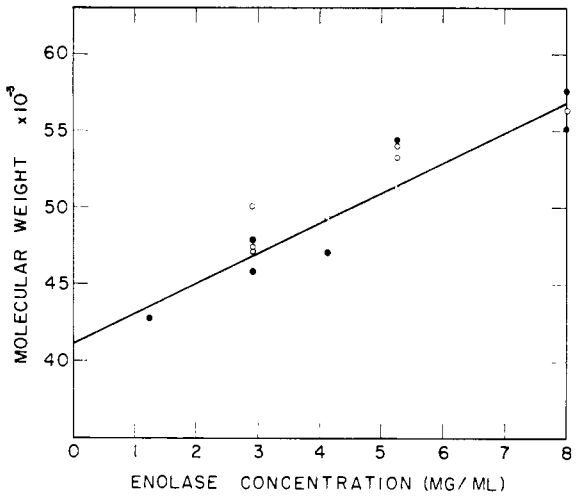


FIGURE 8: Concentration dependence of the molecular weight of enolase in 2 M ammonium sulfate. The data are taken from Table IV. The open circles are for M_m and the closed circles for M_b.

found to be protein concentration dependent, increasing with increasing enolase concentration. The value obtained by extrapolation to infinite dilution was 3 S (Figure 7). If 2 M magnesium sulfate were substituted for ammonium sulfate, the sedimentation coefficient ($s_{20,w}$) of enolase (4 mg/ml) was found to be 5.8 S.

Owing to difficulties in obtaining good boundaries in the synthetic-boundary cell, only the Archibald approach to sedimentation equilibrium method was used for molecular weight determinations in 2 M ammonium sulfate solutions. The molecular weight of enolase in 2 M ammonium sulfate was found to be concentration dependent as indicated in Table IV and Figure 8. The molecular weight determined by extrapolation to infinite dilution was 41,000 which agrees with the expected molecular weight of a monomer. In 1 M ammonium sulfate, molecular weights of 52,300–54,400 were obtained, using a 7.3 mg/ml solution of enolase. These observations suggest that the high concentrations of ammonium sulfate caused dissociation of enolase.

TABLE IV: Molecular Weight of Enolase in 2 M Ammonium Sulfate Determined by the Archibald Approach to Sedimentation Equilibrium Method.

Enolase Concn (mg/ml)	Speed (rpm × 10^{-3})	Time of Picture (min)	Mol Wt M_m	Mol Wt M_b
1.23	29.50	5		42,800
2.89	20.41	14	47,200	
		46	47,300	45,900
		78	50,100	47,900
4.12	20.41	16	49,400	47,100
5.25	20.41	16	53,300	
		48	54,100	
		80	51,300	54,300
8.0	16.20	19		57,600
		51	56,400	55,100

It should be noted that, because of the high density of the solvent, small changes in $\bar{\nu}$ could give large errors in the above data, and it was therefore necessary to determine the apparent $\bar{\nu}$ of enolase in 2 M ammonium sulfate. From several weighings of a single 10-ml sample containing 7.63 mg/ml of enolase, the value obtained at pH 7.8 was 0.725 ± 0.004, which is not significantly different from the value 0.728 in dilute buffer (Holt and Wold, 1961). The $\bar{\nu}$ of bovine serum albumin (Ifft and Vinograd, 1962) and hemoglobin (Kirshner and Tanford, 1964) have also been shown to be essentially unaffected by high concentrations of salt.

Because of the unusual properties of the high ammonium sulfate solutions, it would seem proper to attempt a critical evaluation of some of the experimental procedures which were employed in obtaining the above data.

Most of the sedimentation work was done in conventional cells with aluminum center pieces, and the possibility that artifacts due to dissolved aluminum could confuse the data had to be considered. Several cross checks were therefore carried out using Kel-F cells. The results from the two types of cells were identical in all cases.

The boundary formation in the synthetic-boundary cell gave very poorly reproducible results with the concentrated salt solutions. If the equilibration time allowed for the dialysis of the enolase solution against solvent was insufficient, the expected nongaussian boundaries were observed. But even with well equilibrated samples, the discharge of concentrated salt solutions from the cup did not occur smoothly at a given speed, but seemed rather to take place in a slow flow over a wide range of centrifuge speeds. For this reason the molecular weight of enolase in 2 M ammonium sulfate was determined only by the modified Archibald procedure. This method appears to be technically satisfactory, however, for the following reasons: (a) the results were reproducible, (b) the values obtained by this method for native enolase and for enolase in the dioxane system were in good agreement with values obtained from sedimentation–diffusion data and from short column equilibrium runs, and (c) when the molecular weight of several other proteins in ammonium sulfate were determined by this method, the results were consistent with literature values (Table V).

