Studies on the Structure and Function of Muscle Aldolase

IV. THE ACTION OF DILUTE ALKALI ON PRIMARY STRUCTURE AND ITS EFFECT ON THE DETERMINATION OF SUBUNIT MOLECULAR WEIGHT*

(Received for publication, March 11, 1968)

H. E. Sine‡
From the Department of Biochemistry, State University of New York at Buffalo, Buffalo, New York 14214

L. F. Hass§
From the Department of Biological Chemistry, The Milton S. Hershey Medical Center of the Pennsylvania State University, Hershey, Pennsylvania 17033

SUMMARY

In 0.05 M potassium phosphate buffer (pH 7.0, 20°), native rabbit muscle aldolase has been found to exhibit a molecular weight (Mₘ) close to 160,000, in agreement with previous findings. Upon exposure to cold alkaline borate buffer (pH 12.5, µ = 0.17, 0°), the enzyme spontaneously dissociates into its monomers which subsequently undergo slow hydrolytic degradation. Compensating for electrostatic charge effects and extrapolating the data to zero time in alkali, a subunit molecular weight of 41,400 to 42,000 is obtained. If the native enzyme is exposed to alkaline conditions at 20°, the subunits undergo an initial rapid degradation which eventually yields alkali-resistant material. Similar results are obtained with succinyl aldolase subunits under equivalent conditions.

Acrylamide gel electrophoresis of alkali-treated succinyl aldolase (pH 12.5, 30°) results in the appearance of a limited number of electrophoretic bands. This finding suggests that specific alkali-labile peptide bonds are involved in the degradation process. As a further consequence of the action of alkali, native aldolase shows increased solubility properties. Both native and succinyl aldolase manifest new NH₂-terminal serine, threonine, and glycine residues after treatment with borate buffer at pH 12.5 and 30°. Prolonged exposure to alkali results in increased liberation of the above end groups, but no evidence is obtained for other NH₂-terminal residues except for a small amount of proline.

During a course of study related to the effect of alkali on the structure and function of rabbit muscle aldolase (fructose 1,6-diphosphate d-glyceraldehyde 3-phosphate lyase, EC 4.1.2.13), it was observed that at pH 12.6 and 27° the molecular weight of the native enzyme decreased to what appeared to be a relatively stable value of approximately 22,400 (1). Additional studies on succinyl aldolase subunits (mol wt ~54,000 g per mole) showed that a similar decrease in molecular weight to a value of 27,000 could be obtained under equivalent conditions (2). These observations, coupled with the fact that little evidence could be found for the formation of new NH₂-terminal amino acid residues after treatment of succinyl aldolase with alkali, led to the conjecture that the enzyme might be comprised of six fundamental noncovalently bound subunits (2).

Since recent reports indicate that aldolase is comprised of four subunits instead of six (3-7), an investigation of the stability of the enzyme's primary structure in dilute alkali was undertaken. This report presents data on: (a) the kinetics of subunit degradation in alkali, (b) the change in subunit electrophoretic and solubility properties after treatment with alkali, and (c) the alkali-catalyzed formation of new terminal residues. In addition, substantiating evidence for a four-subunit structure based on equilibrium sedimentation studies in alkali is presented.

MATERIALS AND METHODS

Aldolase—Aldolase was prepared from frozen rabbit muscle (obtained from Pel-Freez Biologicals, Inc., Rogers, Arkansas) by the method of Taylor, Green, and Cori (8). The enzyme was crystallized five times from ammonium sulfate solution and had a specific activity (measured as ΔA₂₅⁰) of 30 to 36 units per mg
per ml at 23° as determined by the hydrazine assay of Jaganathan, Singh, and Damodaran (9). The concentration of the enzyme dissolved in water or neutral buffer was determined by absorption at 280 μm, with the extinction coefficient \( E_{280} = 0.91 \text{ cm}^2 / \text{mg} \) (10). When aldolase was dissolved in 0.1 M potassium borate buffer at pH 12.5, the wave length of maximum absorption shifted from 279.9 μm to 289.5 μm. Under these conditions, aldolase concentration was determined by using the extinction coefficient \( E_{280} = 0.96 \text{ cm}^2 / \text{mg} \) or \( E_{380} = 0.81 \text{ cm}^2 / \text{mg} \).

**Succinyl Aldolase**—Succinyl aldolase was prepared by the method described previously (2), with an 80:1 m ratio of succinic anhydride per lysyl residue in order to assure complete reaction. Succinyl aldolase concentrations were determined by using the extinction coefficient \( E_{280} = 0.82 \text{ cm}^2 / \text{mg} \) (2).

**pH Measurements**—All pH measurements were made with a Leeds and Northrup expanded scale pH meter equipped with an A. H. Thomas combination electrode (No. 4858-160, pH range 0 to 14). Above pH 12.0, the meter was standardized against a saturated Ca(OH)\(_2\) solution which has a pH of 12.52 at 23° and a temperature coefficient (\( \Delta \text{pH}/\Delta T \)) of \(-0.033 \text{ pH unit per deg over the range of 20-35°}\).

