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Aldolase Dissociation into Subunits by Reaction with Succinic Anhydride

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When rabbit muscle aldolase (M_w = 142,000) is reacted with succinic anhydride under neutral conditions, the enzyme readily dissociates into three subunits having a molecular weight (M°_w) of 54,500 and a sedimentation coefficient ($s^\circ_{20,w}$) of 2.48 S. This dissociation is accompanied by a shift in the wavelength of maximum absorption from 279.9 to 276.5 mμ and a concomitant decrease in the extinction coefficient ($E^{0.1\%}_{1\ cm}$). Succinylation also produces a large increase in intrinsic viscosity, [η], from 4.04 to 12.80 ml/g and a significant increase in the frictional ratio, f/f_0, from 1.13 to 2.29. Pycnometric analyses at 20° reveal that succinyl aldolase in Tris-KCl buffer (*p*H 8.0, μ = 0.55) has an apparent specific volume of 0.704 ± 0.008 ml/g. Native aldolase in the same medium has an apparent specific volume of 0.745 ± 0.006 ml/g. When succinyl aldolase subunits are exposed to borate-KCl buffer (*p*H 12.5, μ = 0.52), further dissociation occurs and six polypeptides, having apparently equivalent molecular weights (M°_w = 27,000), are obtained per mole of enzyme. N-Terminal amino acid analyses by Edman degradation yield a minimum of four prolines per mole of native aldolase. Similar amino acid analyses on alkali-treated succinyl subunits show no N-terminal groups, indicating that the six subunits do not result from peptide bond cleavage.

Several reports concerning the structure of muscle aldolase indicate that the native molecule is composed of at least three subunits (Kowalsky and Boyer, 1960; Stellwagen and Schachman, 1962; Deal *et al.*, 1963). Recent investigations by Hass and Lewis (1963), however, show that after exposure to alkaline conditions above *p*H 12.0 the enzyme dissociates into six polypeptide chains having apparently equivalent molecular weights. This finding led to the conjecture that alkali might cause the cleavage of specific covalent bonds while promoting disaggregation of highly negatively charged molecules. Consequently, interest was developed in other modifications which might cause subunit formation through the production of negatively charged peptides under neutral conditions.

Maurer and Lebovitz (1956) and Habeeb *et al.* (1958) have shown that succinic anhydride readily reacts with proteins under relatively mild conditions. As a result, a high negative-charge density is imposed upon the molecule through the elimination of $-NH_3^+$ groups and the introduction of $-COO^-$ ions. When this occurs there is a considerable expansion of molecular structure and, as recently shown with hemerythrin (Klotz and Keresztes-Nagy, 1963), dissociation into subunits is possible.

This report is concerned with the dissociation of rabbit muscle aldolase by reaction with succinic anhydride. The resulting succinyl subunits have been examined at different *p*H values and ionic strengths, and several of their physical properties have been described. N-Terminal amino acid analyses have been performed on native and alkali-treated succinyl aldolase in an effort to establish the actual number of monomeric subunits comprising the native molecule.

Materials and Methods

Materials.—Twice-crystallized aldolase was prepared from rabbit muscle by the method of Taylor *et al.* (1948) as modified by Kowalsky and Boyer (1960). Large preparations of the enzyme were stored at 4° in 0.5 saturated ammonium sulfate. The concentration of dissolved aldolase was determined by absorption at 280 mμ using an extinction coefficient, $E^{0.1\%}_{1\ cm}$ = 0.91 (Baranowski and Niederland, 1949).

Succinic anhydride (mp 120°) was obtained from Eastman Kodak Distillation Products Industries, Rochester, N. Y., and was used without further purification.

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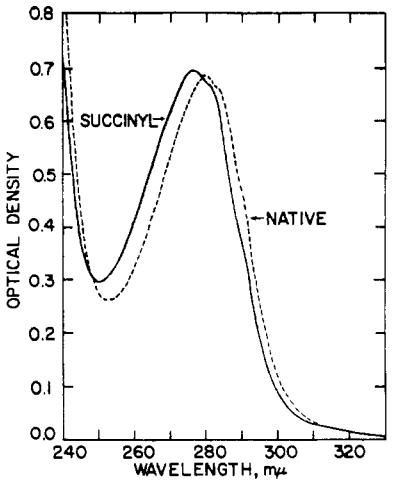

FIG. 1.—Absorption spectra of succinyl and native aldolase in 10^{-3} M PO_4 buffer, *p*H 8.0.

Succinylation Procedure.—Succinylation was performed at 23° between *p*H 7.5 and 8.0. Solid succinic anhydride was added gradually with stirring to a 4% solution of aldolase dissolved in 10^{-3} M potassium phosphate buffer, *p*H 8.0. Unless otherwise stated, a molar ratio of succinic anhydride per free amino group of 20:1 was used. The *p*H of the reaction mixture was automatically maintained by the gradual addition of 1.0 N KOH using a Radiometer autotitrator, Type TTT1. The reaction was terminated 15 minutes after the requirement for KOH had ceased and the *p*H remained constant. Following this, the reaction mixture was dialyzed exhaustively against either the desired buffer or distilled water. If distilled water was used, succinyl aldolase usually was lyophilized to dryness and was stored at 4° for later use.