The data in Table V were also obtained for another reason. Because of the nonideality of the system used several hazards were encountered in calculation and interpretation as well as in experimental procedure. Thus, one must consider the dangers of treating the three-component protein–salt–water system as an ideal two-component system in the calculations. This assumes the absence of preferential interactions and flow

TABLE V: Sedimentation Coefficients and Molecular Weights of Several Proteins in Concentrated Ammonium Sulfate Solutions.

Protein[a]	Conditions: Protein Concn (mg/ml)	Conditions: $(NH_4)_2SO_4$ Concn (M)	Conditions: pH	Results: In Buffer $s_{20,w}$ (S)	Results: In $(NH_4)_2SO_4$ $s_{20,w}$ (S)	Results: In $(NH_4)_2SO_4$ Mol Wt $\times 10^{-3}$	Literature Values: In Buffer $s_{20,w}$ (S)	Literature Values: In Buffer Mol Wt $\times 10^{-3}$	Literature Values: $\bar{\nu}$	Reference
Liver alcohol dehydrogenase	8.4	2.0	6.5	4.46	3.3	166	5.1	84	0.75	Ehrenberg and Dalziel (1958)
Muscle aldolase	5.0	1.8	7.5	7.5	5.5	149	7.4	142	0.742	Stellwagen and Schachman (1962) Taylor and Lowry (1956)
Bovine serum albumin	10.0	2.0	7.1	...	2.1	72.4	4.4	69	0.734	Loeb and Scheraga (1956) Dayhoff *et al.* (1952)
Liver catalase	2.7	1.0	7.0	10.6	9.9	229	11.4	240	0.730	Samejima and Yang (1963)
Yeast enolase	6.0	2.0	7.1	5.7	4.3	66.5	5.8	67	0.735	Malmström (1961)
Liver glutamic dehydrogenase	6.5	1.0	7.2	22.5	28.6	1,300	23.7	990	0.75	Churchich and Wold (1963) Olson and Anfinsen (1952)
Ovalbumin	6.0	2.0	7.1	3.7	1.5	91	3.6	44	0.749	Fox and Foster (1957)

[a] Aldolase was studied in 0.01 M Tris–HCl buffer; all the others in 0.05 M potassium phosphate buffer.

coupling between the solvent components and the protein, an assumption which is clearly hazardous and difficult to support. In the absence of sufficient theoretical arguments on this point, it is felt that the data in Table V lends some empirical support for treating the high salt experiments as ideal two-component systems. Based on a single run at only one protein concentration, the data in Table V show that the molecular weights of aldolase, bovine serum albumin, catalase, and yeast enolase determined by the modified Archibald method in the presence of high concentrations of ammonium sulfate are in excellent agreement with the literature values for the native proteins. The fact that alcohol dehydrogenase and ovalbumin by the same method gave a molecular weight almost exactly double that of the native proteins was in fact taken as a tentative suggestion that these two proteins may form dimers in concentrated ammonum sulfate solutions. Glutamic dehydrogenase which was run in 1 M ammonium sulfate because of its low solubility in salt did not give as consistent results. All of the calculations in Table V used the literature values for partial specific volumes, assuming that these were not affected by salt. Since $(1 - \bar{\nu}\rho)$ enters in both the molecular weight calculations and in the evaluation of $s_{20,w}$, any error due to $\bar{\nu}$ should appear in both quantities, and the fact that $s_{20,w}$ of the proteins in Table V was found to be drastically affected by ammonium sulfate should thus mean that the frictional ratio of these proteins was changed in ammonium sulfate.

In an attempt to get more information about the effect of ammonium sulfate on enolase, other parameters were also studied. Figure 9 gives a comparison of the reduced viscosity of native enolase and enolase in 2 M ammonium sulfate. At pH 7.1 the intrinsic viscosity of enolase was 4.5 ml/g, a value similar to that found for many compact, globular proteins (Tanford, 1958). The addition of 2 M ammonium sulfate at pH 7.8 caused only a small increase in the intrinsic viscosity to 5 ml/g. In the presence of the salt a very large dependence of the reduced viscosity on protein concentration was observed, however.