**Molecular Weight Determinations**—Molecular weights were determined with a Spinco model E analytical ultracentrifuge equipped with a Rayleigh interferometer and rotor temperature indicator and control unit. Interference patterns were developed on Kodak spectrophotographic plates, type H2G, and were measured with a Nikon model 6 microcomparator equipped with a two-dimensional stage.

High speed sedimentation equilibrium experiments were performed with 3.0-mm liquid columns by the technique described by Yphantis (11). Simple 12-mm double sector cells equipped with sapphire windows were used routinely. Corrections for cell window distortion were made by running appropriate water blanks before and after each equilibrium run. In order to reduce the time required to establish equilibrium, the rotor was initially overspeed until the meniscus was depleted of protein (usually 2 to 3 hours) and then gradually decelerated until the desired equilibrium speed was reached. The time required to obtain equilibrium was determined by measuring fringe displacements near the meniscus, center, and bottom of the liquid column until constant values were obtained. Effective reduced molecular weights \( [\omega + Z(x)] \) at various positions within the cell were calculated with the following equation (14):

\[
M_{v,x} = \frac{2RT(d\ln c/3c^2)/(1 - \omega \phi)\omega^2}{1 - \omega \phi^2} \text{cm}^2\text{g}^{-1}
\]

In polydisperse systems, the weight average molecular weight over the entire liquid column was calculated from:

\[
\bar{M}_w = 2RT(c_0 - c_m)/(1 - \omega \phi)\omega^2(\omega^2 + x_m^2)C_0
\]

**Table I**

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Recovery of amino acid derivatives</th>
<th>DPN</th>
<th>PTH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serine</td>
<td>30.9 ± 0.6 (3)</td>
<td>29.4 ± 3.9 (4)</td>
<td></td>
</tr>
<tr>
<td>Threonine</td>
<td>42.7 ± 0.6 (3)</td>
<td>50.0 ± 3.9 (5)</td>
<td></td>
</tr>
<tr>
<td>Glycine</td>
<td></td>
<td>71.7 ± 8.1 (4)</td>
<td></td>
</tr>
<tr>
<td>Proline</td>
<td></td>
<td>39.5 ± 5.5 (9)</td>
<td></td>
</tr>
</tbody>
</table>

* Numbers in parentheses represent the number of determinations made.

The mean \( z \)-average molecular weight \( (\bar{M}_z) \) was calculated from the limiting slopes of plots of ln c against \( z^2 \) at the cell bottom and the meniscus with the relationship, \( \bar{M}_z = (M_{w,x}C_3 - M_{w,m}C_m)/(C_3 - C_m) \) (15).

**Determination of Partial Specific Volume**—Apparent partial specific volumes were determined pycnometrically by the relationship:

\[
\phi' = \rho_0 (1 - \Delta \rho/\phi_c)
\]

where \( \phi' \) is the apparent partial specific volume of the protein in a multicomponent system as defined by Casassa and Eisenberg (16). The \( \phi' \) of aldolase dissolved in 0.1 M potassium borate-0.01 M β-mercaptoethanol at 0° (pH 12.50) was obtained by performing three individual determinations on each of three protein samples at different concentrations ranging from 3.0 to 6.0 mg per ml. The value obtained was 0.717 ± 0.003 ml per g and exhibited no concentration dependence. Because of uncertainty in the third decimal place, a value of 0.72 ml per g was used for all molecular weight determinations at 0°.

Partial specific volumes of 0.742 ml per g at 20° (17) and 0.695 ml per g at 2° (2) were used in calculating the molecular weights of native and succinyl aldolase, respectively.

**NH₂-terminal Analyses**—NH₂-terminal amino acid analyses were performed by both the Edman (18) and the Sanger (19, 20) techniques with the procedures outlined by Freidenreich (21) and Harris and Levy (22). Corrections for losses of NH₂-terminal derivatives were made by performing several experiments with known quantities (0.02 μmole) of either DNP- or PTH-amino acids in the presence of bovine serum albumin. The results of these experiments are given in Table I.

**COOH-terminal Analyses**—Carboxypeptidases A and B were purchased from Worthington. Carboxypeptidase A was a three times crystallized preparation which had been treated with diisopropylphosphorofluoridate. The concentration of the enzyme in 10% LiCl was determined by absorption at 278 μm with the extinction coefficient \( E_{278} = 1.94 \text{ cm}^2 / \text{mg} \) (28). Carboxypeptidase B was obtained as a frozen solution and was treated with DFP by the method of Winstead and Wold (24). The concentration of the enzyme was determined spectrophotometrically with the extinction coefficient \( E_{278} = 2.12 \text{ cm}^2 / \text{mg} \) (25).

