The Dissociation and Reconstitution of Aldolase*

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Rabbit muscle aldolase, which exists in solution as essentially globular, compact, highly organized particles of molecular weight 1.42×10^5, was readily dissociated into three polypeptide chains apparently without the rupture of covalent bonds. This dissociation was effected by treatment with any of the reagents, urea, HCl, acetic acid, or sodium dodecyl sulfate. Detailed studies of the subunits generated in 4 M urea solutions at *p*H 5.5 and in acid solutions (*p*H 2) showed that the polypeptide chains were markedly disorganized as coil-like particles of weight-average molecular weight 0.46×10^5. Upon dissociation and denaturation the reduced viscosity increased from 4.0 ml/g for the native enzyme to 23 ml/g at *p*H 2 and 18 ml/g in 4 M urea solutions. The optical rotation, $[\alpha]_D$, changed from $-23°$ for the native protein to $-62°$ and $-83°$ for the acid-dissociated and urea-dissociated subunits respectively. Similarly there was a marked change in the Drude constant, λ_c, from 283 mμ to 238 mμ and 223 mμ. Accompanying the change in the conformation of the polypeptide chains was a blue shift in the absorption spectrum with λ_{max} decreasing from 279.7 mμ for the native protein to 277.0 mμ for the subunits. Analyses of the shift by difference spectra revealed contributions from chromophoric groups of tyrosine, tryptophan, and phenylalanine due to the altered environment of these chromophores in the dissociated chains as compared to the intact enzyme. Titration of the sulfhydryl groups showed that the 16 groups which are "masked" in the native enzyme reacted readily in the dissociated subunits. No enzymic activity could be detected in the subunits in the presence of urea. However, activity was regained with yields of 65% when the dissociating agents (hydrogen ions or urea) were removed by dilution or dialysis. Reconstitution experiments were performed under different conditions; *p*H 5.5 and concentrations greater than about 50 μl/ml were found to be optimal for the restoration of activity. Kinetic studies showed that extensive annealing was unnecessary, for enzymic activity was regained in only a few minutes. The reconstituted protein was characterized by its reduced viscosity, sedimentation coefficient, molecular weight, optical rotatory dispersion, absorption spectrum, titratable sulfhydryl groups, and specific activity. In all respects the reconstituted macromolecules were virtually identical to the native enzyme.

Current research in molecular genetics and protein biosynthesis has stimulated renewed interest in investigations of the folding of disorganized polypeptide chains to produce biologically active protein molecules with unique three-dimensional conformations and specificities. According to present views (*e.g.* Lumry and Eyring, 1954; Pauling, 1958; Crick, 1958; Zamecnik, 1960; Vaughn and Steinberg, 1959; Berg, 1961) the secondary and tertiary structures of native protein molecules are the direct consequence of the sequential arrangement of amino acids in the polypeptide chains. Thus disorganized polypeptide chains produced by denaturation of proteins should be transformable readily into biologically active macromolecules having the architecture characteristic of the native proteins.

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The denaturation of proteins has been considered by many workers to be an irreversible process because attempts to convert denatured proteins into macromolecules having the original physical, chemical, and biological properties were usually unsuccessful and because the specific refolding of randomly coiled polypeptide chains seemed so improbable (see reviews by Neurath *et al.*, 1944; Anson, 1945; Putnam, 1953; Lumry and Eyring, 1954; Kauzmann, 1954, 1956, 1957). Soybean trypsin inhibitor (Kunitz, 1948) and chymotrypsinogen (Eisenberg and Schwert, 1951) appeared as notable exceptions, for the denatured, inactive proteins could be reactivated and restored to forms resembling the native proteins. Recent experiments with ribonuclease (White, 1961; Anfinsen *et al.*, 1961) have demonstrated that even after denaturation and the rupture of covalent (disulfide) bonds it is possible for the resulting polypeptide chains to regain their original physical, chemical, and enzymic properties when the denaturing agent was removed and the sulfhydryl groups were reoxidized to form disulfide bonds. Similarly, the biological activities and physical properties of lysozyme and taka-amylase

(Isemura *et al.*, 1961), after denaturation and reduction of their disulfide bonds, have been restored in large part upon renaturation and reoxidation.

Many multi-chain proteins and some viruses have been dissociated into subunits and then reconstituted, but as yet the evidence has not been sufficient to justify the conclusion that biological activity in these materials can be restored after the destruction of the secondary and tertiary structures of the subunits. Since it is evident that many enzymes contain more than one polypeptide chain held together largely, if not exclusively, by non-covalent bonds, it was of interest to determine whether this phenomenon of reversible denaturation, with its implications for protein biosynthesis, could be demonstrated for these complex proteins. This report deals with such an investigation on the enzyme, aldolase, a protein of molecular weight about 1.5×10^5 (Taylor and Lowry, 1956) having at least three C-terminal tryosyl residues (Kowalsky and Boyer, 1960), 28 sulfhydryl groups (Swenson and Boyer, 1957), and apparently one "active site" (Grazi *et al.*, 1962). Details are presented to show that it is possible to dissociate the enzyme into three subunits which by a variety of physical and chemical tests appear to be random polypeptide chains devoid of enzymic activity and then to reconstitute the macromolecules into an enzymically active form practically indistinguishable from the native enzyme (Stellwagen and Schachman, 1962; Deal and Van Holde, 1962).

Experimental

Enzymes.—Aldolase was prepared from rabbit muscle by the method of Taylor *et al.* (1948) and twice recrystallized from ammonium sulfate solutions. The enzyme was stored as a suspension in the crystallizing medium at 2° until used. Such preparations had specific activities of 49–56 units/mg when assayed by the method of Sibley and Lehninger (1949) as modified by Swenson and Boyer (1957).

A 10:1 mixture of α-glycerophosphate dehydrogenase–triosephosphate isomerase was purchased from C. F. Boehringer und Soehne through the facilities of the California Corporation for Biochemical Research.

Chemicals.—The barium salt of fructose 1,6-diphosphate (FDP), obtained from Schwarz Laboratories, was converted to the sodium salt by reaction with Dowex 50 (H^+) and subsequent neutralization with sodium hydroxide to obtain a *p*H of 7.4 (Swenson and Boyer, 1957). Phosphocellulose having a capacity of 0.84 meq/g was purchased from the California Corporation for Biochemical Research. Fisher reagent grade urea was twice recrystallized from 70% ethanol solutions by the method of Steinhardt (1938). Solutions of recrystallized urea were made immediately before use.

Aldolase Activity Assays.—Two kinetic assays were employed routinely for the determination of the catalytic activity of aldolase; both were based on the spectrophotometric detection of the triose phosphates produced by the cleavage of fructose 1,6-diphosphate. These changes in absorbance were measured with a Cary Model 11 recording spectrophotometer having a thermostated cell holder. All protein concentrations were determined by the absorbance at 280 mμ, A_{280} (A_{280}/0.91 equals mg aldolase per ml solution [Baranowski and Niederland, 1949; Szabolcsi and Biszku, 1961]).

One of the two kinetic assays was a modification of the hydrazine method described by Jagannathan *et al.* (1956). The reaction mixture in a cuvet with a 1-cm light path contained 7 μM of hydrazine, 12 μM of fructose 1,6-diphosphate, and 1.5 μM of EDTA, at *p*H 7.35 in a 3-ml volume. This solution was maintained at 30° in the spectrophotometer and the change in absorbance at 240 mμ was recorded for a period of 5 minutes. At the end of this period 2–10 μg of aldolase in 10–50 μl of 10^{-3} M EDTA at *p*H 7 were added and the change in absorbance at 240 mμ was again followed. The change in absorbance between the fifth and tenth minutes after addition of the enzyme minus the change noted for the initial 5-minute period in the absence of the enzyme was used to calculate the enzymic activity. A unit of activity was arbitrarily defined as a net change of 1.00 in the absorbance at 240 mμ. Specific activities of 80–100 units/mg were obtained routinely for the aldolase preparations when assayed by this procedure. For some experiments the assay mixture contained 0.05 M sodium phosphate buffer at *p*H 7.35. In this solution the specific activity was found to be 10% lower than in the standard mixture.

The splitting of fructose 1,6-diphosphate was also measured by the α-glycerophosphate dehydrogenase procedure described by Richards and Rutter (1961a). All solutions contained EDTA at a concentration of 10^{-3} M. A specific activity of 13.0 μM fructose 1,6-diphosphate cleaved/minute/mg protein was found for the aldolase preparation assayed by this procedure at 25°.