Determination of the Extinction Coefficient of Succinyl Aldolase.—Succinylation of native aldolase by different molar excesses of succinic anhydride should have a negligible effect on the value of the extinction coefficient of the acylated enzyme provided the smallest amount of reagent used combines with most of the reactive protein groups. This was tested by preparing succinyl aldolase using molar ratios of succinic anhydride per free amino group of 20:1 (sample A) and 60:1 (sample B). These samples were exhaustively dialyzed against cold distilled water and lyophilized. A portion of the lyophilized samples was dried *in vacuo* at 100° overnight. After correcting for the ash content (constant weight at 800°), 16.7% (sample A) and 16.6% (sample B) Kjeldahl nitrogens were obtained. These values were used in calculating the protein concentrations of several aqueous solutions which were analyzed for milligrams of nitrogen (micro-Kjeldahl) after determining their absorption spectra between 240 and 340 mμ. Absorption spectra were obtained on a Cary Model 14 recording spectrophotometer after the establishment of a constant base line over the entire spectral range investigated. At λ_{max} (276.5 mμ), the average extinction coefficients for a 0.1% succinyl aldolase solution were 0.81 and 0.83 for samples (A) and (B), respectively. A value, $E^{0.1\%}_{1\ cm} = 0.82$ was used for all subsequent determinations regardless of the amount of succinic anhydride used.

Determination of Apparent Specific Volume.—The apparent specific volumes ($\bar{V}_{app}$) of native and succinyl aldolase were obtained from density determinations of protein solutions (approximately 0.7%) in 0.5 M KCl, 0.1 M Tris, and 0.01 M mercaptoethanol, *p*H 8.0. Densities were measured in a 6.0-ml pycnometer equilibrated in a constant temperature bath at 19.99° ± 0.01°. Bath temperature fluctuations were established using a Beckman differential thermometer. Three separate determinations were made on each protein solution and $\bar{V}_{app}$ was calculated from the following relationship (Schachman, 1957):

$$\bar{V}_{app} = 1/\rho_0 - 1/c(\rho/\rho_0 - 1)$$

where ρ_0 and ρ are the densities of the solvent and the solution, respectively, and c is the concentration in g/ml. The $\bar{V}_{20}$ obtained for native aldolase was 0.745 ± 0.006 ml/g, in good agreement with the value of 0.742 ml/g reported by Taylor *et al.* (1948). The $\bar{V}_{20}$ for succinyl aldolase was found to be 0.704 ± 0.008 ml/g. When the latter $\bar{V}$ was calculated (Cohn and Edsall, 1943a) from the amino acid composition of aldolase (Velick and Ronzoni, 1948) assuming complete succinylation of the 93 lysyl groups, a value of 0.734 ml/g was obtained. Since succinylation denatures aldolase, it is evident that the apparent specific volume of the unfolded protein must be lower than that of the native molecule. This observation is consistent with the findings by Linderstrøm-Lang (1949) for unfolded β-lactoglobulin, Harrington and Schellman (1956), and Holcomb and VanHolde (1962) for unfolded ribonuclease, and Kielley and Harrington (1960) for myosin denatured in 5.0 M guanidine·HCl.

Sedimentation Experiments.—Sedimentation equilibrium and velocity experiments were performed with a Spinco Model E analytical ultracentrifuge equipped with a phase-plate schlieren diaphragm, a Rayleigh interference optical system, and a rotor-temperature indicator and control unit. Photographic plates were measured with a Nikon Model 6 optical comparator equipped with Mann lead screws.

Sedimentation velocity experiments were performed at a constant temperature of 20° employing a rotor speed of 59,780 rpm. $s_{20,w}$ values were calculated and extrapolated to infinite dilution to obtain $s^\circ_{20,w}$.

Sedimentation equilibrium experiments were performed at 2° with 1.6 to 1.7-mm liquid columns using the technique described by Richards and Schachman (1959). Three samples were run simultaneously in a Spinco J rotor. Two 12-mm double-sector wedge-centerpiece cells were used with the more concentrated protein solutions and a 30-mm double-sector cell was used with the most dilute solution. Initial protein concentrations, c_0 in fringes, were determined in a synthetic-boundary cell. In all the experiments described equilibrium conditions were established by initially overspeeding the rotor to 17,250 rpm for 2 hours followed by gradual deceleration to 9945 rpm, and allowing the rotor to run at the latter speed for 18–20 hours. The apparent molecular weight of the protein was obtained from slopes of ln c vs. x^2 plots using the equation:

$$M_{app} = 2RT(d \ln c/dx^2)/(1 - \bar{V}\rho)\omega^2$$

If polydispersity was indicated by concave ln c vs. x^2 plots, the weight-average molecular weight over the entire liquid column was calculated from

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

In these equations M, R, T, $\bar{V}$, ρ, and ω have their usual designations (Schachman, 1959), x is the distance from the rotational axis, c_0 is the initial protein concentration, and c_m and c_b refer to the protein concentrations at the meniscus and bottom of the liquid column, respectively. Making the appropriate temperature corrections for 2°, a $\bar{V}$ of 0.695 ml/g was used for all molecular weight calculations involving succinyl aldolase (Svedberg and Pedersen, 1940).