In preparation for carboxypeptidase digestion, aldolase in alkaline solution (0.1 M potassium borate-0.01 M β-mercapto-

The abbreviations used are: DNP-, 2,4-dinitrophenyl-; PTH, phenylthiodyantoin; DFP, diisopropylphosphorofluoridate; TCA, trichloroacetic acid.
After incubation at 25°, ratios of enzyme to aldolase of 1:18 and 1:45, respectively.

Molecular weights plotted as a function of protein concentration 5% TCA. The resulting supernatant solutions were pooled and lyophilized three times to remove most of the TCA. The final remainder of the run (23 hours).

To 9945 rpm for 1 hour and then reduced to 6995 rpm for the remainder of the run (23 hours).

FIG. 1. Molecular weight determinations of native muscle aldolase in 0.05 m potassium phosphate buffer (pH 7.0) at 20° by low speed equilibrium centrifugation. The left-hand ordinate gives the logarithm of the protein concentration in fringes, the right-hand ordinate depicts the protein concentration directly in milligrams per ml, and the abscissa represents the square of the distance from the rotational axis. Three samples were run simultaneously in the Spinco An-J rotor. The rotor speed was adjusted to 9945 rpm for 1 hour and then reduced to 6995 rpm for the remainder of the run (23 hours).

Fig. 2. Molecular weight determination of native muscle aldolase in 0.05 m potassium phosphate buffer (pH 7.0) at 20° by high speed equilibrium centrifugation. The left-hand ordinate depicts the effective reduced number, weight, and z-average molecular weights plotted as a function of protein concentration (measured as fringe displacement) across the centrifuge cell. The right-hand and upper coordinates indicate the actual molecular weights and protein concentrations, respectively. O and ●, initial protein concentrations of 0.10 and 0.21 mg per ml, respectively. The rotor speed was adjusted to 15,220 rpm and allowed to run for 20 hours.

ethanol, pH 12.5) was neutralized to pH 8.0 with 0.1 N HCl. Carboxypeptidases A and B were then added to give molar ratios of enzyme to aldolase of 1:18 and 1:45, respectively. After incubation at 25°, 1.5-ml aliquots (14.6 mg of aldolase) were withdrawn at appropriate time intervals (including zero time) and the reaction was stopped by adding an equal volume of 10% trichloracetic acid. The precipitated protein was removed by centrifugation and was washed twice with 1.0 ml of 5% TCA. The resulting supernatant solutions were pooled and lyophilized three times to remove most of the TCA. The final lyophilized residue was dissolved in 0.2 N sodium citrate buffer (pH 2.2) and stored at -20° for future analysis.

Amino Acid Analyses—Quantitative amino acid analyses were performed on the Spinco model 120 B automatic amino acid analyzer according to the method described by Moore, Spackman, and Stein (26).

Polyacrylamide Gel Electrophoresis of Alkali-treated Succinyl Aldolase—Approximately 20 mg of succinyl aldolase were dissolved in 2.25 ml of 0.1 m potassium borate buffer (pH 12.6) containing 0.01 m 3-mercaptoethanol. Incubation of 1.25 ml of this solution was conducted at 20°, while the remainder was incubated at 2-4°. At successive time intervals, 0.1-ml aliquots were removed from each incubation mixture and neutralized to pH 8.5 by the addition of 0.2 N HCl. These samples were subjected to electrophoretic analysis with the Canalco model 12 disc electrophoresis apparatus and the procedure described by Davis (27). Approximately 0.3 mg of succinyl aldolase was added to each of 12 tubes containing 7% cross-linked polyacrylamide gel.

RESULTS

Fig. 1 illustrates typical plots of \( \ln c \) against \( x^2 \) obtained during a low speed centrifugation experiment performed at three different initial protein concentrations in 0.05 m potassium phosphate buffer at pH 7.0. Each of the plots is perfectly rectilinear, indicating that the macromolecular material is apparently homogenous (15). When the slopes of these lines are used for calculating the weight average molecular weights, a mean value of 156,000 ± 3,600 g per mole is obtained. This value is significantly larger than most of those reported previously (1, 17, 28, 29), but is in accord with the value of 158,000 determined earlier by Kawahara and Tanford (3).

Since low speed centrifugation does not readily lend itself to macromolecular fractionation, the high speed sedimentation equilibrium technique of Yphantis (11) was used in order to provide supporting evidence for the molecular weight given above. Fig. 2 depicts the results obtained when native aldolase, at two different initial loading concentrations, is allowed to come to equilibrium at 15,220 rpm. In this figure, the point average molecular weights (\( \bar{M}_n \), \( \bar{M}_w \), and \( \bar{M}_z \)) are plotted against the fringe displacements across the centrifuge cell. Within the limits of error, each of the curves at different loading concentrations is almost coincident, indicating that the point average molecular weights are identical functions of the concentrations at \( x \) and that the protein is essentially homogeneous. The monodispersity of the aldolase preparation is also borne out by the close agreement in \( M_n, M_w, \) and \( M_z \) values. Furthermore, the values in Fig. 2 indicate that the molecular weight of native aldolase is close to 160,000, which is in good agreement with that obtained by low speed equilibrium centrifugation.