Attempts to measure the viscosity of enolase in the 20% dioxane system were unsuccessful because of the tendency of the protein to precipitate in the viscometer capillary.

In a final attempt to compare the molecular dimensions of native enolase to those of enolase in ammonium sulfate and in dioxane, the rotational relaxation time was determined from depolarization of fluorescence measurements on a sample of enolase labeled with DNS. The activity of DNS–enolase immediately after the labeling was found to be 90–98% that of the native enzyme; however, after dialysis and column chromatography on Dowex 2 the activity usually decreased to approximately 70%. The extent of labeling in the DNS–enolase was calculated from the optical density readings at 280 and 340 mμ and gave an average value of 1.2–1.9 DNS molecules/molecule of enolase. The $s_{20,w}$ for the DNS–enolase was determined to be 5.80 S at a concentration of 2.5 mg/ml. This is essentially

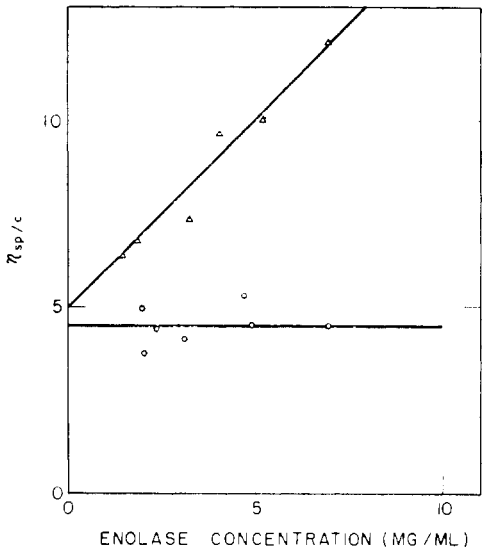


FIGURE 9: The effect of protein concentration on the reduced viscosity of enolase in 0.05 M imidazole buffer, pH 7.1 (circles), and in 0.05 M imidazole buffer, pH 7.8, containing 2 M ammonium sulfate (triangles). The measurements were carried out at 4° with Cannon-Fenske viscometers with a flow time of 300 seconds.

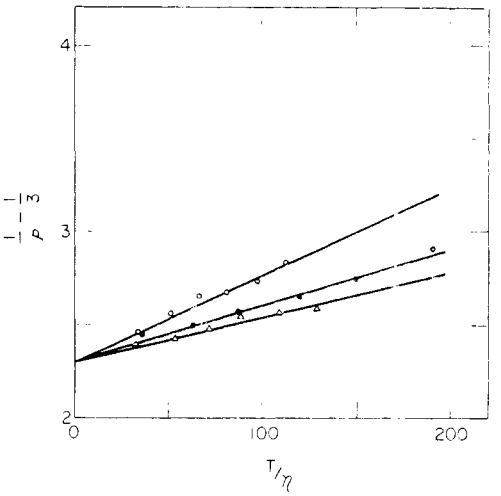


FIGURE 10: Fluorescence polarization of native and dissociated enolase. Results with DNS–enolase (1 mg/ml) in 0.05 M potassium phosphate, pH 7.1, are represented by closed circles; results for DNS–enolase (1 mg/ml) in 0.05 M potassium phosphate, pH 7.1, containing 0.2 M ammonium sulfate, 5×10^{-3} M Versene, and 20% dioxane, are shown by open circles; results for DNS–enolase (1 mg/ml) in 0.05 M potassium phosphate–2 M ammonium sulfate, pH 7.8, are illustrated by triangles. All measurements were made at 5° and the viscosities were varied by addition of sucrose.

the same as that for native enolase at the same concentration. DNS–enolase and enolase also had the same sedimentation coefficients in the dioxane system. In 2 M ammonium sulfate, however, a 1-mg/ml sample of DNS–enolase gave an $s_{20,w}$ of 3.5, which is almost 0.5 S higher than that for unlabeled enolase under the same conditions.