Sedimentation Studies.—Sedimentation velocity and sedimentation equilibrium experiments were performed in a Spinco Model E ultracentrifuge equipped with a phase plate as a schlieren diaphragm and a rotatable light source for Rayleigh optics. Double sector cells with a 12-mm optical path and sapphire windows were used routinely. Photographic plates (Spectroscopic II G) were analyzed with the aid of a Gaertner microcomparator.

All sedimentation velocity experiments were conducted at constant temperature (22°–25°) with rotor speeds of 59,780 rpm. The observed sedimentation coefficients were corrected to values corresponding to a solvent with the viscosity and density of water at 20° ($s_{20,w}$).

Sedimentation equilibrium experiments were performed with 4-mm liquid columns using Rayleigh optics, as described by Richards and Schachman (1959). The initial concentrations, c_0 in fringes, of the protein solutions were determined by either an experiment in a synthetic boundary cell or a preliminary sedimentation velocity experiment. For the latter the number of fringes observed was corrected for radial dilution to give the initial concentration. The rotor velocity was selected so that at equilibrium the protein concentration at the cell-bottom was 3 to 5 times the concentration at the air-liquid interface. The position of the zero order fringe in the equilibrium pattern was determined from the location of the white light fringe with the appropriate amount of 1,3-butanediol added to the solvent compartment or by calculations based on equations expressing the conservation of mass within the cell. The apparent weight average, M_w, molecular weight of the protein at various positions in the cell at equilibrium, was calculated from the slopes of log c vs. x^2 plots according to the equation

$$M_w = \frac{2RT}{(1 - \bar{V}\rho)\omega^2} \frac{d(\ln c)}{d(x^2)}.$$

The z average molecular weight, M_z, was calculated from the equation

$$M_z = \frac{M_b c_b - M_m c_m}{c_b - c_m}$$

where M_b and M_m were evaluated from the limiting slopes of $\ln c$ vs. x^2 at the cell bottom and meniscus, respectively. The weight average molecular weight for the entire contents of the cell was calculated from

$$M_w = \frac{2RT}{(1 - \bar{V}\rho)\omega^2(x_b^2 - x_m^2)} \frac{c_b - c_m}{c_0}.$$

In these equations R is the gas constant, T the absolute temperature, c the concentration in fringes, ω the angular velocity in radians/second, x the distance from the axis of rotation (in cm) with the subscripts, b and m, referring to the liquid bottom and meniscus, respectively, ρ the density of the solution, and $\bar{V}$ the partial specific volume of the protein. The value, 0.742 ml/g for $\bar{V}$ (Taylor and Lowry, 1956) was used in all calculations.

Viscosity Measurements.—Viscosity measurements were made in an Ostwald viscometer with an average shear gradient of 265 sec.$^{-1}$ and an outflow time of 108.4_1 seconds for 1 ml of water. Measurements were made at 25.35 ±0.005° and the outflow times were determined to ±0.03 seconds (Schachman, 1957). An average of four determinations was obtained for each sample; utflow times differed by no more than ±0.05 seconds. All viscosity results are reported as reduced viscosities, η_{sp}/c, where η_{sp} is the specific viscosity and c is the protein concentration in g/ml.

Optical Rotation Measurements.—All rotations were measured with a Rudolph polarimeter coupled to a Beckman monochromator equipped with a tungsten light source. The analyzer prism was driven by an automatic electromechanical null detection system built by Mr. Robert Johnson of this laboratory. Rotations were measured at selected intervals over the range 350–680 mμ in 1 or 2 dm polarimeter cells. Successive determinations differed by no more than ±0.006°. The observed optical rotations were corrected for the rotation and the refractive index of the solvent. The rotatory dispersion data were plotted according to the simple Drude equation as $-1/[\alpha]_\lambda$ *vs.* λ^2 or as $[\alpha]_\lambda\lambda^2$ *vs.* $[\alpha]_\lambda$ where $[\alpha]_\lambda$ is the rotation at the wave length, λ. The Drude constants, λ_c, were evaluated from the intercepts of the first type of plot and from the slopes of the latter.

Difference Spectra Titration.—The *p*H of the titrated aldolase solution was measured at appropriate intervals with Radiometer microcalomel and glass electrodes coupled to a Radiometer Type TTTlc *p*H meter with a scale expander. The temperature of the jacketed titration vessel was maintained at 25° by circulation of water from a Haake constant temperature bath. The aldolase solution was agitated by a glass paddle stirrer at speeds sufficiently low that no surface denaturation occurred. In order to vary the *p*H, 1 M HCl was added from an Agla microsyringe by means of a glass capillary. The volume of acid added was measured by a micrometer attached to the plunger of the syringe. Aliquots from the titration vessel immediately were placed in a glass-capped 4-ml quartz cuvet with a light path of 1 cm and the spectrum was scanned from 320–250 mμ in a Cary Model 11 Spectrophotometer. Corrections in the recorded absorbancies were made for the dilution of the aldolase solution by the addition of HCl and for the scattering due to the turbidity which appeared at intermediate *p*H values. The scattering was corrected for by assuming a linear dependence of turbidity on wave length in the region between 320 and 250 mμ.

Sulfhydryl Titration.—Mercaptide formation with *p*-mercuribenzoate (Sigma Chemical Company) was measured by the spectrophotometric method of Boyer (1954). The protein and *p*-mercuribenzoate were allowed to react 90 minutes prior to the determination of the absorbance at 250 mμ.

Acid Dissociation.—For the preparation of acid-dissociated aldolase, 1 M HCl was added rapidly to aldolase in 0.05–0.10 M NaCl solutions to obtain a *p*H of 2.0. These acidified solutions were allowed to stand for at least 15 minutes at room temperature prior to an examination of their physical properties or their ability to regain enzymic activity.

Urea Dissociation.—Aldolase was dissociated in 4 M urea by mixing equal volumes of a solution

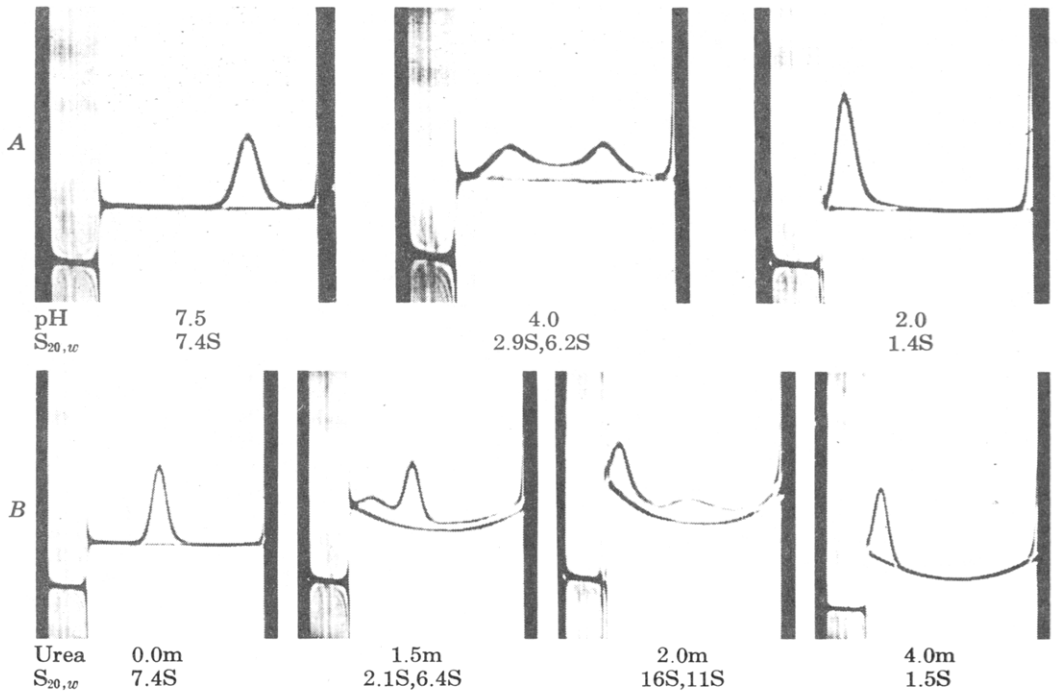

FIG. 1.—Sedimentation velocity patterns for aldolase solutions in the presence of increasing concentrations of acid (*A*) and urea (*B*). *A*, solutions contained aldolase at 7.5 mg/ml. All pictures were taken 60 minutes after reaching a speed of 59,780 rpm at angles of the schlieren diaphragm of 50–60°. Reading from left to right the media contained: 0.04 M NaCl and 0.01 M Tris buffer at *p*H 7.5; 0.01 M acetate buffer at *p*H 4.0; and 0.05 M NaCl and 0.01 M HCl. *B*, solutions contained 9.1 mg/ml of aldolase in 0.2 M NaCl, 0.05 M acetate buffer at *p*H 5.0. The patterns were obtained 30–60 minutes after attaining a speed of 59,780 rpm at angles of 50–70° for the schlieren diaphragm.

of 8 M urea, 0.4 M NaCl, 0.1 M NaOAc at *p*H 5.5, and a solution of aldolase in 10^{-3} M EDTA. This mixture was maintained at room temperature for at least one hour.