Determination of Subunit Molecular Weight in Alkali by High and Low Speed Equilibrium Centrifugation—Fig. 3 illustrates the results obtained during a low speed sedimentation equilibrium run performed at pH 12.5 (\( \mu = 0.1\mu \)) and 0° on three protein samples at different concentrations. After a total time in alkali of 21 hours (centrifugation plus dialysis time) (Fig. 3A), the samples appear to be homogenous as indicated by the rectilinearity of plots of \( \ln c \) against \( x^2 \). Calculation of the apparent average molecular weights (\( \bar{M}_{n,app} \)) leads to values which are obviously dependent upon protein concentration (Table II). If the reciprocals of these values are plotted against the initial...
loading concentrations (C₀) and extrapolated to infinite protein dilution to overcome thermodynamic nonideality, a weight average molecular weight (Mₖₒ) of 40,200 is obtained.

Since the above molecular weight could represent an erroneously low value because of alkali-catalyzed peptide bond hydrolysis, centrifugation was carried out for a prolonged period of time to determine any change in extrapolated Mₖₒ values at selected time intervals. The results of this experiment are summarized in Fig. 3B, Fig. 4, and Table II. After 103 hours in alkali at 0°, a molecular weight of 34,200 is obtained at infinite protein dilution, indicating that some hydrolysis had occurred. Hydrolytic action is also reflected in the molecular weight values procured over the entire time course of this study but, if the Mₖₒ values are extrapolated to zero time in alkali (Fig. 4), a subunit weight average molecular weight of 41,400 is obtained.

During the above experiments, the plots of ln c against x² appeared to be perfectly rectilinear despite the heterogeneity which undoubtedly developed as the result of hydrolysis (compare Fig. 3, A and B). This experiment, therefore, shows the inherent danger involved in determining the molecular weight of an apparently monodisperse material when low speed centrifugation methods are used.

If the subunit molecular weight in alkali (pH 12.5, 0°) is determined by high speed equilibrium centrifugation (33,450 rpm) at three different initial loading concentrations, the results shown in Fig. 5 are obtained. After a total time in alkali of 11 to 15 hours, most of the points for each plot describe a perfectly straight line over 80% of the cell. At the higher initial protein concentration (0.3 mg per ml), however, there is an obvious deviation from linearity near the meniscus, indicating a small amount of contamination by low molecular weight material which remains undetectable at the lower protein concentrations. As illustrated in Fig. 5, the apparent molecular weights are somewhat concentration-dependent. If the charge nonideality is eliminated by extrapolation of the data to infinite protein dilution, respective Mₖₒ and Mₖᵣ values of 41,100 and 41,700 are obtained.

Other methods for overcoming thermodynamic nonideality include the use of charge-independent moments which in essence eliminate the second virial coefficient. The charge-independent moments as defined by Yphantis² are: Mᵥ₁ = (Mᵥₙ Mᵥₐ /2 Mᵥₒ - Mᵥₙ) and Mᵥ₂ = (Mᵥₒ Mᵥₐ / Mᵥₙ). For completely monodisperse material, a plot of Mᵥ₁ or Mᵥ₂ (σᵥ₁ or σᵥ₂) as a function of protein concentration is equivalent to that shown in Fig. 1, A. The results obtained after a total time in alkali of 21 hours; B, the results obtained after 103 hours in alkali. The lower plots (1/Mᵥₙ against C₀) illustrate the dependence of the apparent molecular weight on protein concentration. The rotor speed was adjusted to 17,250 rpm for 1½ hours and then reduced to 9945 rpm for the remainder of the run (103 hours).

**Table II**

Analysis of change in molecular weight of dissociated aldolase after exposure to alkali

The following results were obtained during continuous low speed equilibrium centrifugation under the conditions cited in Fig. 3. Photographs were taken at the indicated time intervals and analyzed for Mₖₒ.

<table>
<thead>
<tr>
<th>Initial protein concentration</th>
<th>Mᵥₒ, app X 10⁻⁴ after exposure to pH 12.5 borate buffer at 0° for</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg/ml</td>
<td>21 hrs</td>
</tr>
<tr>
<td>4.15</td>
<td>30.6</td>
</tr>
<tr>
<td>2.85</td>
<td>33.2</td>
</tr>
<tr>
<td>1.40</td>
<td>36.2</td>
</tr>
</tbody>
</table>

Extrapolated Mₖₒ

40.2 39.4 37.8 35.7 34.2

*Personal communication.*
Studies on Structure and Function of Muscle Aldolase. IV

Vol. 244, No. 2

Fig. 5. Molecular weight determination of dissociated aldolase in 0.1 M potassium borate-0.01 M β-mercaptoethanol (pH 12.5, μ = 0.17) at 0° by high speed equilibrium centrifugation. The left-hand ordinate depicts the reciprocals of the effective reduced number and weight average molecular weights obtained at three different initial loading concentrations (○, 0.11; ●, 0.21; ▽, 0.30 mg per ml). The remaining coordinates are the same as those given in Fig. 2. The rotor speed was adjusted to 39,400 rpm for 3 hours and then reduced to 33,450 rpm for the remainder of the run (~5 hours). The total time in alkali for each sample ranged from 11 to 15 hours (dialysis plus centrifugation time).