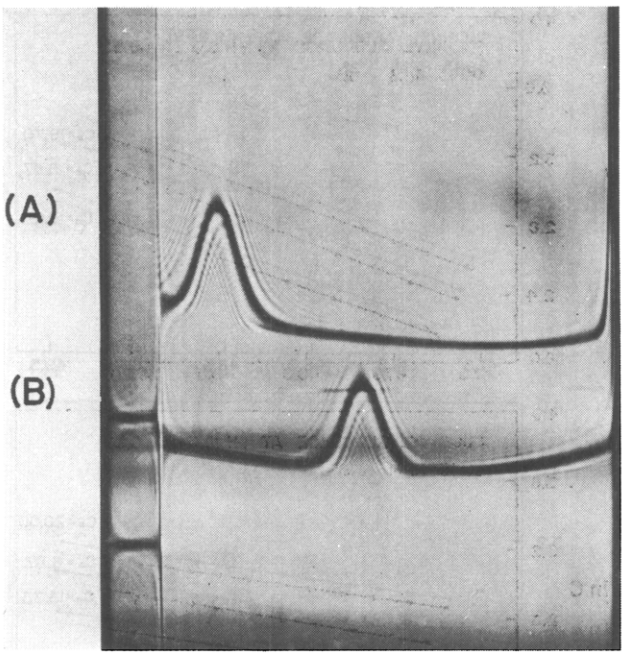

FIG. 2.—Sedimentation velocity patterns at 20° of succinyl(A) and native(B) aldolase in 0.5 M KCl, 0.1 M Tris-Cl, 0.01 M mercaptoethanol, *p*H 8.0. Photographs were taken at 64 minutes and a phase-plate angle of 65° after reaching a rotor speed of 59,780 rpm.

Viscosity Measurements.—Viscosity measurements were made with a 2.0-ml Ostwald-type viscometer having a water-outflow time of 184.18 seconds at 20.00° ± 0.01°. Prior to all determinations protein samples were dialyzed for at least 48 hours against 0.5 M KCl–0.1 M Tris-Cl buffer, *p*H 8.0, containing 0.001 M mercaptoethanol (Eastman Kodak Distillation Products Industries, Rochester, N.Y.). Five or more determinations were made on each protein sample with an average outflow-time deviation of ± 0.04 second. Viscosities were calculated relative to the solvent, neglecting small kinetic energy corrections (Schachman, 1957).

Determination of Free Amino and Sulfhydryl Groups After Succinylation.—Free sulfhydryl groups were measured by mercaptide formation with *p*-mercuribenzoate according to the method of Boyer (1954). The protein and *p*-mercuribenzoate were allowed to react in 0.05 M phosphate buffer, *p*H 7.0, for 90 minutes prior to measuring the change in absorbancy at 250 mμ.

Estimation of free amino groups was made by colorimetric analyses at 570 mμ using the method of Moore and Stein (1948) as modified by Fraenkel-Conrat (1957). Solutions of unmodified aldolase were used as standards for comparison. Prior to analysis, solutions of native and succinyl aldolase were dialyzed exhaustively against 0.1 M KCl. During analyses, samples were read against a blank containing an appropriate volume of dialysate.

N-Terminal Amino Acid Analysis.—N-Terminal amino acid analyses were performed by the Edman (1950) procedure using phenylisothiocyanate (Eastman Kodak Distillation Products Industries, Rochester, N. Y.) and the paper-strip technique developed by Fraenkel-Conrat (1954). Paper chromatography employing the various solvent systems developed by Edman and Sjöquist (1956) was used for isolating N-terminal derivatives. Known amino acid phenylthiohydantoins (Mann Research Laboratories, Inc., New York, N. Y.) were used as identification markers.

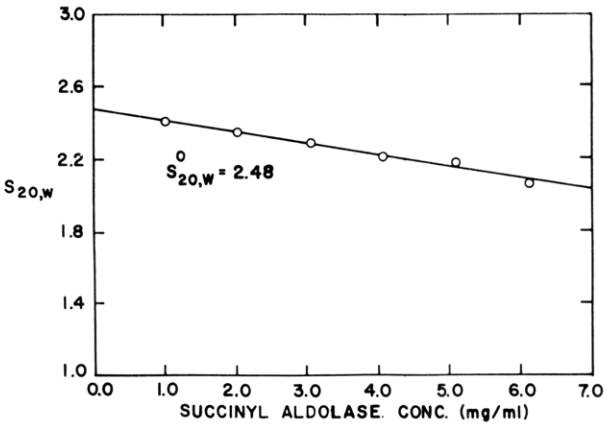

FIG. 3.—Concentration dependence of the sedimentation coefficient of succinyl aldolase at 20° in Tris-KCl buffer (*p*H 8.0, μ = 0.55) containing 0.01 M mercaptoethanol.