Aldolase was dissociated in 8 M urea by dissolving sufficient amounts of urea in a mixture of aldolase and concentrated salt solutions to give a final solution of 8 M urea, 0.2 M NaCl, and 0.05 M NaOAc at *p*H 5.5. These solutions likewise were allowed to stand at room temperature for at least one hour.

RESULTS

Dissociation into Subunits.—As shown in Figure 1, the addition of acid or urea to a solution of aldolase caused a progressive and marked change in the sedimentation velocity pattern of the native protein. The disappearance of material from the boundary corresponding to native aldolase was accompanied by the formation of material sedimenting more slowly.

In the acid series, Figure 1A, single symmetrical boundaries were observed at both *p*H 7.5 and 2.0, with sedimentation coefficients of 7.4 *S* and 1.4 *S* respectively. At *p*H 4.0, however, two diffuse boundaries were detected. Although the sedimentation coefficients corresponding to these boundaries were markedly different, 2.9 *S* and 6.2 *S*, the pattern did not resolve completely into two distinct boundaries as would be expected for a mixture of non-interacting components with those sedimentation coefficients. The experiments illustrated by Figure 1A were limited to solutions of ionic strength of 0.1 or less. Increasing the salt concentration led to diffuse boundaries and aggregation of the protein.

The sedimentation patterns for aldolase in urea solutions are shown in Figure 1B. These experiments were performed at *p*H 5.0 in order to minimize oxidation of the sulfhydryl groups. In the absence of urea the pattern showed a single symmetrical boundary (7.4 *S*). Similarly, a single component (1.5 *S*) was observed in 4 M urea. At intermediate urea concentrations, 1.5 M and 2.0 M, the pattern was characteristic of an interacting system. In 2.0 M urea, the system showed a marked tendency toward aggregation with a considerable fraction of the protein having a sedimentation coefficient of 11 *S*. The transformation caused by urea at a concentration of 4 M appeared to be complete, for neither the shape of the boundary nor its sedimentation coefficient was altered when the urea concentration was increased to 8 M.

The changes in the ultracentrifuge pattern illustrated in Figure 1 were also observed in

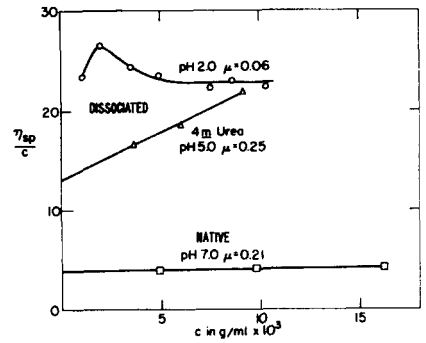

FIG. 2.—The reduced viscosity of native and dissociated aldolase. The reduced viscosity, η_{sp}/c, on the ordinate has the units ml/g, and the concentration is in g/ml. Results with native aldolase in 0.2 M NaCl–0.01 M phosphate buffer, *p*H 7, represented by □; results for acid-dissociated aldolase in 0.05 M NaCl–0.01 M HCl, *p*H 2, shown by O; results for urea-dissociated aldolase in 4 M urea, 0.2 M NaCl–0.05 M acetate buffer, *p*H 5, illustrated by △.

sedimentation experiments on aldolase solutions containing various concentrations of sodium dodecyl sulfate (Ramel *et al.*, 1961). At a detergent concentration of 10 mg/ml the ultracentrifuge pattern revealed only a single component (2.2 *S*). Similarly, the addition of acetic acid (0.83 M) caused the principal component (7.4 *S*) to disappear with the formation of a single component (1.9 *S*).

Since a decrease in sedimentation coefficient can be attributed to a dissociation of macromolecules into compact subunits, to an increase in the frictional coefficient resulting from swelling or elongation of the particles, or to combinations of dissociation and disorganization, viscosity studies were also performed. Figure 2 gives the results of these experiments for solutions of aldolase at *p*H 7, *p*H 2, and in the presence of 4 M urea at *p*H 5. At neutral *p*H the reduced viscosity of aldolase was 4.0 ml/g, a value found for many compact, globular proteins (Tanford, 1958). Lowering the *p*H to 2 or adding urea at *p*H 5 caused a large increase in the reduced viscosity and in its dependence on concentration. Not only was the intrinsic viscosity, *i.e.* the reduced viscosity at infinite dilution, markedly increased in 4 M urea, but also the slope of the plot of η_{sp}/c *vs.* c was elevated. In acid solutions, a maximum was observed in the plot of η_{sp}/c *vs.* c.

These drastic alterations in the hydrodynamic behavior of aldolase upon treatment with acid or urea were also shown by the marked increase in the concentration dependence of the sedimentation coefficient (Schachman, 1959). For native aldolase the sedimentation coefficient was found to vary as a function of concentration according to the relation, $s_{20,w} = 7.9\ (1 - 0.006\ c)$ where c is in mg/ml. The results for the urea-dissociated material were found to fit the relation $s_{20,w} = 1.6\ (1 - 0.015\ c)$; for the acid-dissociated material the best fitting linear relation was $s_{20,w} = 2.0\ (1 - 0.039\ c)$.

Although molecular weights can be calculated, in principle, from combinations of sedimentation coefficients and intrinsic viscosities, such determinations are dependent on the assumption of a hydrodynamic model. In order to eliminate such uncertainties sedimentation equilibrium experiments were performed. Figure 3 shows plots of log c *vs.* x^2 for aldolase at *p*H 7, *p*H 2, and in the presence of 6 M urea. For homogeneous material such plots give straight lines (Svedberg and Pedersen, 1940). Indeed, the experimental data for aldolase in neutral solution could be described precisely by a straight line and the molecular weight[1] calculated from the slope of the line was 1.42×10^5. In acid solution at a concentration of 2.5 mg/ml the weight average molecular weight was 0.46×10^5, a value one-third the molecular weight of the enzymically active protein. As seen in Figure 3B the plot was not straight, indicating heterogeneity with respect to molecular weight. At *p*H 2 the polypeptide chains are highly charged. Consequently, the ionic strength employed here (0.06) may not have been sufficient to eliminate electrostatic effects, which would lead to a measured molecular weight less than the true value (Svedberg and Pedersen, 1940). Evidence for this was provided by another determination at a higher concentration, 5 mg/ml, which yielded a lower value of 0.42×10^5.

In urea solutions at a protein concentration of 5 mg/ml the apparent weight average molecular weight, 0.37×10^5 (Fig. 3C), was about one-fourth that of native aldolase. However, a determination at a lower concentration of protein (2.5 mg/ml) gave a value of 0.42×10^5, and it is likely that the value at infinite dilution of protein would be even higher. No corrections were made for possible preferential interactions between the protein and either water or urea (Schachman, 1960).

Taken together the hydrodynamic and thermodynamic studies strongly indicate that aldolase is dissociated into three subunits upon acidification or exposure to concentrated urea solutions.

Properties of the Dissociated Polypeptide Chains.—The marked disorganization of the macromolecular structure of aldolase in solutions of low *p*H or in a medium containing high concentrations of urea, as evidenced by the viscosity and sedimentation velocity studies, was also manifested in the alteration of other physical and chemical properties. Figure 4 shows optical rotatory dispersion data for both native aldolase and the subunits in acid and in urea. The value, −23°, for $[\alpha]_D$ is in agreement with that obtained by others (Jirgensons, 1959; Drechsler *et al.*, 1959) for native aldolase and is in the range observed for many compact, globular proteins (Schellman

[1] This experiment was performed by E. Glen Richards, and the authors are indebted to him for these results.