Fig. 6. The influence of total time in alkali (pH 12.5, μ = 0.17, 0°) on the point distribution of the effective reduced charge-independent moment of dissociated aldolase. Reading from top to bottom, each frame depicts the results obtained during successive time intervals in alkali up to 67 hours. The initial protein concentration was 0.3 mg per ml. Rotor speed adjustments were the same as those described in Fig. 5. Centrifugation was continuous for 67 hours.

Distribution within the centrifuge cell should describe a perfectly horizontal line; however, if the material is polydisperse, the plot should manifest downward curvature because of solute fractionation and a difference in values for \( M_n \), \( M_w \), and \( M_z \).

Use was made of the \( \sigma_{1L} \) moment to show the alkaline lability of aldolase during a prolonged high speed sedimentation equilibrium run. The results of this experiment are summarized in Fig. 6, which shows the point distribution of \( \sigma_{1L} \) at various time intervals in alkali up to 67 hours. With increased time in alkali, there is a definite increase in downward curvature near the meniscus, indicating the progressive formation of low molecular weight material. Degradation is also reflected in the time-dependent decrease in \( \sigma_{1L} \) near the bottom of the cell. If the protein is initially monodisperse, the \( \sigma_{1L} \) moments at the bottom, center, and meniscus of the cell should extrapolate to a common ordinate intercept when plotted as a function of time in alkali. Fig. 7 shows that, within the limits of error, this is essentially the case. Thus, by the method described, a subunit molecular weight of 42,000 is obtained.

Kinetics of Subunit Degradation in Dilute Alkali—In an effort to gain additional information about the alkaline stability of aldolase subunits, studies were performed on the rate of change in molecular weight of the native and succinylated proteins exposed to alkaline conditions at elevated temperatures.

Fig. 8 illustrates the decrease in \( \bar{M}_{p0} \) of nondialyzable succinyl aldolase peptides as a function of hours in alkali at pH 12.6 and 20°. It should be noted that, during this experiment, the alkaline incubation medium was neutralized prior to exhaustive dialysis against pH 8.0 Tris-KCl buffer in preparation for ultracentrifugation. Under these conditions, the observed changes take place very slowly, indicating that the succinyl subunit is hydrolytically degraded rather than simply dissociated. During this study, a moderate degree of polydispersity has been observed as indicated by curvilinear plots of ln c against \( \bar{M} \) (not shown). Since molecular association should be minimal as a result of the
repulsive forces generated by the high net negative charges imposed by the large number of covalently linked succinyl groups, heterogeneity probably results solely from hydrolysis.

Treatment of native aldolase in a manner similar to that just described gives approximately equivalent results (Fig. 9). During this study, however, hydrolysis could not be stopped by lowering the pH of the reaction medium since protein precipitates from solution when this procedure is used, particularly after short exposure of the enzyme to alkali (see Fig. 11). Nevertheless, hydrolysis could be strongly impeded by increasing the ionic strength and lowering the temperature of the reaction medium, as illustrated in Fig. 9. Thus, during 91 hours of centrifugation at pH 12.6 and 0° (μ = 0.67), the subunit molecular weight remained substantially unchanged.

**Electrophoretic and Solubility Properties of Alkali-treated Aldolase**—The action of dilute alkali on muscle aldolase was also followed by electrophoretic and solubility studies. Fig. 10A illustrates the results obtained by acrylamide gel electrophoresis

![Fig. 7](image-url)

**Fig. 7.** Plots of the charge-independent moments of dissociated aldolase as a function of total time in alkali. The values of $M_{x1}$ were obtained from points in Fig. 6 corresponding to the meniscus (O), midpoint (●), and bottom (△) of the liquid column.

![Fig. 8](image-url)

**Fig. 8.** The decrease in $M_{x1}$ of succinyl aldolase as a function of time in alkali. Succinyl aldolase was incubated at 20° in 0.1 M potassium borate (pH 12.6) containing 0.01 M β-mercaptoethanol. At successive time intervals, aliquots were removed from the incubation mixture, neutralized, and finally dialyzed against 0.1 M Tris-0.5 M KCl-0.01 M β-mercaptoethanol (pH 8.0). $M_{x1}$ was determined at 2° as described previously (13).

![Fig. 9](image-url)

**Fig. 9.** The decrease in $M_{x1}$ of native aldolase subunits as a function of time in alkali. Aldolase was incubated at pH 12.6 under the conditions described in Fig. 1. At appropriate time intervals, aliquots were removed from the incubation mixture, cooled, and finally dialyzed at 0° against 0.1 M potassium borate-0.5 M KCl-0.01 M β-mercaptoethanol (pH 12.6). A control experiment conducted at pH 12.6 and 0° (μ = 0.67). In this case, the centrifuge was run continuously for 91 hours and $M_{x1}$ was determined at selected time intervals (13).