The fluorescence polarization of DNS–enolase was compared in buffer, in 2 M ammonium sulfate, and in the dioxane system. The viscosity was varied by the addition of sucrose and the temperature was kept constant. It was assumed that the viscosity of each system was the simple sum of the viscosity of each component in the system. The plots of $1/p - 1/3$ *vs.* T/η are presented in Figure 10. The relaxation times were calculated according to the following equation developed by Perrin (1926).

$$(1/p - 1/3) = (1/p - 1/3)(1 + 3\tau_o/\rho_h)$$

Here p is the observed fluorescence polarization, p_o is the limiting polarization, τ_o is the lifetime of the excited state of the fluorescent oscillator, and ρ_h is the harmonic mean of the two principal relaxation times of the rotation of ellipsoidal molecule under the physical conditions of the experiment. The value of 1.2×10^{-8} second was used for τ_o for DNS–enolase (Lewis and Kashi, 1945). The relaxation times at 5°, calculated from the data in Figure 10, were 90×10^{-9} second for DNS–enolase in buffer, 103×10^{-9} second for DNS–enolase in 2 M ammonium sulfate, and 53×10^{-9} sec for DNS–enolase in the dioxane system. This suggests that the species of enolase in the dioxane system are either smaller or much more compact than those of native enolase in buffer. It also demonstrates that the molecular species in 2 M ammonium sulfate are different from those in the dioxane system. Assuming that the monomer is the predominant form in 2 M ammonium sulfate, the data suggest that a substantial loss of molecular symmetry is associated with the dissociation in ammonium sulfate.

The difference between the molecular species of native enolase and enolase in dioxane and in ammonium sulfate is also illustrated by the optical rotatory dispersion data shown in Table VI. The value −36° for $[\alpha]_D$ is in agreement with that reported by Malmström (1962). Since the measured optical rotations were low, ranging from − 0.09 to 0.82°, the accuracy of the data is not

TABLE VI Specific Rotation and Drude Constant of Enolase.

Buffer System	$[\alpha]_D^3$ (deg)	λ_c (mμ)
0.05 M imidazole, pH 7.8	−36	243
0.05 M potassium phosphate, 0.2 M ammonium sulfate, and 20% dioxane, pH 7.1	−55	214
0.05 M imidazole and 2 M ammonium sulfate	−74	214

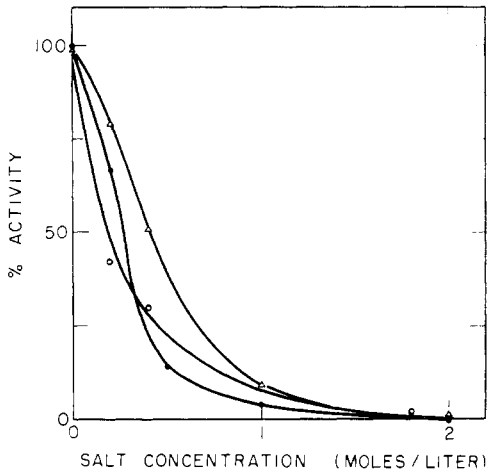


FIGURE 11: The effect of salt concentration on the activity of enolase. Enolase (8×10^{-3} mg/ml) aliquots were assayed in 3-ml reaction mixtures containing 10^{-3} M glyceric acid 2-phosphate, 10^{-3} M magnesium sulfate, 0.05 M imidazole, pH 6.7, and the above salt concentrations. The open circles and triangles are for enolase assayed at 25° in magnesium sulfate and ammonium sulfate solutions, respectively. The closed circles represent enolase assayed at 7° in the presence of ammonium sulfate.