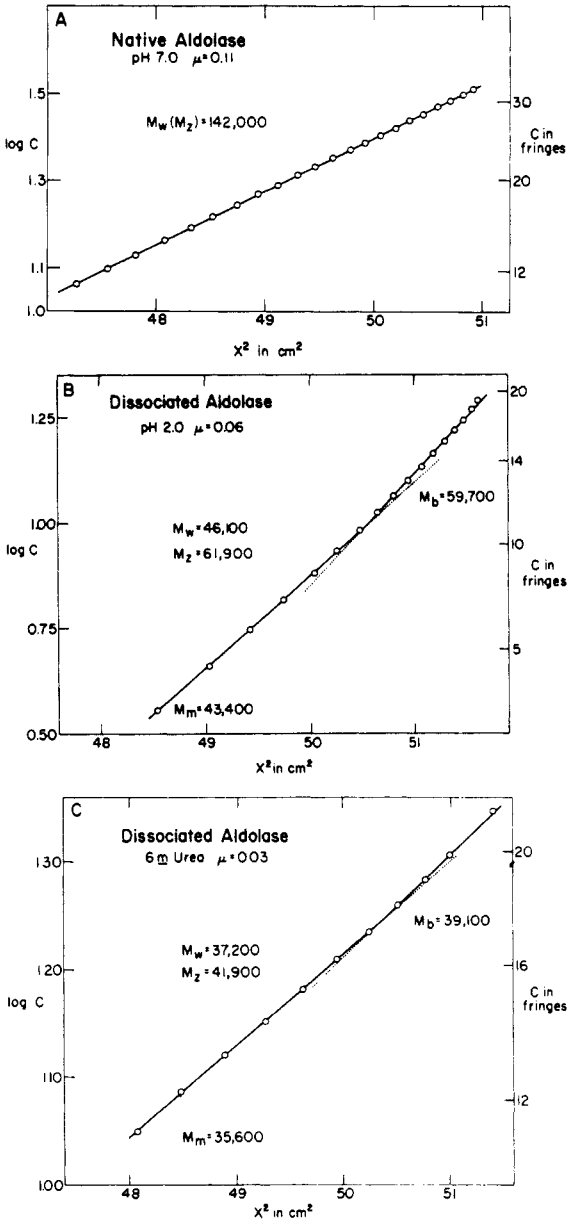

FIG. 3.—Molecular weight determinations from sedimentation equilibrium experiments on native and dissociated aldolase. The left ordinate gives the logarithm of the protein concentration in fringes and the abscissa represents the square of the distance (cm^2) from the axis of rotation. The right ordinate gives the protein concentration directly in fringes (40 fringes ≅ 10 mg/ml). *A*, native aldolase at 5 mg/ml in 0.05 M phosphate buffer *p*H 7.0. The speed was 5,784 rpm and the time of sedimentation was 21.5 hours. *B*, aldolase at 2.5 mg/ml in 0.05 M NaCl–0.01 M HCl, *p*H 2.0, was sedimented at 14,290 rpm for 24 hours. *C*, aldolase at 4 mg/ml in 6 M urea and 0.05 M imidazole buffer, *p*H 6.2, was sedimented for 22 hours at 11,573 rpm. The terms M_m, M_b, M_w, and M_z are described in the experimental section.

and Schellman, 1961; Jirgensons, 1961a). As with other proteins, upon denaturation the

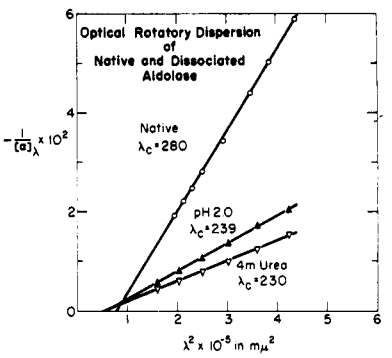

FIG. 4.—The optical rotatory dispersion of native and dissociated aldolase. Results with native aldolase, 8.4 mg/ml, in 0.2 M NaCl–0.01 M phosphate buffer at *p*H 7, represented by O; results for acid-dissociated aldolase, 5.4 mg/ml, in 0.05 M NaCl–0.01 M HCl at *p*H 2, shown by ▲; results with urea-dissociated aldolase, 5.2 mg/ml, in 4 M urea, 0.2 M NaCl–0.05 M acetate buffer at *p*H 5 illustrated by ▽. All measurements were made at 26°.

levorotation increased to −62° at *p*H 2 and −83° in 4 M urea. Similarly, the Drude constant changed from 283 mμ for the native enzyme to 238 mμ and 223 mμ for aldolase at *p*H 2 (*cf.* Jirgensons, 1961b) and in 4 M urea, respectively. According to current views, these results show that the polypeptide chains had undergone a marked conformational change from an organized structure to one with greater randomness.

The environment of the chromophoric groups in the protein also is altered as a result of treatment with hydrogen ions or urea. Difference spectra for both the acid and urea-dissociated aldolase compared to native aldolase as a reference solution are shown in Figure 5A. A similar acid difference spectrum has been reported previously (Drechsler *et al.*, 1959). In these difference spectra maxima occurred at 279 and 287 mμ, which are normally associated with tyrosyl residues; at 291 mμ, attributable generally to tryptophanyl residues; and at 265 and 269 mμ, corresponding to phenylalanyl residues. Apparently all of the chromophores of these residues have an altered environment in the dissociated chains as compared to the intact enzyme. The greater difference extinction coefficients observed for the acid-dissociated subunits result presumably from charge effects on the chromophoric groups. The change in the environment of the tyrosyl residues as a function of *p*H is shown in Figure 5B. At *p*H 2 the transition from the native structure to the disorganized subunits is complete, and the midpoint of the titration curve is about *p*H 4.2.

Swenson and Boyer (1957) have found that 11 sulfhydryl groups were titratable with *p*-mercuribenzoate in native aldolase and that an additional 16 groups[2] could be titrated in the presence of 6 M urea. This has been confirmed in the present

[2] This value has been corrected to correspond to a molecular weight of 1.42×10^5.

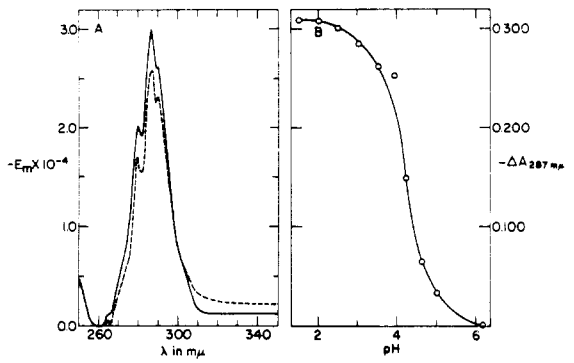

FIG. 5.—*A*, difference spectra of aldolase. The ordinate, E_M, represents the molar extinction coefficient of the difference spectra with a molecular weight of 1.42×10^5. ———, Acid difference spectrum. An aldolase solution containing 2.0 mg/ml in 0.05 M NaCl–0.01 M HCl at *p*H 2.0 was compared with a reference solution of aldolase at *p*H 6.1 in 0.05 M NaCl. – – – –, Urea difference spectrum. An aldolase solution containing 2.1 mg/ml in 4 M urea, 0.2 M NaCl, and 0.05 M acetate buffer at *p*H 5.0 was compared with a reference solution of aldolase in the same solvent without urea. Corrections were made for the absorbance of the urea. *B*, acid difference spectrum titration. The ordinate, ΔA_{287}, represents the difference in absorbance between the solution at a specific *p*H as compared to the control at *p*H 6.2. The reference cell contained aldolase at 1.9 mg/ml in 0.1 M NaCl at *p*H 6.2. To the other cell containing aldolase at 1.9 mg/ml in 0.1 M NaCl were added small increments of 1 M HCl to give the *p*H values indicated. The observed ΔA_{287} after each addition of acid was corrected for dilution of the aldolase solution. The maximum dilution was 2%.

studies, as shown in Table I, which summarizes the physical and chemical data for the intact enzyme and the polypeptide chains dissociated in acid and urea. Table I also contains data for the enzymic activity of aldolase in the absence and presence of urea (4 M). In agreement with the results of Swenson and Boyer (1957), it was found that the activity was destroyed in urea solutions. It should be noted that urea does not interfere with the reaction between hydrazine and dihydroxyacetone phosphate, one of the products of the enzymically catalyzed splitting of fructose 1,6-diphosphate. Therefore, it can be concluded that the enzyme is inactivated by treatment with urea. A comparable demonstration of the inactivation of the enzyme in acid solutions was not feasible because of the lack of suitable assay procedures at low *p*H values.

