Alkali-induced Structural Changes in Muscle Aldolase

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Received May 21, 1963

The physical and enzymatic properties of rabbit muscle aldolase have been investigated over the *p*H range from 8.2 to 12.6. At *p*H 12.6, six subunits having molecular weights of approximately 22,400 are rapidly formed from the native enzyme (mw 140,000). This process is accompanied by a marked change in the Drude constant λ_c from 265 mμ to 222 mμ. Similarly, there is a decrease in the sedimentation coefficient $s^0_{20,w}$ from 7.43 to 1.81. Detailed studies within the *p*H range between 10.7 and 12.6 indicate that molecular expansion precedes a dissociation process in which a 55,000 molecular weight intermediate is formed. Differential spectral analyses reveal that thirty-one to thirty-three of the forty-four tyrosyl groups in aldolase titrate anomalously; however, if aldolase is exposed to 4.0 M urea for 1 hour, all the tyrosyl groups titrate normally. Kinetic studies on the rate of tyrosinate ion formation indicate that molecular unfolding is biphasic and that both rates obey the first-order rate law. After a 1-minute exposure to *p*H 12.6 buffer at room temperature, aldolase recovers 70–75% of its initial activity upon neutralization. Longer periods of exposure at *p*H 12.6 result in greater losses in regainable activity. The kinetics of reactivation and irreversible denaturation are both first order.

Recent experiments indicate that under the proper conditions completely denatured, single-chained, low molecular weight proteins (e.g., ribonuclease, White, 1961; lysozyme; and taka amylase, Isemura *et al.*, 1961) reassume their active native conformations as a result of the intrinsic thermodynamic properties of their primary amino acid sequences (White, 1961; Haber and Anfinsen, 1961). Several examples of the reversible dissociation of multicomponent proteins also appear in the literature (e.g., see Deal *et al.*, 1963a); however, several reports show that after exposure to certain denaturing conditions similarly complex molecules do not renature or have not been successfully renatured (e.g., glutamic dehydrogenase, Frieden, 1962; pyruvate kinase, Morawiecki, 1960). Other proteins, as indicated by various physical and hydrodynamic parameters, appear to be renatured, but are devoid of biological activity (e.g., myosin, Young *et al.*, 1962; catalase, Samejima *et al.*, 1961).

Current investigations on rabbit muscle aldolase (mw 142,000) show that the native molecule can be reconstituted in large part after dissociation into three polypeptide chains (mw 45,000) following treatment with urea, HCl, or acetic acid (Stellwagen and Schachman, 1962; Deal *et al.*, 1963b). These findings, coupled with the report that aldolase has three C-terminal tyrosyl residues that can be removed by treatment with carboxypeptidase (Kowalsky and Boyer, 1960), appear to indicate that the native molecule is composed of three basic subunits. The results presented here, however, show that when aldolase is exposed to an environment above *p*H 12.0 at 23°, the enzyme rapidly and irreversibly disrupts into six stable subunits having apparently equivalent molecular weights of approximately 22,400 (Hass and Lewis, 1963). This process of alkali-induced subunit formation has been studied by ultracentrifugation, polarimetry, and spectrophotometry. Reconstitution after brief exposures to alkaline *p*H values has also been examined with the view of obtaining a better understanding of the requirements necessary for the formation of the intricate molecular structure essential for catalytic activity.

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Materials and Methods

Materials.—Twice-recrystallized 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 in 0.5 saturated ammonium sulfate at 4° until used. Overnight dialysis against a continuous flow of cold distilled water followed by lyophilization completely removed ammonium sulfate from the enzyme preparation and reduced the triose phosphate isomerase contamination to less than 1.0%. Dissolved portions of lyophilized aldolase were used in most of the experiments reported.

α-Glycerophosphate dehydrogenase was purchased from Boehringer und Soehne. The tetrasodium salt of fructose-1,6-diphosphate was purchased from the Sigma Chemical Company and reduced nicotinamide adenine dinucleotide was obtained from the Pabst Laboratories. D,L-Glyceraldehyde-3-phosphate was kindly supplied by Dr. A. H. Mehler and was used for the determination of triose phosphate isomerase activity.