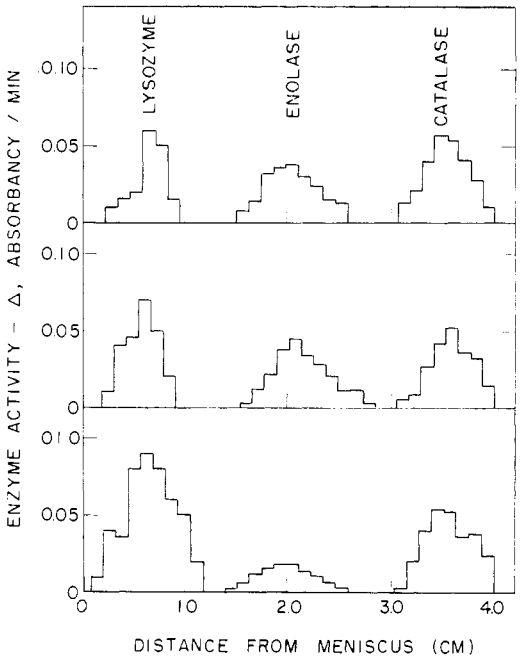


FIGURE 12: Enzyme activity distribution in a sucrose density gradient. Lysozyme (0.25 mg), catalase (0.2 mg), and rabbit muscle enolase (6.8×10^{-3} mg) mixed in 0.10 ml of 0.05 M imidazole containing 10^{-3} M magnesium sulfate, pH 7.0, were layered on a sucrose gradient. After 14 hours of centrifugation at 38,000 rpm, 4°, the gradient was fractionated and analyzed (bottom graph). Two other gradients centrifuged at the same time contained 3.4×10^{-2} mg of rabbit muscle enolase (center graph) and 0.02 mg of yeast enolase (top graph) as well as the standards.

great, but a change in the salt and dioxane system is still apparent.

The effect of ammonium sulfate on enolase activity determined at both 7° and 25° and the effect of magnesium sulfate at 25° are shown in Figure 11. As should be expected from these results, only slight activity (0.1–0.2%) could be detected when the crystals of enolase were assayed in 70% saturated ammonium sulfate. These data together with the sedimentation data and molecular weights suggest that enolase crystallizes from approximately 2.3 M ammonium sulfate in an inactive form. If this is correct, the process of inactivation must be completely reversible as the enzyme can be stored for several months suspended in 70% saturated ammonium sulfate saturated with magnesium sulfate without any loss of activity. A sample stored in solution in 2 M ammonium sulfate containing 10^{-2} M Versene for 5 days was also found to be fully active after dilution into buffer.

Estimation of Molecular Weight under the Conditions of the Activity Assays. The data presented thus far have suggested that the di ner of rabbit muscle enolase is the biologically active form. It must be emphasized that the physical studies were made on solutions containing approximately 4 mg/ml of enolase which is 4.7×10^{-5} M, while the enolase concentration in the average assay is 4.8×10^{-9} M. To obtain physical data on the enzyme at concentrations approaching those used in the activity assays, a sucrose density gradient experiment was conducted in which two concentrations of rabbit muscle enolase were compared to yeast enolase with lysozyme and catalase as reference proteins in all tubes. Yeast enolase was chosen for comparison because it has a suitable molecular weight (67,000) and sedimentation coefficient (5.8 S) and is accepted as a single-chain enzyme. The activity distribution in the gradient tubes is given in Figure 12 and the calculated sedimentation coefficients in Table VII. It should be pointed out that

TABLE VII: Sedimentation Coefficients Calculated from Sucrose Density Gradient Centrifugation.

Standard[a]	Yeast Enolase	Rabbit Muscle Enolase 6.8×10^{-3} mg	Rabbit Muscle Enolase 3.4×10^{-2} mg
Catalase	6.6	6.6	6.6
Lysozyme	6.6	6.1	6.1
Yeast enolase		5.5	5.7

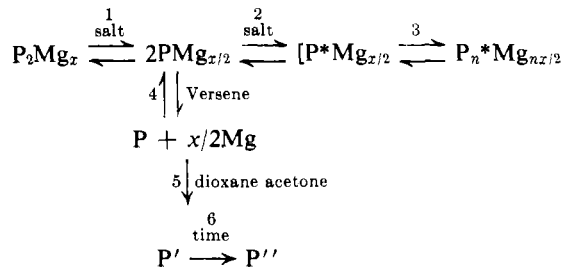
[a] The following $s_{20,w}$ were used for calculations: catalase, 11.3 (Martin and Ames, 1960); lysozyme, 1.9 (Fox and Foster, 1958); and yeast enolase, 5.8 (Malmström, 1961).

enolase was found to be fully active when tested in an assay medium containing 15% sucrose (this is approximately the sucrose concentration at which the enolase zone appeared in the gradient).