Reconstitution of Enzymically Active Material.—Initial attempts to regenerate enzymic activity from acid-dissociated aldolase solutions involved direct neutralization of concentrated solutions (5 mg/ml). This led invariably to aggregation of the polypeptide chains to form turbid solutions containing rapidly sedimenting material with little or no enzymic activity. In order to reduce this random aggregation of the dissociated polypeptide chains an alternative procedure was adopted. The dissociating agent (hydrogen ions) was effectively removed by rapid dilution of the dissociated aldolase (*p*H 2) into buffers of the desired *p*H. As seen in Figure 6, 65% of the initial enzymic activity was regained by this means. From studies with buffers of different *p*H values it was found that restoration of enzymic activity proceeded optimally at *p*H 5.5 Changing the salt concentration from 0.05 to either lower or higher values had little influence on the yield of enzymic activity (Table II). Similarly

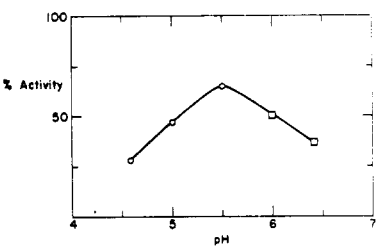

FIG. 6.—Effect of *p*H on the recovery of enzymic activity. The ordinate represents the total yield (in per cent of the original activity) after reconstitution of acid-dissociated aldolase. The abscissa gives the *p*H of the solution into which the acid-dissociated aldolase was diluted. Aliquots (100 μl) of aldolase, 5 mg/ml, in 0.05 M NaCl at *p*H 2, were diluted rapidly to 5 ml with 0.05 M acetate or phosphate buffer at 25°. After 15 minutes, aliquots of the diluted solution were assayed for enzymic activity by the hydrazine method. O, acetate buffer; □, phosphate buffer.

TABLE I

PHYSICAL AND CHEMICAL PROPERTIES OF NATIVE AND DISSOCIATED ALDOLASE

Sample	$s^{\circ}_{20,w}$ (S)	η_{sp}/c^{a} (ml/g)	M.W.[b] (× 10^{-5})	Sp. Act. (Units/mg)		SH (mole^{-1})	λ_{max}[c] (mμ)	$[\alpha]_D^{25°}$ (deg.)	λ_c[f] (mμ)
Native	7.9	4	1.42	73[e]	13[d]	10.6	279.7	−23	283
Acid dissociated	2.0	23	0.46	—	—	—	277.0	−62	238
Urea dissociated	1.6	18	0.42	<1	—	27.1	277.0	−83	223

[a] Results are given for solutions at a concentration of 5×10^{-3} g/ml rather than at infinite dilution because of the uncertain extrapolation of the data for acid-dissociated aldolase. [b] These are apparent weight average molecular weights obtained from sedimentation equilibrium experiments. The concentrations were 5.0, 2.5 and 2.5 mg/ml for the native, acid-dissociated, and urea-dissociated aldolase respectively. [c] Hydrazine assay. [d] α-Glycerophosphate dehydrogenase assay. One unit is 1 μM fructose 1,6-diphosphate cleaved/minute. [e] These are the maxima in the absorption spectra. [f] These were evaluated from plots of $[\alpha]_\lambda \lambda^2$ *vs.* $[\alpha]_\lambda$.

TABLE II

EFFECT OF VARIOUS REAGENTS ON RESTORATION OF ENZYMIC ACTIVITY[a]

Acid-Dissociated Aldolase

Reconstitution Medium	Yield of Enzymic Activity (%)
0.01 M Acetate, *p*H 5.5	72
0.025 M Acetate, *p*H 5.5	79
0.05 M Acetate, *p*H 5.5	65
0.05 M Acetate, *p*H 5.5	62[b]
0.1 M Acetate, *p*H 5.5	66
0.03 M Succinate, *p*H 5.5	73
0.03 M Pyrophosphate, *p*H 5.6	8
0.05 M Phosphate, *p*H 6.0	50
0.05 M Histidine, *p*H 5.6	56
0.006 M Fructose 1,6-diphosphate, 0.05 M Acetate, *p*H 5.5	61
0.1 M Mercaptoethanol, 0.05 M Acetate, *p*H 5.5	69

Urea-Dissociated Aldolase

Dissociation Medium	Reconstitution Medium	Yield of Enzymic Activity (%)
4 M Urea	0.05 M Acetate, *p*H 5.5	62
4 M Urea	0.05 M Acetate, *p*H 5.5	63[c]
8 M Urea	0.05 M Acetate, *p*H 5.5	60
8 M Urea	0.05 M Acetate, *p*H 5.5	38[b]

Acetic Acid–Dissociated Aldolase

Dissociation Medium	Reconstitution Medium	Yield of Enzymic Activity (%)
0.83 M Acetic acid	0.05 M Acetate, *p*H 5.0	63

[a] Solutions were maintained in the dissociation media for at least 15 minutes for acid-dissociated aldolase and 60 minutes for the urea-dissociated material. Dissociation was carried out at 25° and at a concentration of 5 mg/ml. Reconstitution was effected by dilution to give a concentration of 100 μg/ml in the media listed. Solutions were then maintained with stirring at 25° for 15 minutes prior to assaying for enzymic activity by the hydrazine procedure. [b] These samples were maintained in the dissociating media, acid or 8 M urea, for 8 hours at about 24° prior to reconstitution. [c] Reconstitution was effected by dialysis of the urea-dissociated aldolase (5 mg/ml) against the buffer indicated. The dialysis was performed at 2° for 12 hours.

the nature of the buffer ions, with the exception of pyrophosphate, as seen in Table II, proved to be without marked influence; 60–70% of the enzymic activity was restored whenever *p*H 5.5 solutions were used as the diluting medium. The addition of the substrate, fructose 1,6-diphosphate, had no effect on the yield, nor did the presence of 2-mercaptoethanol at *p*H 5.5. Even for acid-dissociated aldolase maintained at *p*H 2 and 25° for 8 hours, the yield of enzymic activity was 62%.

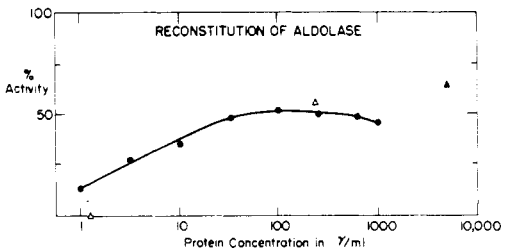

FIG. 7.—Effect of protein concentration on the recovery of enzymic activity. The ordinate gives the total yield (in per cent of the original activity) after reconstitution at the concentrations given on the abscissa. A solution of aldolase containing 5 mg/ml in 0.05 M NaCl was titrated by addition of 1 M HCl to *p*H 2.0. ●, activity recovered after rapid dilution of aliquots of dissociated aldolase with appropriate volumes of 0.05 M acetate buffer at *p*H 5.5. The reconstitution was done at 25°. ▲, activity recovered after dialysis of acid-dissociated aldolase against 0.05 M acetate buffer at *p*H 5.5 for 12 hours at 2°. Data designated by Δ were obtained by the dialysis procedure. The solutions of acid-dissociated aldolase were first diluted to the concentrations indicated with 0.05 M NaCl–0.01 M HCl and then dialyzed against 0.05 M acetate buffer at *p*H 5.5. Enzymic activity was measured by the α-glycerophosphate dehydrogenase assay.