Aldolase Assay Procedures.—Aldolase was assayed by the method of Racker (1947) using α-glycerophosphate dehydrogenase. Protein was determined by absorption at 280 mμ using an extinction coefficient, $E^{0.1\%}_{1\text{ cm}} = 0.91$ (Baranowski and Niederland, 1949). Specific activity was measured at 340 mμ and gave an absorbancy change of 90–95/min/mg of aldolase/ml at 23° and *p*H 8.0.

Polarimetric Measurements.—Optical rotatory dispersion data were obtained with a Rudolph Model 80 photoelectric spectropolarimeter utilizing an oscillating polarizer and the method of symmetrical angles. The polarimeter tube (100 mm × 8.5 mm) was filled with an 0.1% enzyme solution and was maintained at 23° during all determinations. Measurements were performed at wavelengths corresponding to the strong mercury lines between 600 and 300 mμ. A fixed symmetrical angle of 5° was used together with a slit opening of usually less than 0.1 mm. Repeated measurements remained stable within ±0.003° after the enzyme had been exposed to the solvent for at least 0.5 hour. Corrections were made for the rotation of the solvent, but small corrections for the solvent-refractive index were neglected (Gordon and Jencks, 1963). The

rotatory dispersion data were observed to fit the one-term Drude equation, $[\alpha]_\lambda = k/(\lambda^2 - \lambda^2_c)$, where $[\alpha]_\lambda$ is the specific rotation at wavelength λ. The Drude constants λ_c were calculated from the slopes of the plots of $[\alpha]_\lambda^2\lambda$ vs. $[\alpha]_\lambda$ (Yang and Doty, 1957).

Ultracentrifuge Determinations.—Sedimentation coefficients and molecular weights were determined with a Spinco Model E analytical ultracentrifuge equipped with a rotor temperature indicator and control unit, a phase plate schlieren diaphragm, and a Rayleigh interference optical system. Photographic plates were measured with a Nikon Model 6 optical comparator equipped with Mann lead screws.

Sedimentation coefficients were determined at 20° using a rotor speed of 59,780 rpm. Kel-F centerpieces were employed because of the high alkalinity of several of the buffers used. Sedimentation coefficients were calculated by the usual method and were corrected to values in water at 20°. Reciprocal plots of values obtained at several protein concentrations were extrapolated to infinite dilution to obtain $s^0_{20,w}$.

Sedimentation equilibrium experiments were performed by using either the short-column technique as described by Richards and Schachman (1959) or the high-speed equilibrium technique as described by Yphantis (1962). Liquid column heights of 1.6–1.7 mm were used in the short-column experiments. Three samples were run simultaneously in the Spinco J rotor. Two 12-mm wedge centerpiece cells were employed with the more concentrated protein solutions and a 30-mm cell was used with the most dilute solution. The initial protein concentration was determined in the centrifuge using a synthetic-boundary cell; less concentrated solutions were obtained by serial dilution. Graphs of ln c vs. x^2 were constructed. If the linearity of the plots indicated homogeneity, the values of $d \ln c/dx^2$ were calculated and the apparent molecular weights were obtained from the equation:[1]

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

If the material appeared polydisperse, the apparent weight average molecular weight was obtained from the equation:

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

In cases where the molecular weight was found to be a function of protein concentration, the reciprocals of the apparent molecular weights were extrapolated to infinite dilution to obtain M_w^0.

The high-speed equilibrium technique described by Yphantis (1962) was modified by using a double-sector synthetic-boundary centerpiece. This permitted layering buffer on a small volume of very dilute protein solution. When used in conjunction with an initial overspeeding of the rotor, a very significant saving in the time required to attain equilibrium was accomplished.