RESULTS

Extent of Aldolase Succinylation.—When native aldolase was succinylated using a 20:1 ratio of succinic anhydride per free amino group, about 92–95% of the free amino groups reacted along with approximately 14 of the 27 sulfhydryl groups. When determining sulfhydryl groups, no spectral shift was noticed during mercaptide formation indicating that all of the unsuccinylated —SH groups were immediately available for reaction with *p*-mercuribenzoate (Swenson and Boyer, 1957). If larger ratios of succinic anhydride were used (e.g., 60:1), essentially no free amino groups and only 3–4 —SH groups were detected. Thus upon complete succinylation, the molecular weight of aldolase should increase from 1.42×10^5 to approximately 1.54×10^5 for the intact molecule.[1] Tyrosyl groups are not reactive (Habeeb *et al.*, 1958; Fraenkel-Conrat, 1957).

Succinyl Aldolase Spectral Properties.—When the secondary and tertiary structures of proteins are disrupted, the ultraviolet-absorption maxima of the molecules undergo a "blue shift" to shorter wavelengths and show a slight decrease in intensity (Beaven and Holiday, 1952). Similar changes have been observed after succinylating aldolase. As indicated under Materials and Methods, the extinction coefficient ($E_{276.5}$ = 0.82 cm²/mg) of succinyl aldolase is approximately 10% lower than that of native aldolase (E_{280} = 0.91 cm²/mg). A greater difference (13%) is obtained when both proteins are compared at 280 mμ. As illustrated in Figure 1 a "blue shift" in the maximum wavelength from 279.9 mμ to 276.5 mμ also occurs. Although these phenomena might be explained by field effects due to the interaction of charges with aromatic groups, it appears more likely from accompanying observations that the effects are probably due to the destruction of hydrophobic bonding by strong coulombic forces thereby exposing the aromatic residues to a predominantly different environment from that in the intact molecule (Yanari and Bovey, 1960).

Hydrodynamic Properties of Succinyl Aldolase.—Figure 2 illustrates the large velocity differences in the sedimentation patterns of succinyl (A) and native (B) aldolase under identical conditions. Despite the expected microheterogeneity of succinyl aldolase, it migrates as a single well-defined boundary even during extended periods of sedimentation.

The dependence of succinyl aldolase sedimentation

[1] Based on 93 lysine and 27 cysteine groups per 1.42×10^5 mw.

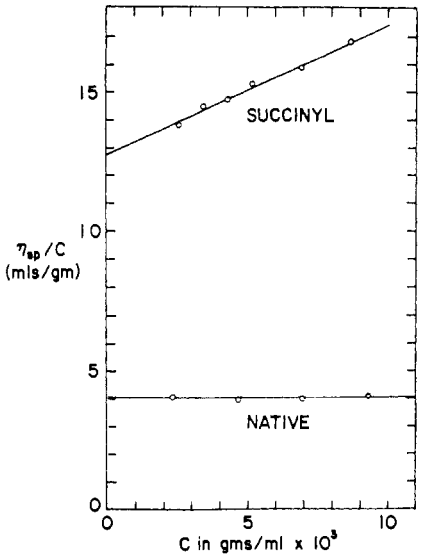

FIG. 4.—Reduced viscosity of succinyl and native aldolase in Tris-KCl buffer (*p*H 8.0, μ = 0.55) containing 0.001 M mercaptoethanol.

on concentration in Tris-KCl buffer (*p*H 8.0, μ = 0.55) is shown in Figure 3. Fitting of the data by least squares analysis gave the following relationship:

$$s_{20,w} = 2.48 - 0.064c$$

where c is the protein concentration in mg/ml. The $s^\circ_{20,w}$ value (2.48 S) is much lower than 7.43 S reported for native aldolase at *p*H 8.2 (Hass and Lewis, 1963). Succinylation, however, can cause a drastic decrease in the sedimentation coefficient by promoting isotropic swelling and molecular unfolding (Habeeb *et al.*, 1958). Nevertheless, an $s^\circ_{20,w}$ value of 2.48 S is close to the value of 1.9–2.0 S for acid-dissociated aldolase (mw 46,000–51,000) found by Deal *et al.* (1963) and Stellwagen and Schachman (1962). This suggests that succinic anhydride is capable of causing the formation of aldolase subunits.

Viscosity measurements were made to compare other hydrodynamic properties of succinyl and native aldolase. Figure 4 illustrates plots of the reduced viscosity (η_{sp}/c) vs. protein concentration at *p*H 8.0 and high ionic strength. Under these conditions the reduced viscosity of native aldolase shows no concentration dependence and has a value of 4.04 ml/g, in excellent agreement with 4.0 ml/g reported by Stellwagen and Schachman (1962) for aldolase at *p*H 7.0. The reduced viscosity of succinyl aldolase, however, shows a strong concentration dependence and extrapolates to a markedly increased intrinsic viscosity, $[\eta]$, of 12.80 ml/g at infinite dilution. At high ionic strengths the ion atmosphere tends to shield polyelectrolyte charges so that the polymer behaves like an uncharged molecule (Yang, 1961; Hermans and Overbeek, 1948). Thus, under the conditions employed here, succinyl aldolase presumably resembles a highly unfolded structure uncomplicated by large electrostatic properties.