The tube exhibiting the maximum enolase activity from the most dilute rabbit muscle enolase experiment had an active enolase concentration of 3.8×10^{-8} M. This is only 5–10 times higher than the normal concentration of enolase in an assay medium, compared to a discrepancy of 6000–12,000-fold in the other physical studies. Only 41% of the activity was recovered, but this is not too suprising since it has been observed that dilute solutions of rabbit muscle enolase lose activity on standing. The sedimentation coefficients vary depending on which standard is used for calculations, but the value for rabbit muscle enolase agrees well with that for yeast enolase. It is concluded from this experiment that the 82,000 mol wt dimer is the active form of rabbit muscle enolase in dilute solution. The same conclusion was arrived at from experiments in which substrate and magnesium were added to the dilute enzyme in the sucrose density gradient experiment.[3]

Discussion

The results of the physical studies on rabbit muscle enolase (summarized in Table VIII) can best be discussed in terms of the following proposed model.[4] In the model



P_2Mg_z is native enolase, $PMg_{z/2}$ is a compact monomer (with a frictional ratio very similar to that of the native enzyme), $P^*Mg_{z/2}$ is a hypothetical unfolded or solvated monomer, and $P_n{}^*Mg_{nz/2}$ is an aggregate (n-mer) of this modified monomer; P is a compact monomer from which Mg^{2+} has been removed, and in organic solvents this monomer is converted to P′, which is the form observed in the dioxane and acetone systems. P″ is the insoluble and irreversibly denatured aggregate found after prolonged exposures of the protein to the two solvent systems. It is postulated that P_2Mg_z is the only enzymatically active form in this model.

There are two reasons for including magnesium in the different intermediates in this scheme. One is the fact that the enzyme is crystallized from solvent systems containing magnesium, and the second is the observed effect of Versene in the dioxane and acetone systems. It is interesting to note that the enzyme shows some activity when assayed in the absence of added magnesium, and that this activity is eliminated by the addition of Versene. The requirement for 10^{-3} M magnesium in the assay medium to obtain optimal rates may in fact suggest that magnesium has a role as a structural component of the active enzyme and also is required at a higher concentration as a participant in the formation of the enzyme–substrate complex. This dual role of Mg^{2+} has recently been demonstrated in yeast enolase by Brewer and Weber (1965).

The series of reactions leading to P′ is consistent with the data obtained in the acetone and dioxane systems. It was shown that all three components (salt, Versene, and organic solvent) were required for rapid dissociation. Thus at low salt concentrations only a small amount of $PMg_{z/2}$ would be present, and the rate of formation of P′ should be very low. Similarly, in the absence of Versene or even more pronounced in the presence of Mg^{2+}, the form P could be present only in small amounts and again the formation of P′ should be slow. All the data indicate that salt and Versene affect the *rate* of dissociation only, and, since the salt effect and Versene effect were shown to be readily reversible, it is logical to propose that step 5 is the one which is not readily reversible, and that P′ is the stable monomer form observed in the solvent systems. Reaction 6 is included to explain the slow irreversible loss of activity and the concomitant appearance of insoluble protein on prolonged exposure to the organic solvent systems. The molecular weights in the dioxane and acetone system (Table VIII) clearly show that P′ is indeed a monomer. Based on the sedimentation coefficients in the dioxane and acetone systems and the diffusion coefficient in the dioxane system (Table VIII), it appears that P′ is a partially unfolded monomer unit.

The behavior of enolase in ammonium sulfate can best be explained by a series of reactions (1–3) in rapid dynamic equilibrium, and is certainly not compatible with a simple equilibrium between P_2Mg_z and $PMg_{z/2}$. The two reasons why an asymmetrical form ($P_n{}^*Mg_{nz/2}$) must be proposed are the low sedimentation coefficients in Figure 7 and the high reduced viscosities in Figure 9. If the concentration-dependent change in molecular weight (Figure 8) were due only to a shift of the equilibrium in reaction 1 toward the dimer form, it should be possible to calculate the change in the sedimentation coefficient (using $s_{20,w} = 3.0$ S for $PMg_{z/2}$ and $s_{20,w} = 5.7$ S for P_2Mg_z) corresponding to the equilibrium mixture observed in Figure 8 (using mol wt 41,000 for $PM_{z/2}$ and mol wt 82,000 for P_2Mg_z). Such a calculation gives a steeper slope for the dependence of $s_{20,w}$ on protein concentration than the one observed in Figure 7, indicating that the frictional ratio of the high molecular weight component in ammonium sulfate is larger than that of the native dimer. This is completely consistent with the viscosity data in Figure 9 showing a large increase in reduced viscosity (increased frictional

[3] M. J. Cardenas, personal communication.

[4] It must be re-emphasized that the proposed model, the discussion, and the conclusions drawn from the hydrodynamic data are based on the assumption that there is no preferential interaction or flow coupling in the high salt system, and thus must be considered tentative until the assumption can be completely tested.