![Fig. 10](image-url)

**Fig. 10.** Polyacrylamide gel electrophoresis of alkali-treated succinyl aldolase. The upper figure (A) represents the results obtained after incubating succinyl aldolase for various time intervals at pH 12.6 and 20°. The lower figure (B) represents the results obtained from a similar experiment conducted at 2-4°. Experimental conditions are described under "Materials and Methods."
Studies on Structure and Function of Muscle Aldolase. IV

Vol. 244, No. 2

ALDOSE ALKALI (hours)

Fig. 11. Solubility of aldolase as a function of time in alkali. Aldolase samples (8 mg per ml) were incubated in 0.1 M borate buffer (pH 12.5) at the indicated temperatures in the presence (upper) and absence (lower) of 0.01 M β-mercaptoethanol. Aliquots (0.5 ml) were removed at successive time intervals and neutralized to pH 8.5 by the addition of 1 M HCl. The samples were incubated at 25° for 30 min and then centrifuged at 27,000 X g for 15 min at 25° to remove the protein precipitate. The supernatant solution was subsequently analyzed for ultraviolet-absorbing material at 280 μm.

of succinyl aldolase after exposure to pH 12.6 borate buffer at 20° for various lengths of time. After prolonged exposure to alkali, a limited number of electrophoretic bands is detectable. Furthermore, the electrophoretic pattern indicates that the disappearance of one major band results in the specific appearance of another major band (e.g., Band c → e and Band d → f), presumably with the concurrent formation of low molecular weight material which accumulates at the extreme anodic end of the gel (Band b). These observations suggest that the hydrolysis of specific peptide bonds occurs since a broad spectrum of electrophoretic species is not evident. It should be pointed out that during this study any O- and S-succinyl bonds would be rapidly cleaved (30), but this by itself would result in electrophoretic migration of the protein toward the cathode.

Fig. 10B illustrates the electrophoretic results obtained at low temperature (24°) under identical pH conditions. In this case, gross breakdown of peptide structure apparently does not take place. Instead, there is an initial band spreading followed by separation into two distinct species, possibly as a result of the slow hydrolysis of alkali labile succinyl ester and thioester bonds (30).

Fig. 11 illustrates the solubility properties of aldolase at pH 8.5 and 25° after exposing the enzyme to alkaline conditions (pH 12.5) at various temperatures in the presence and absence of

β-mercaptoethanol (pH 12.5) for 24 hours at 30°. The samples were then neutralized to pH 8.5 by the addition of 1 M HCl prior to reaction with fluorodinitrobenzene or phenylisothiocyanate (21). A represents a descending chromatogram of PTH-amino acids obtained from alkali-treated native aldolase, while B and C represent ascending chromatograms of DNP-amino acids obtained from alkali-treated native and succinyl aldolase, respectively. Additional experimental details are outlined under “Materials and Methods.”

Fig. 12. Chromatography of NH₂-terminal amino acid derivatives obtained from alkali-treated aldolase. Aldolase samples (30 to 30 mg) were incubated in 0.1 M potassium borate-0.01 M

Downloaded from http://www.jbc.org/ by guest on August 28, 2017
β-mercaptoethanol. An increase in the time or the temperature parameter results in an increase in protein solubility. This observation suggests that primary bonds are ruptured, resulting in the formation of relatively small peptides which are capable of remaining in solution by virtue of their inability to form large aggregates. In the absence of β-mercaptoethanol, peptide solubility is somewhat depressed, presumably a result of ---S--- bridge formation which increases the size of certain fragments formed during hydrolysis. Thus, 100% solubility is obtained in 24 and 36 hours at 30° in the presence and absence of sulphydryl reagents, respectively.

End Group Analyses—Previous attempts to show peptide bond hydrolysis by end group analyses on alkali-treated aldolase gave negative results (2). The results reported here, however, show that aldolase primary structure is destroyed in dilute alkali and that a limited number of peptide bonds are involved in the degradation process. Thus, when aldolase is treated with pH 12.5 borate buffer for 24 hours at 30° and then subjected to NH₂-terminal analyses by either the Sanger (19, 20) or the Edman (18) technique, three amino acids (serine, threonine, and glycine) are found in addition to native NH₂-terminal proline. This is illustrated by the chromatographic results obtained for both the native and the succinylated enzymes (Fig. 12).

Inspection of Fig. 12A clearly shows that the phenylthiohydantoin derivatives of the NH₂-terminal residues liberated by alkali have the same migration properties as authentic PTH-serine, threonine, and glycine when subjected to descending chromatography. The unidentified spot with an R₂ value of 0.83 represents a rearranged product of PTH-proline which arises during exposure to acid. When the dinitrophenyl derivatives of the NH₂-terminal amino acids are subjected to ascending chromatography (Fig. 12B), completely predictable results are obtained, thus providing additional support for the identities of the new end groups arising in alkali. Fig. 12C indicates that NH₂-terminal serine, threonine, and glycine are also formed when succinyl aldolase is treated at high pH values. DNP-proline is not observed in either of the latter two figures since it is extremely refractory to isolation as the DNP-derivative (21).