Molecular Weight Determinations.—Molecular weights were obtained from sedimentation equilibrium experiments as outlined under Materials and Methods. Figure 5 shows typical ln c vs. x^2 plots of succinyl aldolase at *p*H 8.0 (top), *p*H 12.5 (middle), and *p*H 8.0 after exposure to alkali (bottom). The virtually rectilinear plots illustrated in the top and middle graphs indicate essentially homogeneous material. The experimental points in the lower graph, however, definitely show upward concavity, indicating molecular

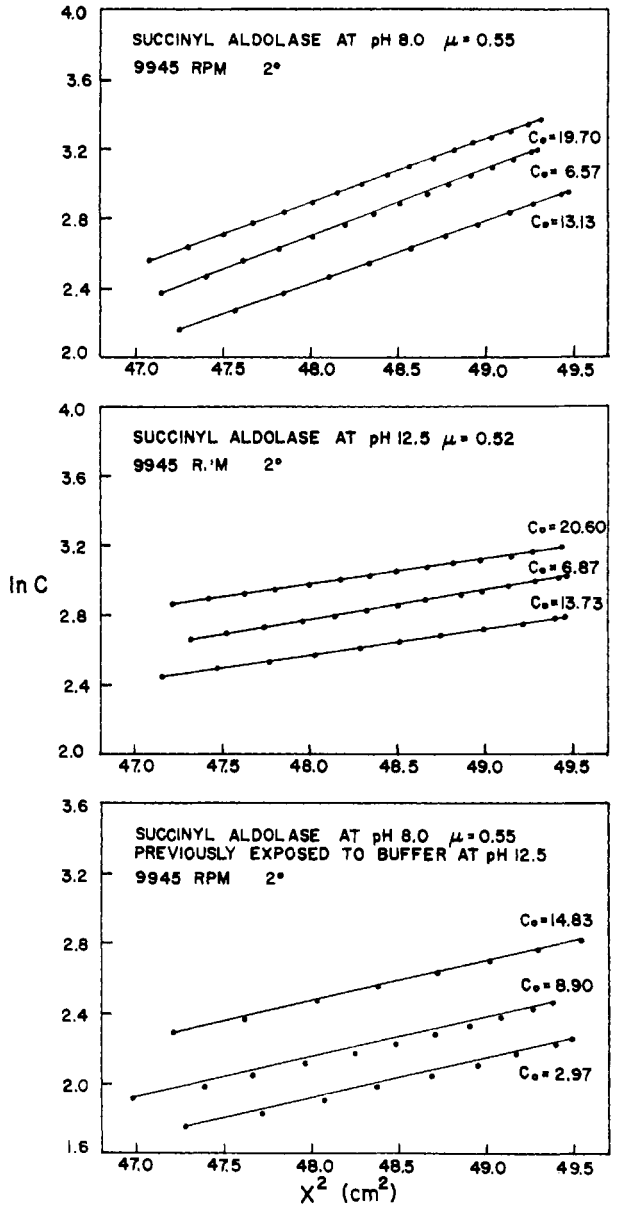

FIG. 5.—Typical ln c vs. x^2 plots of succinyl aldolase obtained from sedimentation equilibrium experiments at 2°. The ordinate represents the logarithm of the protein concentration in fringes and the abscissa represents the square of the distance from the center of rotation. (Top) Succinyl aldolase in Tris-KCl buffer (*p*H 8.0, μ = 0.55). (Middle) Succinyl aldolase in borate-KCl buffer (*p*H 12.5, μ = 0.52). (Bottom) Succinyl aldolase in Tris-KCl buffer (*p*H 8.0, μ = 0.55) after exposure to borate-KCl buffer, *p*H 12.5, for 43 hours. All solutions contained 0.01 M mercaptoethanol.

polydispersity (Svedberg and Pedersen, 1940). This polydispersity probably represents a slow aggregation (over a period of 3–4 days) of peptides following neutralization of the alkali-treated material.

Figure 6 illustrates the influence of ionic strength and protein concentration on the apparent molecular weight of succinyl aldolase at *p*H 8.0. At an ionic strength (μ) of 0.25, the molecular weight shows a distinct concentration dependence which can be attributed to highly negatively charged polypeptides (Pedersen, 1940). Analysis of the data by least squares gave the following relationship:

$$1/M_{app} = 1.77 \times 10^{-5} + 2.45 \times 10^{-6}c$$
$$M^\circ = 56{,}400$$