TABLE VIII: Physical Properties of Native and Dissociated Enolase.

Sample	$s_{20,w}$ (S)	η_{sp}/c (ml/g)	Mol Wt $\times 10^{-3}$	$D_{20,w}$ (cm²/sec) $\times 10^{7}$	ρ_h (sec) $\times 10^{9}$	$[\alpha]_D^{5-7}$ (deg)	λ_c (mμ)
Native enolase	5.7	4.5	82	6.1	90	−36	243
Enolase in 2 M ammonium sulfate	3.0[a]	5.0[a]	41[a]		103	−74	214
Enolase in the dioxane system	3.60		44	7.4	53	−55	214
Enolase in the acetone system	3.70		39.5				

[a] These data were obtained by extrapolation to infinite dilution.

ratio) with increased protein concentration, and also with the high relaxation time for enolase in ammonium sulfate in Figure 10.

These data thus support the proposal that the unfolded or solvated *n*-mer $P_n{}^*Mg_{nx/2}$ is the high molecular weight form of the enzyme in 2 M ammonium sulfate at high protein concentrations. The intermediate $P^*Mg_{x/2}$ is hypothetical and would according to the model never exist in any measurable concentration, so that the observed equilibrium in 2 M ammonium sulfate would be between $PMg_{x/2}$ (predominant at infinite protein dilution) and $P_n{}^*Mg_{nx/2}$ (predominant at high protein concentration).

Although consistent with this model, the activity measurements cannot be used in direct support. The results in Figure 11 clearly show a rapid loss of activity as the concentration of the salt is increased. It must be noted, however, that the salt inhibition may be completely unrelated to dissociation and affect only the catalytic function of the enzyme. Thus, 2 M magnesium sulfate, which did not cause dissociation, strongly inhibited the enzyme.

Recently Kirshner and Tanford (1964) have reported dissociation of hemoglobin by inorganic salts [NaCl, $MgCl_2$, and $(NH_4)_2SO_4$]. The sedimentation coefficient was found to decrease as the salt concentration was increased and was found to be concentration dependent just as has been observed with enolase. However, Kirshner and Tanford (1964) found that the molecular weight was the only factor which appreciably influenced the sedimentation coefficient. This does not seem to be the case with enolase. It is also difficult to explain the effect of ammonium sulfate on the sedimentation behavior of several other proteins without consideration of symmetry changes induced by the high salt concentration (Table V). A recent report by von Hippel and Wong (1964) that ammonium sulfate stabilizes the native conformation of several biopolymers as deduced from its effect on the helix-coil transition temperature of the polymers also implies a conformational difference between the protein in buffer and in high salt when compared at a given temperature. Direct evidence for structural changes induced by high salt concentration has recently been reported by Rupley (1964). He found that the properties of tyrosine residues in methemoglobin vary significantly in going from physiological conditions to concentrated salt solutions. The fact that the tyrosine ionization of methemoglobin crystals and methemoglobin in solution at the same (high) ionic strength were found to be identical furthermore indicates that the protein crystallized as the structurally altered form.

The relaxation times obtained from fluorescence polarization studies show that the molecular species in 2 M ammonium sulfate have lost molecular symmetry when compared to the dissociated species in the dioxane system. The fact that the sedimentation coefficient for the DNS–enolase in 2 M ammonium sulfate was 3.5 S (1 mg/ml), which is approximately 0.45 S higher than for native enolase at this concentration, may suggest that the presence of DNS induced a shift in the monomer–*n*-mer equilibrium toward $P_n{}^*Mg_{nx/2}$, thus giving the long relaxation times for DNS–enolase in 2 M ammonium sulfate.

The optical rotatory dispersion data support the other evidence in showing that the molecular species in the dioxane system (P′) and in 2 M ammonium sulfate ($PMg_{x/2} \rightleftharpoons P_n{}^*Mg_{nx/2}$) are different. By the criterion of increased negative rotation, both forms are denatured, showing less ordered structure than the native enzyme. Assuming ideal behavior in all the solvent systems, all the evidence is thus consistent with the proposed model, and it appears reasonable to consider the possibility that the form of enolase which crystallizes from ammonium sulfate solutions is the modified and inactive form $P_n{}^*Mg_{nx/2}$.

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