Since the restoration of enzymic activity involved presumably the association of three chains through a series of concentration-dependent reactions, the effect of concentration during reconstitution was investigated. Figure 7 shows the results obtained when the *p*H of acid-dissociated aldolase solutions was raised from 2.0 to 5.5. Most of the data were obtained by rapid dilution of the dissociated subunits into acetate buffer at *p*H 5.5. Some of the experiments were performed by dialyzing the acid-dissociated material against the acetate buffer. The latter procedure, in contrast to that involving direct neutralization, produced marked restoration of activity (65%) even when the concentration was as high as 5 mg/ml. At low concentrations (below 40 μg/ml) the recovery of enzymic activity was substantially less than that obtained when the protein concentration during reconstitution was 100 μg/ml or greater. It should be noted that native aldolase, itself, becomes inactivated when the enzyme concentration is 10 μg/ml or less. This latter inactivation can be prevented by the addition of bovine serum albumin to the dilute enzyme solutions (Richards and Rutter, 1961b). No studies were conducted on the effect of an inert protein during the reconstitution process.

Enzymic activity was also recovered from urea-dissociated aldolase, as shown in Table II. It should be noted that comparable yields were obtained by both the dilution and dialysis procedures. Swenson and Boyer (1957) had found previously that solutions of aldolase in 4 M urea (devoid of activity) regained activity upon dilution to 0.08 M urea at *p*H 7.2. However,

they found no restoration of activity if the aldolase was exposed first to urea at concentrations of 5.6 M or higher. In contrast, the results presented in Table II show that 60% of the enzymic activity was recovered even from aldolase dissociated and denatured in 8 M urea solutions. However, prolonged exposure (8 hours) of the polypeptide chains to 8 M urea solutions led to a marked diminution in the recovery of enzymic activity, for only 38% of the activity was regained upon dilution.

Preliminary kinetic studies of the restoration of activity are shown in Figure 8. The kinetics

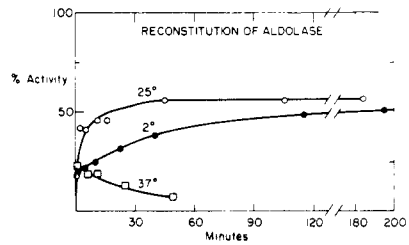

FIG. 8.—Kinetics of the recovery of enzymic activity. The ordinate gives the total yield (in per cent of the original activity) after reconstitution and the abscissa gives the time. Aldolase at 5 mg/ml in 0.05 M NaCl was dissociated by the addition of 1 M HCl to *p*H 2. Aliquots (100 μl) of this solution were diluted to 5.0 ml with 0.05 M acetate buffer at *p*H 5.5 at time zero at the indicated temperatures and maintained at these temperatures for the duration of the reconstitution. At appropriate intervals 25 μl samples of the diluted protein solutions were removed and assayed by the α-glycerophosphate dehydrogenase procedure. The assay was conducted at 25°, and the reaction time during the assay was 3 minutes.

were identical, at 25°, for aldolase which had been dissociated by acid, 4 M urea, or 8 M urea. The maximum activity recovered (60%) remained constant for at least 5 hours at 25° or several days at 2°, indicating that the recovered enzymically active product was stable. Enzymic activity was regained rapidly, with the rate at 25° substantially greater than that at 2°. At 37° there was a rapid initial restoration of enzymic activity followed by inactivation.[3]

All attempts thus far to regenerate aldolase activity from material which was dissociated in sodium dodecyl sulfate solutions have been unsuccessful. The dissociated subunits produced by acetic acid, however, were reconstituted to active enzyme with a yield of 63%, as shown in Table II.

The Nature of the Reconstituted Enzyme.—In order to characterize the enzymically active product by physical chemical means relatively concentrated solutions were required. These were obtained from the reconstitution experiments involving large dilutions by selectively removing the active product by adsorption on phosphocellulose followed by elution with a buffer of high ionic strength. The material recovered in this manner sedimented as a single component in the ultracentrifuge (Fig. 9D) with a sedimentation coefficient of 7.5 *S*. For comparison the ultracentrifuge pattern for native aldolase (7.5 *S*) is shown in Figure 9A. The products obtained by dialysis were examined directly in the ultracentrifuge. Figures 9B and 9E show the patterns observed for the material reconstituted after urea and acid dissociation respectively. The major component in each pattern had the sedimentation coefficient characteristic of the native protein. Both preparations contained heterogeneous material which sedimented more rapidly than the principal component. When the *p*H of these solutions was raised from 5.5 to 7.5 the aggregated material was precipitated and the supernatant solutions when examined in the ultracentrifuge showed only a single, symmetrical boundary. The patterns for these proteins reconstituted from urea-dissociated and acid-dissociated aldolase are shown in Figures 9C and 9F respectively.

Detailed studies of physical and chemical properties as well as the specific activities of the reconstituted proteins are summarized in Table III. For comparison, the results obtained with the native enzyme are also included. Although the sedimentation coefficients of the reconstituted proteins are slightly greater than that for the native enzyme, the agreement is good. That the reconstituted protein exists as a compact macromolecule is shown by the low value of the reduced viscosity, 4.2 ml/g. The molecular weights, 1.5 –1.6 $\times$ 10^5, indicate that the reconstitution process involved the association of the three polypeptide chains produced during the treatment with urea or hydrogen ions. Although the restoration of total enzymic activity upon reconstitution was only about 65%, the protein had the same specific activity as native aldolase after the aggregated material seen in the ultracentrifuge patterns (Figs. 9B and 9E) was removed. In addition the 16 sulfhydryl groups which are masked in the native enzyme and made available by urea dissociation (presumably also by acid dissociation) were masked once again after reconstitution. The spectral properties of the reconstituted protein were identical to those of the native enzyme, since the blue shift accompanying the dissociation was completely reversed upon reconstitution. Finally, the conformation of the polypeptide chains in the reconstituted protein, as revealed by the optical rotatory dispersion data, appeared to be identical with that in the native enzyme.

DISCUSSION

The physical properties summarized in Table I show that the native protein exists in solution as compact, essentially globular particles of molecular weight 1.42 $\times$ 10^5 (*cf.*, Taylor and Lowry, 1956). The hydrodynamic data, when combined with the molecular weight from sedi-

[3] Under comparable conditions the native enzyme is stable.

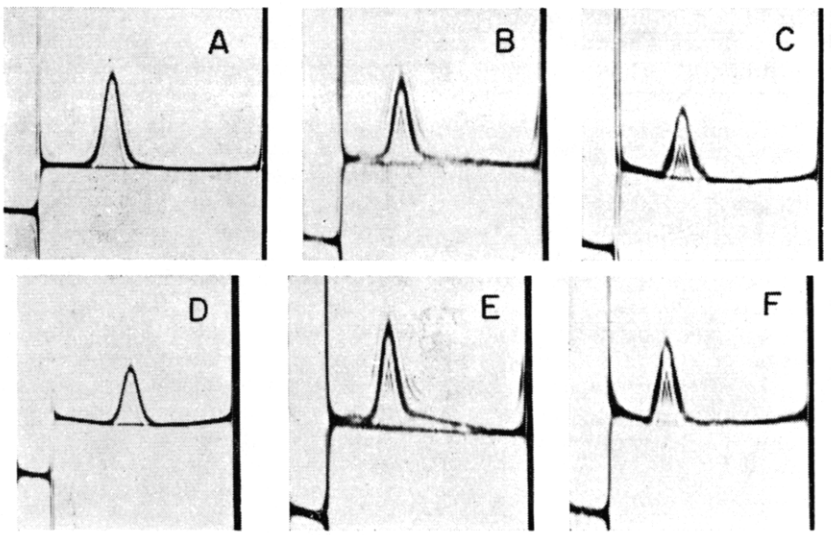

FIG. 9.—Sedimentation velocity patterns of native and dissociated aldolase. *A*, native aldolase at *p*H 7.5. *B*, aldolase reconstituted from 4 M urea by dialysis versus 0.05 M sodium acetate buffer at *p*H 5.5. *C*, supernatant solution obtained by raising the *p*H of *B* from 5.5 to 7.5. *D*, aldolase reconstituted from acid by dilution and subsequently purified on phosphocellulose. *E*, aldolase reconstituted from acid by dialysis versus 0.05 M sodium acetate buffer at *p*H 5.5. *F*, supernatant solution obtained by raising the *p*H of *E* from 5.5 to 7.5. Photographs were taken 24–40 minutes after attainment of 59,780 rpm at schlieren diaphragm angles of 50-65°.