It is noteworthy that, even after incubating native aldolase for 120 hours at pH 12.5 and 30°, no evidence is found for NH₂-terminal amino acids other than those already mentioned. This suggests that certain seryl and threonyl residues are involved in the alkaline degradation of aldolase, although the participation of glycine in the hydrolytic mechanism is also a possibility.

Listed in Table III are quantitative estimates of the NH₂-terminal residues found after exposing aldolase to alkali for various time intervals. After 24 hours, two to three bonds are cleaved and after 120 hours this value increases to approximately nine to 11. In view of the large number of serine (94), threonine (89), and glycine (112) residues in native aldolase (31), the lack of random degradation is surprising (Fig. 10A). This apparent specificity might arise from effects caused by neighboring groups or particular amino acid pairs, or both. In an effort to determine whether a unique amino acid in peptide linkage with serine, threonine, and glycine was involved, alkali-treated aldolase was subjected to digestion with a mixture of carboxypeptidases A and B. The results of this experiment indicated that there was an initial rapid release of a large variety of COOH-terminal amino acids (e.g. tyrosine, alanine, serine, valine, leucine, and lysine), making it difficult to select a specific residue which might have influenced the hydrolysis.

### DISCUSSION

Although dilute alkaline solutions are routinely used as solvents for proteins, knowledge concerning the stability of the peptide bond at elevated pH values is meager. Warner (32) studied the hydrolysis of egg albumin at various temperatures and at several concentrations of hydroxide ion; however, his results indicate that the peptide bond should be quite stable under the conditions cited in this paper. Therefore, the purpose of this report is to point out that protein primary structure can be extremely labile in dilute alkali and, as a result, several difficulties may be encountered in determining subunit molecular weights when alkali is used as a dissociating medium. An explanation is also provided for the earlier conjecture that rabbit muscle aldolase might be comprised of six subunits (1) rather than four as recently suggested by other investigators (3-7).

The experiments described here show that caution must be exercised in attempting to determine the subunit composition of proteins by equilibrium centrifugation methods at elevated pH values. Thus, in addition to creating large electrostatic charge effects, there is the inherent danger of promoting peptide bond hydrolysis under seemingly mild conditions. This is illustrated by the continuous drop in the mass of the aldolase subunit under the conditions cited in this paper. Therefore, the purpose of this report is to point out that protein primary structure can be extremely labile in dilute alkali and, as a result, several difficulties may be encountered in determining subunit molecular weights when alkali is used as a dissociating medium. An explanation is also provided for the earlier conjecture that rabbit muscle aldolase might be comprised of six subunits (1) rather than four as recently suggested by other investigators (3-7).

### TABLE III

<table>
<thead>
<tr>
<th>Method</th>
<th>Experiment</th>
<th>NH₂-terminal amino acids found²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Pro</td>
</tr>
<tr>
<td>Edman</td>
<td>Untreated</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>2.89</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>3.71</td>
</tr>
<tr>
<td></td>
<td>Alkali-treated (20 min)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>4.00</td>
</tr>
<tr>
<td></td>
<td>Alkali-treated (20 hrs)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>5.43</td>
</tr>
<tr>
<td>Sanger</td>
<td>Untreated</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1.52</td>
</tr>
<tr>
<td></td>
<td>Alkali-treated (120 hrs)</td>
<td>6.51</td>
</tr>
</tbody>
</table>

² Corrected values based on a molecular weight of 159,000 for native aldolase.

 Evelyn H. Sine and L. F. Hass 437
weight at several expanded time intervals. The resulting values may then be extrapolated to zero time in alkali to obtain a valid estimate of the actual molecular weight. In order to use this procedure, however, the rate of hydrolytic degradation must be appreciably less than the rate of attainment of sedimentation equilibrium. One can be assured of this by showing, with interference optics, that there is little or no change in fringe displacement ($M$) over a reasonable period of time (several hours) under conditions in which complete centrifugal equilibration would normally be expected (see "Materials and Methods").

Another potential source of error which could have yielded low estimates of the aldolase subunit molecular weight involves the extrapolation of $1/M_{app}$ against $c$ to overcome thermodynamic nonideality. This type of extrapolation is theoretically appropriate when applied to uncharged macromolecules at equilibrium (33) but, currently, there is no satisfactory theory which allows for similar treatment of charge-dependent systems (33, 34). Consequently, the molecular weight averages obtained from Figs. 3, A and B, and 5 could be low estimates of the average masses of the protein. On the other hand, it is theoretically possible to surmount charge nonideality through use of the charge-independent moments ($M_1$ and $M_2$) defined by Yphantis (see text). When this is done, it is found that there is good agreement in the subunit molecular weights arrived at by both methods after compensating for hydrolytic degradation (Figs. 4 and 7). Thus, it is believed that the subunit values of 41,400 to 42,000 reported here are essentially correct. Moreover, these values are in excellent agreement with those obtained by other investigators (3, 7). Based on these findings, the ratio of the native molecular weight to that of the subunits is approximately 4:1, strongly supporting the concept that aldolase is comprised of four polypeptide chains (3–7).