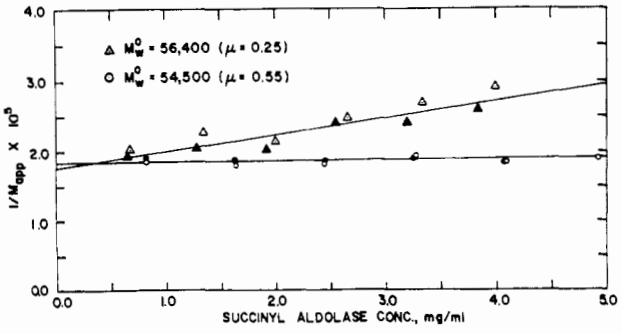

FIG. 6.—The influence of ionic strength and protein concentration on the apparent molecular weight of succinyl aldolase. △, experiments in Tris-KCl buffer (*p*H 8.0, μ = 0.25); ○, experiments in Tris-KCl buffer (*p*H 8.0, μ = 0.55). All solutions contained 0.01 M mercaptoethanol. Solid points represent parallel experiments.

Dampening of the protein charge by virtually doubling the ionic strength to μ = 0.55 considerably diminishes the concentration effect as indicated graphically and by least squares analysis.

$$1/M_{app} = 1.83 \times 10^{-5} + 5.35 \times 10^{-6}c$$
$$M_w^\circ = 54{,}500$$

Due to the scatter of experimental points at μ = 0.25, the molecular weight obtained at μ = 0.55 is considered more reliable. When this value is corrected for the contribution of approximately 40 succinyl groups, a molecular weight of 50,500 is obtained. Thus, under the conditions used, succinic anhydride dissociates native aldolase into three subunits. The value of 50,500 is in excellent agreement with 51,000 reported by Deal *et al.* (1963) for acid-dissociated aldolase. The data imply that the molecular weight of native aldolase is closer to 1.50 × 10^5 than the low values of 1.42 × 10^5 and 1.40 × 10^5 reported by Stellwagen and Schachman (1962), and Hass and Lewis (1963), respectively. Assuming that the lower values for native aldolase are correct, a subunit molecular weight of 50,500 for dissociation into thirds represents an experimental error of only 6.0%.

Since native aldolase dissociates into six subunits when exposed to alkali, attempts were made to obtain the same number of polypeptides under more neutral conditions. Consequently, sedimentation equilibrium experiments were performed on succinyl aldolase subunits dissolved in 5.0 M guanidine·HCl.[2] Under these conditions, a weight-average molecular weight (M_w°) of 47,200 was obtained, indicating no further dissociation. The low value of 47,200 probably reflects a change in $\bar{V}$ due to the binding of guanidine·HCl.

The stability of succinyl aldolase subunits during and after alkali treatment was also investigated. For these experiments a protein sample was dialyzed against borate-KCl buffer (*p*H 12.5, μ = 0.52) for 43 hours. A similar sample was redialyzed for 48 hours against Tris-KCl buffer (*p*H 8.0, μ = 0.55) after exposure to alkali. The results are presented in Figure 7 and analysis of the data by the method of least squares yields the following equations:

$$1/M_{app} = 3.70 \times 10^{-5} + 2.39 \times 10^{-6}c$$
$$M_w^\circ = 27{,}000 \text{ (at } p\text{H } 12.5)$$
$$1/M_{app} = 3.04 \times 10^{-5} + 7.97 \times 10^{-7}c$$
$$M_w^\circ = 32{,}900 \text{ (at } p\text{H } 8.0 \text{ after exposure to alkali)}$$

[2] 5.0 M guanidine·HCl causes native aldolase to dissociate into three subunits (L. F. Hass and M. S. Lewis, unpublished data).

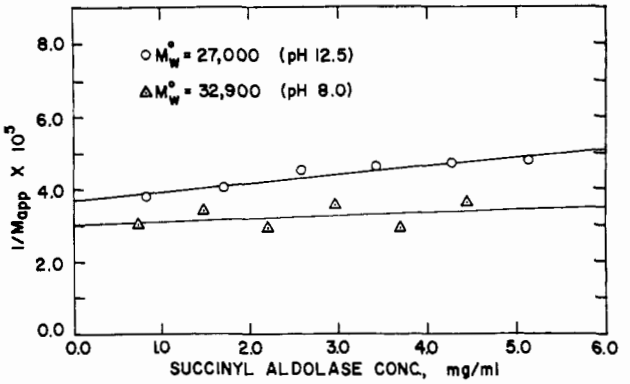

FIG. 7.—Concentration dependence of the apparent molecular weight of succinyl aldolase during and after exposure to alkali. ○, experiments in borate-KCl buffer (*p*H 12.5, μ = 0.52); △, experiments in Tris-KCl buffer (*p*H 8.0, μ = 0.55) after exposure to borate-KCl buffer, *p*H 12.5, for 43 hours.

The molecular weight of 27,000 obtained at *p*H 12.5 is almost half that obtained at *p*H 8.0 (Fig. 6). The concentration dependence of the apparent molecular weight at this *p*H value undoubtedly reflects the influence of increased molecular charge.