TABLE III

PHYSICAL AND CHEMICAL PROPERTIES OF NATIVE AND RECONSTITUTED ALDOLASE

Sample[a]	$s_{20,w}$[b] (*S*)	η_{sp}/c (ml/g)	M.W.[b] ($\times 10^{-5}$)	Sp. Act. (U/mg)		SH (mole^{-1})	λ_{max}[f] (mμ)	$[\alpha]_D^{26°}$ (deg.)	λ_c[g] (mμ)
Native	7.6	4.0[c]	1.42	73[d]	13.0[e]	10.6	279.7	−23	283
Reconstituted from acid by dilution	7.5	4.2[c]	1.48	73	13.2	11.5	279.7	−22	290
Reconstituted from acid by dialysis	7.8	—	1.60	—	13.5	11.4	279.7	−21	292
Reconstituted from urea by dialysis	7.8	—	1.59	—	11.5	10.4	279.7	−20	287

[a] All physical chemical measurements were made on solutions of aldolase in 0.2 M NaCl–0.01 M phosphate buffer, *p*H 7. [b] The concentrations were 5.0, 4.0, 2.5, and 2.5 mg/ml respectively. [c] The concentrations were 4 mg/ml. [d] Hydrazine assay. [e] α-Glycerophosphate dehydrogenase assay. One unit is 1 μM fructose 1,6-diphosphate cleaved/minute. [f] These are the maxima in the absorption spectra. [g] These were evaluated from plots of $[\alpha]_\lambda\lambda^2$ *vs.* $[\alpha]_\lambda$.

mentation equilibrium, give $2.2_7 \times 10^6$ for the parameter, β, which describes the shape of the kinetic units in solution (Scheraga and Mandelkern, 1953). Although this value is slightly larger than the theoretical value for rigid, spherical particles, it should be noted that β is an insensitive function of particle shape and slight alterations in the experimental quantities lead to values of β corresponding to ellipsoidal particles of modest axial ratio.[4] Evidence for the compactness of the macromolecules in solution is provided by the low value, 3.8 ml/g, of the intrinsic viscosity. Calculation of the radius of the hydrodynamic units leads to 44 A, a value only 25% larger than that of solid, uniform spheres having the weight of aldolase molecules and density equal to the reciprocal of the partial specific volume.

Addition of hydrogen ions or urea to solutions of aldolase produces marked changes in both the hydrodynamic behavior and the molecular weight determined by sedimentation equilibrium. As yet the corrections for the non-ideality of the solutions at *p*H 2 and in 4 M urea solutions are not adequate. Nor has there been a conclusive evaluation of the possible complicating effects of preferential interactions in the solutions containing large amounts of urea (Schachman, 1960). Nonetheless it is unlikely that the corrections for these interactions will cause sufficient change in the value, 0.46×10^5, for the molecular weight

[4] The calculated value of β led to an axial ratio of 6 (for prolate ellipsoids of revolution), but no attempt has been made in this work to obtain the precision necessary for a detailed consideration of particle shape.

of the subunits to require modification of the conclusion that the native enzyme molecules are composed of three subunits. It is important to note that the studies of Kowalsky and Boyer (1960) on the action of carboxypeptidase on aldolase in H_2O^{18} showed that aldolase molecules contained at least three C-terminal tyrosines. Proline has been identified as an N-terminal amino acid in aldolase (Udenfriend and Velick, 1951). Only two N-terminal prolines per molecule of aldolase were found, but the reagent employed, *p*-iodophenylsulfonyl chloride, does not give quantitative results and this finding cannot be considered as being in conflict with the determination of the number of C-terminal amino acids. It can be concluded, therefore, from the physical and chemical data presented here and from the enzymic studies of Kowalsky and Boyer, that aldolase molecules are composed of three polypeptide chains folded into compact, relatively rigid particles.

Since such widely diverse and mild reagents as urea, hydrogen ons, acetic acid, and sodium dodecyl sulfate all caused the breakdown of the macromolecular structure into subunits, the three polypeptide chains in aldolase must be held together by secondary forces only (non-covalent bonds). The dissociation by acid can be attributed to the large intramolecular repulsive forces (Kauzmann, 1954) which develop as the net charge is increased due to the titration of the carboxyl groups. At an ionic strength of 0.05 the transition from the native to the dissociated structure, as revealed by both the sedimentation velocity studies (Fig. 1A) and the difference spectrum titration (Fig. 5B), occurred with a mid-point about *p*H 4. This *p*H corresponds to the *pK* normally assigned to carboxyl groups in proteins. When the electrostatic repulsion is partially damped at *p*H 2 in solutions containing NaCl at higher ionic strengths, aggregation occurs. Whether the disruptive effect of urea is the result of the breaking of intra- and inter-chain hydrophobic or hydrogen bonds is still uncertain (Kauzmann, 1954, 1959; Klotz, 1960; Bruning and Holtzer, 1961; Levy and Magoulas, 1962; Whitney and Tanford, 1962) despite its use as a denaturant for many years. Aldolase appears to be particularly sensitive to urea, for even 2.0 M urea caused a partial disorganization and dissociation. When the urea concentration was not sufficient to cause complete dissociation the partially disordered protein molecules aggregated (Fig. 1B) to particles larger than the native enzyme. Apparently the partial unfolding of the polypeptide chains made available interacting groups which, in the absence of sufficient urea, caused aggregation. At higher urea concentrations (4 M) these inter-chain interactions were reduced and the isolated, single polypeptide chains represented the stable conformation. In the dilute acetic acid solutions (0.83 M, *p*H 2.6) the dissociation is attributable to the rupture of hydrophobic bonds in addition to the intramolecular repulsive forces generated at the *p*H of the solutions. Sodium dodecyl sulfate, as found with many proteins (Putnam, 1948), was effective as a denaturant even at low concentrations. This can be attributed in part to the repulsive forces produced, as the net negative charge (even at *p*H 7) was increased owing to the binding of the anions and in part to the rupture of inter- and intra-chain hydrophobic bonds in the protein through interaction with the long hydrocarbon chain of the detergent.

Calculations of the shape and the effective volume of the dissociated polypeptide chains are not given here because of the lack of satisfactory corrections for the charge effects (in acid solutions) and for possible preferential interactions (in urea solutions). Nonetheless it is clear from the hydrodynamic properties of the subunits that they resemble disorganized, flexible, coil-like chains. The large reduced viscosity in urea (Fig. 2) is similar to that found for many proteins (Neurath *et al.*, 1942; Kauzmann, 1954). Further evidence is provided by the large dependence of sedimentation coefficient on concentration (Schachman, 1959). This is characteristic of random chain polymers in good solvents (Flory, 1953). The maximum in the reduced viscosity of the subunits in acid solutions (Fig. 2) is similar to that found for polyelectrolytes at low concentration (Fuoss and Strauss, 1949; Harrap and Woods, 1961) and is indicative of the deformability of the randomly coiled chains depending upon the local ionic environment.

As shown in Figures 3B and 3C the plots of log c *vs.* x^2 were curves upward, indicating that the dissociated material was heterogeneous with respect to molecular weight. Although this may be a reflection of chain length differences among the dissociated subunits, it is more likely that the polydispersity is the result of slight aggregation of the polypeptide chains. The finding of three C-terminal tyrosines (Kowalsky and Boyer, 1960) is consistent with the view that the three chains are identical. On the other hand, these workers found only two alanines released from the interior of the aldolase chains through the continued action of carboxypeptidase; this led them to suggest that alanine was the penultimate residue (at the C-terminal end) in at least one of the chains and that all three chains were not identical. Since there appears to be only one "active site" in the enzyme (Grazi *et al.*, 1962; Stellwagen and Schachman, in preparation), it is important to determine whether there are chemical differences among the polypeptide chains. Fractionation experiments, amino acid analyses, and spectrophotometric titration of abnormal tyrosyl residues (Stellwagen, Donovan, and Schachman, unpublished observations) thus far have not been definitive, partly because of the large molecular weight, and an answer as to the identity or non-identity of the three polypeptide chains must await the results of further research.