When aldolase (or succinyl aldolase) is exposed to alkaline conditions at elevated temperatures, there is an initial rapid decrease in the molecular weight of nondialyzable protein for a period of approximately 60 hours. Following this, the rate of degradation slows down considerably so that, after 288 hours, a molecular weight of 15,000 is still obtained. This resistance to further alkaline degradation suggests that hydrolysis is not completely random. In fact, the occurrence of "hard core" material could indicate that alkali-treated aldolase is capable of maintaining (or forming) certain structural features which impede its complete destruction. In this regard, Aune et al. (35) recently reported that many proteins are capable of retaining regions of ordered structure in acid. Additional evidence in favor of alkali-resistant material is provided by the electrophoresis experiments (Fig. 10.1) which show (within the limits of detection) that very few hydrolytic species are formed after long term exposure of aldolase to elevated pH values. Since equivalent conditions were used for both the electrophoresis and the kinetic studies, the less anodic bands in Fig. 10.1 probably represent the hard core material found by ultraconcentration.

The formation of new NH$_2$-terminal residues during alkaline degradation is restricted to serine, threonine, glycine, and a small amount of proline (see Table III). The lability of proline peptide bonds is not surprising since tertiary amides exhibit more carbonyl character than either primary or secondary amides and are more susceptible to nucleophile attack by OH$^-$. Presumably, hydrolysis results as a consequence of the restricted resonance contribution by the amide nitrogen resulting from the formation of the dipolar ionic hybrid.

The alkaline lability of peptide bonds containing serine and threonine suggests that dilute alkali (like acid) is capable of promoting an $N \rightarrow O$ peptidyl shift through the formation of a hydroxysuccinimine intermediate (II) (36).

![Diagram](http://www.jbc.org/)

The resulting ester (III) should then undergo rapid hydrolysis to produce fragmentation of the peptide chain. Bergmann and Brand (37) have established that the $N$-acetyl derivative (I) is favored under neutral or slightly alkaline conditions, but it should be noted that all of the degradation studies described here were performed in the presence of borate buffer. This may have special significance in light of the recent report of Cunningham and Schmir (38) which illustrates that the alkaline hydrolysis of 4-hydroxybutyranilide is greatly accelerated in the presence of either phosphate or bicarbonate buffers. In order to account for the catalysis, the following intermediates were proposed:

![Diagram](http://www.jbc.org/)

On the basis of this proposal, it seems possible that borate might also function as a catalyst by participating in the formation of a stabilizing intermediate which allows the hydroxyl group of either serine or threonine to become involved as an intramolecular nucleophile in the selective breakdown of polypeptides. In this regard, Bruice and Marguardt (39) have shown that the hydrolysis of primary amides over the pH range 7.8 to 9.1 is substantially accelerated in the presence of both borate and phosphate salts. The same investigators have also shown that the introduction of a hydroxyl group in the $\gamma$ position of butyramide greatly increases alkaline catalysis of the amide bond.

The appearance of glycine as a new NH$_2$ terminal amino acid is puzzling since the involvement of this residue in selective bond cleavage is difficult to visualize. Earlier studies by other investigators (40, 41) indicate that serine and threonine in peptide linkage are readily degraded to glycine and a variety of other products when exposed to strong alkali at elevated temperatures. Whether or not degradation of the hydroxyamino acids to glycine occurs under the conditions described here remains to be investigated.

Acknowledgments—We are grateful to Dr. D. A. Yphantis and Mr. Dennis Roark for providing us with their high speed sedimentation equilibrium computer program and for the many

References

438 Studies on Structure and Function of Muscle Aldolase. IV Vol. 244, No. 2
helpful discussions regarding equilibrium centrifugation techniques. We would also like to thank Mr. Joseph Morganti and Mrs. Catherine Whaley for their expert technical assistance.

REFERENCES

Studies on the Structure and Function of Muscle Aldolase: IV. THE ACTION OF DILUTE ALKALI ON PRIMARY STRUCTURE AND ITS EFFECT ON THE DETERMINATION OF SUBUNIT MOLECULAR WEIGHT

H. E. Sine and L. F. Hass


Access the most updated version of this article at [http://www.jbc.org/content/244/2/430](http://www.jbc.org/content/244/2/430)

Alerts:
- When this article is cited
- When a correction for this article is posted

[Click here](http://www.jbc.org/content/244/2/430.full.html#ref-list-1) to choose from all of JBC’s e-mail alerts

This article cites 0 references, 0 of which can be accessed free at [http://www.jbc.org/content/244/2/430.full.html#ref-list-1](http://www.jbc.org/content/244/2/430.full.html#ref-list-1)