Neutralization of alkali-treated succinyl subunits results in a molecular weight increase (M_w° = 32,900) which is apparently due to aggregation as indicated in Figure 5. This tendency to form higher molecular weight material after neutralization was unexpected, since the high negative charge density of the succinyl polypeptides should not have been altered appreciably during exposure to alkali. Unaltered succinyl aldolase subunits maintained at *p*H 8.0 show no tendency to aggregate under equivalent conditions of ionic strength.

Analysis of Hydrodynamic and Molecular-Weight Data.—By combining hydrodynamic and molecular weight data further insight concerning molecular size and shape can be obtained. Thus, under standard conditions for substances dissolved in water at 20°, the frictional ratio (f/f_o) can be calculated from the following relationship (Pedersen, 1940):

$$f/f_o = 1.19 \times 10^{-15}[M_w^{2/3}(1 - \bar{V}\rho)/s^\circ_{20,w}\bar{V}^{1/3}]$$

where f is the molecular frictional coefficient and f_o the frictional coefficient of a sphere of equivalent molecular weight. Using this relationship, frictional coefficients of 2.29 and 1.13 were determined for succinyl and native aldolase, respectively. These data have been incorporated into Table I and may be used to determine anhydrous axial ratios from Perrin's tables (Cohn and Edsall, 1943b). Table I summarizes the physical and hydrodynamic properties of native and succinyl aldolase.

N-Terminal Amino Acid Analyses.—Unfortunately no satisfactory method has been described for the quantitative determination of N-terminal amino acids of highly complex proteins. Using ^{131}I-*p*-iodophenylsulfonyl chloride, Udenfriend and Velick (1951) found approximately two N-terminal prolines per mole of aldolase, but apparently quantitative results were not obtained.

Since proline is extremely refractory to quantitative determination using the dinitrofluorobenzene method of Sanger (1945, 1949) (see Porter, 1957), the Fraenkel-Conrat (1954) procedure was used for the N-terminal amino acid analyses reported here.[3] One advantage

[3] For a detailed description of this method see Fraenkel-Conrat *et al.* (1955).

TABLE I
COMPARISON OF THE PHYSICAL PROPERTIES OF NATIVE AND SUCCINYL ALDOLASE

Sample	$M_w^\circ \times 10^{-5}$ at pH 8.0	11.9	12.5	$s^\circ_{20,w}$[a] (S)	$[\eta]$ (ml/g)	f/f_0	λ_{max}[b] (mμ)	$E_{\lambda_{max}}$ (cm^2/mg)	$\bar{V}_{app}$[c] (ml/g)
Native	1.42[d]	0.550[e]	0.224[e]	7.43	4.04	1.13	279.9	0.91	0.745 ± 0.006
Succinyl[f]	0.545		0.270	2.45	12.80	2.29	276.5	0.82	0.704 ± 0.008

[a] Values at pH 8.0–8.2. [b] Maxima in absorption spectra at neutral pH. [c] Values obtained at 20° in Tris-KCl buffer (pH 8.0, μ = 0.55) containing 0.01 M mercaptoethanol. [d] This value was obtained by E. G. Richards and probably represents the best weight-average molecular weight for native aldolase in neutral solution (see Stellwagen and Schachman, 1962). [e] Values reported by Hass and Lewis (1963). [f] Succinyl aldolase was prepared using a ratio of succinic anhydride per free amino group of 20:1 as outlined under Materials and Methods. Under these conditions approximately 95% of the free amino groups and 14 of the 27 /SH groups found in native aldolase are succinylated.

of this method is that the same protein sample can be used for repeated determinations.

Initial analysis of native aldolase gave 1.39 moles of proline phenylthiohydantoin(proline-PTH)[4] per mole of enzyme. Repeating the procedure on the same sample gave 2.59 moles of proline-PTH and approximately 0.03 mole of an unidentified PTH. When the sample was analyzed for a third time, proline-PTH was not found; instead, a very small amount of unidentified material was obtained. Thus, a total of 3.98 moles of proline was obtained per mole of aldolase. Proline-PTH was observed to be unstable under the conditions used; therefore, the moles of proline found must be considered minimal.

End-group analysis was also performed to determine whether alkali caused the formation of six succinyl aldolase subunits through peptide-bond cleavage (Fig. 7). Since succinyl peptides do not tend to precipitate from neutral solution, this material is ideal for manipulation between neutral and highly alkaline pH values. To test for bond hydrolysis, succinyl aldolase subunits were dialyzed first for 42 hours against 0.1 M potassium borate buffer, pH 12.5, and then redialyzed against distilled water for 62 hours. End-group analysis on the material after final dialysis gave completely negative results.

DISCUSSION

Aldolase has been dissociated into subunits by exposure to extreme pH values and by a number of reagents including urea, guanidine·HCl, and sodium dodecylsulfate (Stellwagen and Schachman, 1962; Deal *et al.*, 1963; Hass and Lewis, 1963). The findings reported here support the concept that coulombic forces play an important role in maintaining the structure of the native enzyme. Thus, when negatively charged succinylate ions are introduced as covalently bound groups into the aldolase molecule, the enzyme readily dissociates into three polypeptide chains. This effect is accomplished near neutral pH values and undoubtedly results from cancellation of the 93 positively charged NH_3^+ groups which are present in the native protein. Steric effects due to the introduction of foreign molecules may also play a role in the denaturation process.