At intermediate stages of the dissociation of the

macromolecules in either acid or urea solutions the sedimentation velocity patterns (Fig. 1A, *p*H 4; Fig. 1B, 1.5 M and 2.0 M urea) exhibited some of the characteristics of interacting systems involving association-dissociation equilibria (Gilbert, 1955, 1959; Bethune and Kegeles, 1961). Until more data become available, however, an interpretation of the patterns in terms of these theories would be premature. The transition from the native to the disorganized structure upon the addition of excess dissociating agent (hydrogen ions or urea) was very rapid, but it has not been established that the interconversion from the native form to the dissociated subunits at some intermediate concentration of hydrogen ions (or urea) is equally rapid. If these interconversions, at *p*H 4 or 1.5 M urea for example, prove to have reaction times comparable to the duration of the sedimentation experiments, the aforementioned theories would be inapplicable and some modifications and extensions of the theoretical treatments of Scholten (1961) and Cann and Bailey (1961) must be applied. It is important to note that Swenson and Boyer (1957) found that some of the masked sulfhydryl groups reacted slowly with *p*-mercuribenzoate in dilute urea solutions while reacting extremely rapidly at higher urea concentrations. A detailed evaluation of these patterns would prove of considerable value in probing the details of the dissociation process. For example, the component with a sedimentation coefficient of 6.2 *S* may represent a partially disordered macromolecule possessing the same weight as the native enzyme. Alternatively the lowered sedimentation coefficient (6.2 *S* as compared to 7.4 *S*) may be the result of the interactions in the solution (Gilbert, 1959) or it may be caused, in part, by the viscosity contributed by the slow component. Similarly the 2.9 *S* boundary may represent a single chain structure that is relatively compact and not as disorganized as the 1.4 *S* component. However, the evidence as yet does not preclude the existence of a relatively stable two-chain structure.

Despite the apparent randomness of the three polypeptide chains in acid and urea solutions, the reconstitution produced macromolecules with physical, chemical, and enzymic properties almost identical to those of native aldolase (Table III). It could be argued, of course, that the dissociated chains were not completely disordered and that some local interactions were sufficently strong to withstand the denaturing action of the reagents described above. In the light of existing knowledge regarding non-covalent bonds in proteins as well as the diversity of the denaturing agents employed, this seems unlikely; but it must be recognized that the highly specific reconstitution process may have been initiated at a small region of the polypeptide chains in which some "ordered" structure persisted even though the bulk of the specific secondary and tertiary structures was destroyed. Although this reservation must be entertained it seems clear that, for aldolase at least, the native structure has the conformation of lowest free energy and that the refolding and association reactions are dictated by the type and sequential arrangement of the amino acids in the polypeptide chains.

The restoration of enzymic activity was accomplished generally with yields of 60–75%. Even for aldolase maintained at *p*H 2 for 8 hours, the recovery was 62%. Similarly, 60% of the initial activity was regained after total destruction of the active units by incubation of the enzyme in 8 M urea for one hour. Although the yields were high it is likely that, as yet, the optimal kinetic conditions such as temperature, *p*H, and ionic environment have not been found for the reconstitution reactions. It should be recognized also that the inability to achieve yields greater than about 75% may be the result of irreversible side-reactions occurring during the dissociation and denaturation of the native enzyme. Although 60% of the activity was regained after incubation of the enzyme in 8 M urea for one hour, prolonged exposure (8 hours) followed by reconstitution led to only 38% recovery. This lower yield may be attributable to carbamylation reactions from the cyanate formed in concentrated urea solutions (Stark *et al.*, 1960). Similarly, the interplay of competing reactions may be responsible for the observed effect of *p*H on the recovery of enzymic activity from acid-dissociated aldolase. At the lower *p*H values, dissociation is favored (the conformation of the protein changes markedly at *p*H 4); at the higher *p*H values some oxidation of the unmasked sulfhydryl groups may occur. The formation of disulfide bonds by oxidation of the exposed sulfhydryl groups also may have been responsible for the finding of Swenson and Boyer (1957) that no enzymic activity could be recovered from aldolase exposed to urea at concentrations above 5.6 M. It should be noted that in their experiments the dissociation and reconstitution were performed at *p*H 7.2–7.4, whereas a *p*H of 5.5 was employed in the work described here. Since oxidation of sulfhydryl groups is more likely to occur at the higher *p*H values, the lack of restoration of activity in the experiments at *p*H 7.4 may be attributable to the formation of disulfide bonds which interfered with the specific refolding of the three polypeptide chains to give active molecules. The inability to recover enzymic activity from the subunits produced by sodium dodecyl sulfate is probably due to the strongly bound detergent ions which interfere with reconstitution.

As seen by the rapidity of the restoration of activity (only a few minutes are required) extensive annealing is unnecessary. A preliminary analysis of the kinetics of the restoration of activity as well as the change in the difference spectrum during the reconstitution (Donovan, Stellwagen, and Schachman, unpublished observations) indicates that the process is first order with respect to concentration. If further experimentation supports this tentative conclusion, the

association process involving the three independent chains would be eliminated from consideration as the rate-determining step in the over-all reconstitution. A relatively slow conformational change within each subunit followed by a more rapid association process could satisfy first order kinetics. Alternatively, the final annealing of the structure after association of the three chains may be rate determining. It is of interest that similar kinetics have been obtained thus far for the creation of the "active site" and for the reestablishment of that environment of the chromophoric groups which was characteristic of the native enzyme. Further characterization of some of the components observed during the dissociation (Fig. 1A, 1B) along with kinetic measurements using other physical properties should provide the data necessary for a detailed understanding of the reconstitution process.

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Bovine Pancreatic Procarboxypeptidase B. I. Isolation, Properties, and Activation*

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A chromatographic procedure has been developed for the isolation in pure form of bovine procarboxypeptidase B. The purified zymogen is homogeneous, as judged by chromatography, sedimentation analysis, moving boundary electrophoresis, and potential enzymatic activity. The activation of the zymogen is mediated by trypsin and presumably occurs in two steps. An initial fast reaction leads to the appearance of 60–70% of the maximal activity without any significant change in the sedimentation coefficient of the protein. The second step occurs more slowly and leads to the formation of a protein with a lower sedimentation coefficient and full enzymatic activity. Crystalline carboxypeptidase B has been isolated from activation mixtures. This enzyme is active toward the basic substrate benzoylglycyl-L-arginine but also hydrolyzes substrates for carboxypeptidase A, such as hippurylphenyllactic acid and carbobenzoxyglycyl-L-phenylalanine. Evidence is presented that the activities toward basic and aromatic compounds reside in the same enzyme.

Aqueous extracts of acetone powder of bovine pancreas contain zymogens for several of the proteolytic enzymes known to occur in the exocrine secretions of the gland. The most acidic of the zymogens, and hence the one which emerges last when such extracts are subjected to chromatography on DEAE-cellulose, is procarboxypeptidase A (Keller *et al.*, 1958b). This protein has recently been shown to be a complex of three subunits (Brown *et al.*, 1961) and the precursor of two different enzymes, carboxypeptidase A and an endopeptidase similar in specificity to chymotrypsin (Keller *et al.*, 1958a).

This protein, however, is not the only potential source of carboxypeptidase A activity in aqueous extracts of pancreatic acetone powder. Another fraction, much less strongly absorbed onto DEAE-cellulose, also gives rise, after tryptic activation, to activities against the ester hippurylphenyllactic acid (Snoke and Neurath, 1949) and the peptide carbobenzoxyglycyl-L-phenylalanine (Hofmann and Bergmann, 1940), which are typical substrates of carboxypeptidase A. However, this protein fraction, which is the subject of the present report, also shows potential activity against benzoylglycyl-L-arginine and thus possesses also the specificity characteristics of procarboxypeptidase B (Folk, 1956). This pancreatic procarboxypeptidase has been isolated in pure form and characterized, and a crystalline carboxypeptidase has been prepared from the purified zymogen after tryptic activation. Evidence has also been obtained that the active enzyme possesses in fact dual specificity and hydrolyzes substrates for both carboxypeptidases A and B. Although this dual specificity has not been previously recognized, there are indications that the procarboxypeptidase described here is identical with the procarboxypeptidase B partially purified by Folk and Gladner (1958). For this reason, this proenzyme will be referred to as bovine pancreatic procarboxypeptidase B.

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[1] The following abbreviations are used here: DEAE = diethylaminoethyl-, Tris = tris(hydroxymethyl)aminomethane, DFP = diisopropylphosphofluoridate, HPLA = hippuryl-*d,l*-phenyllactic acid, BGA = benzoylglycyl-L-arginine, CGP = carbobenzoxyglycyl-L-phenylalanine.

Materials and Methods

Acetone powders, prepared from fresh bovine pancreas glands by the method described by Stein (1954) and by Keller *et al.* (1956), were obtained from Worthington Biochemical Corporation.

DEAE-cellulose[1] with a capacity of 0.9 meq per gram of resin was a reagent grade product