As indicated in Figures 5 and 7, succinyl aldolase exposed to alkaline conditions dissociates into six subunits having apparently equivalent molecular weights (M_w = 27,000). Under these conditions the tyrosyl and free cysteinyl groups are ionized, thereby increasing the negative-charge density by approximately 14–17 units per peptide. Neutralization of the alkali-treated material results in a slow aggregation of peptides and an increase in particle molecular weight (M_w° = 32,900). This phenomenon is surprising, particularly if it is assumed that each peptide has a random distribution of charges. Under equivalent ionic strength conditions (μ = 0.55), unaltered succinyl peptides (M_w° = 54,500) remain completely stable. Aggregation, however, could indicate the association of monomeric subunits through hydrophobic bonding[5] which is overcome only by ionizing aromatic residues. If this is the case, it would be interesting to determine what effect nonpolar solvents would have on succinyl or native aldolase dissociation. Attempts to obtain six subunits under neutral conditions through the combined use of succinic anhydride and guanidine·HCl were unsuccessful.

End-group analyses indicated that at least 4 moles of proline-PTH could be obtained per mole of aldolase. It was also pointed out that proline-PTH was unstable under the conditions employed and that the moles of proline found must be considered minimal. Very little evidence for other amino acids was found, immediately suggesting that aldolase may be composed of three subunits terminating in proline with the possibility that two or all three chains terminate in prolyl-proline.

Due to the compact structure of native aldolase, two cycles of the Fraenkel-Conrat procedure may have been necessary (see Results section) to permit all the N-terminal groups to react with phenylisothiocyanate. The fact that small amounts of phenylthiohydantoins, other than proline-PTH, were observed could indicate that the penultimate residues were resistant to hydantoin formation for steric or other reasons. Glycine peptides, for example, are comparatively resistant to hydantoin formation and have been known to interfere with clear-cut stepwise degradation (Fraenkel-Conrat *et al.*, 1955). If this is the case, the results suggest that aldolase may be composed of six subunits, all terminating in proline.

Unlike unaltered aldolase subunits, high concentrations of succinyl peptides resist precipitation from solution over a wide pH range. Consequently, stable neutral succinyl peptide solutions can be regained after exposure to a variety of alkaline pH values. With appropriate manipulation, succinyl aldolase should make ideal material for determining whether alkali causes the formation of six subunits through peptide-bond cleavage. Prior blocking of all free amino groups by succinylation provides a unique method for the detection of newly formed N-terminal groups resulting from chemical action.

Using the Fraenkel-Conrat modification of the Edman procedure, N-terminal residues were not found after exposing succinyl aldolase to alkali at pH 12.5 for 42 hours. Thus, it appears that highly alkaline conditions do not promote the formation of six sub-

[4] Abbreviation used in this work: PTH, phenylthiohydantoin.

[5] In this case, a random distribution of charges cannot be assumed.

units through peptide-bond cleavage. It should be pointed out that the procedure used here will not detect hydrolysis of either ester or thioester bonds, but the presence of these bonds in aldolase has not been demonstrated. There is also the possibility that N-terminal residues in succinyl peptides are refractory to isolation and identification by the method described. This may be true, particularly if the N-terminal residue happens to be ϵ-succinyl lysine.

Calculated axial ratios for anhydrous native aldolase are 3.3 (for a prolate ellipsoid) and 3.5 (for an oblate ellipsoid), indicating molecular compactness. In contrast, the succinyl aldolase subunit has corresponding axial ratios of 28 and 44. The latter values can be interpreted as indicating the highly unfolded state of the molecule; otherwise, it must be assumed that the subunit resembles a rodlike model with completely unrealistic dimensions.

Further speculation concerning the effective sizes and shapes of proteins in solution is provided by a β-function (Scheraga and Mandelkern, 1953) defined as follows:

$$\beta = \frac{S[\eta]^{1/3}\eta_o N}{M^{2/3}(1 - \bar{V}\rho)}$$

where N is Avagadro's number and η_o is the solvent viscosity. Calculation of the β-function for native and succinyl aldolase gave anomalously low values of 2.09×10^6 and 1.92×10^6, respectively. The minimum theoretical value, 2.12×10^6, reported by Scheraga and Mandelkern represents a spherical molecule having equal axial ratios. Thus it appears that native aldolase could resemble a sphere with an axial ratio of 1.0 and an effective volume (V_e) close to that of the partial specific volume ($\bar{V}$). Assuming that the β value for succinyl aldolase represents a sphere with a frictional ratio of 1.0 and a viscosity increment (ν) of 2.5 (see Scheraga, 1961), it can be shown that the molecule would have an effective volume 7–12 times greater than its partial specific volume (Kominz *et al.*, 1962; Lewis *et al.*, 1963). This suggests that succinylation may cause isotropic swelling of the subunit with marked solvent penetration of the molecular domain. It also provides a realistic model for explaining the hydrodynamic behavior of the succinyl subunit.

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