Spectrophotometric Measurements.—Ultraviolet difference spectra, tyrosine titrations, and the kinetics of abnormal tyrosine ionization were obtained with a Cary Model 14 recording spectrophotometer. *p*H measurements were performed on a Beckman Model 76 expanded scale *p*H meter equipped with a Beckman combination electrode No. 39183. For determinations above *p*H values of 7.0, 9.0, and 11.5, the *p*H meter was standardized against reference buffers having *p*H values of 7.00, 10.00, and 12.52, respectively. All spectral measurements were made at a constant temperature of 23°.

[1] In the above equations (Richards and Schachman, 1959) R = the gas constant, T = the absolute temperature, $\bar{V}$ = the partial specific volume of the protein (assumed to be 0.740 for all conditions of temperature and *p*H, Taylor *et al.*, 1948), ρ = the density of the solution, c = protein concentration, ω = the angular velocity, c_0 = the initial protein concentration, and m and b refer to the meniscus and bottom of the liquid column, respectively.

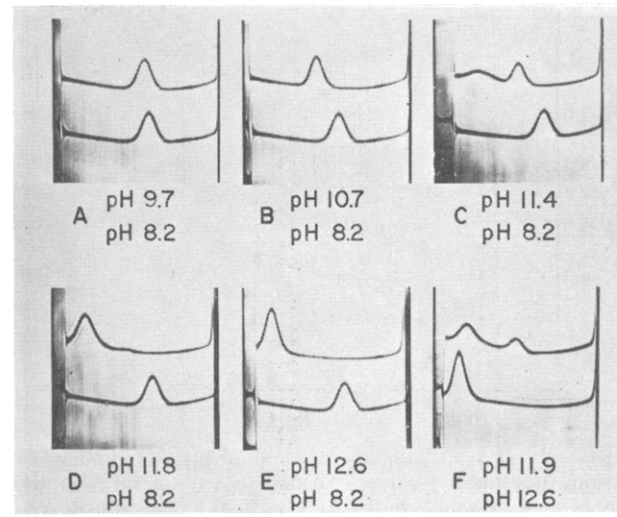

FIG. 1.—Sedimentation patterns of aldolase at various alkaline *p*H values. All solutions contain approximately 0.5% aldolase in KCl-borate buffer at a constant ionic strength of 0.26. Photographs were taken at 64 minutes and a phase plate angle of 65° after reaching a rotor speed of 59,780 rpm. All sedimentation runs were performed at 25° with the exception of that shown in F. Frame F represents enzyme solution maintained at 0° for 0.5 hour prior to sedimentation at 4°.

Spectrophotometric tyrosine titrations were performed at various *p*H values by adding appropriate amounts of approximately 1.0 N KOH to the diluted protein solutions so that a constant volume of 1.0 ml was maintained. Readings were taken within 30 seconds after the addition of alkali. To insure against *p*H changes due to CO_2 absorption, all samples were maintained in tightly stoppered cuvets.

For the reverse titration experiments, aldolase samples were added to a *p*H 12.6 KCl-borate solution (initial vol, 1.0 ml) and allowed to remain at room temperature for approximately 1 to 2 minutes. These samples were titrated to lower *p*H values by the addition of appropriate volumes of 1.0 N HCl. Small optical density corrections were made for the various volume changes, assuming application of Beer's law.

For titrations in urea, the aldolase samples were exposed to a 4.0 M urea solution for at least 1 hour at room temperature prior to the addition of KOH. Corrections for optical density changes at different *p*H values due to the presence of 4.0 M urea were unnecessary. If sufficient time were not allowed for the relatively slow denaturation of aldolase by urea, anomalous tyrosine titration was observed as explained in the Results section.

Aldolase Dissociation and Reactivation.—Alkali-dissociated aldolase was prepared at room temperature by adding an equal volume of KCl-borate buffer, at various *p*H values, to the enzyme dissolved in distilled H_2O. The final concentration of borate was 0.2 M with sufficient KCl present to give an ionic strength of 0.26; above *p*H 12.0 the borate concentration was 0.1 M with no change in ionic strength. Preparations were usually checked for exact *p*H values before performing physical measurements.

All buffers used in the reactivation experiments were saturated with nitrogen, and mercaptoethanol (final concentration, 0.01 M) was added to prevent the rapid

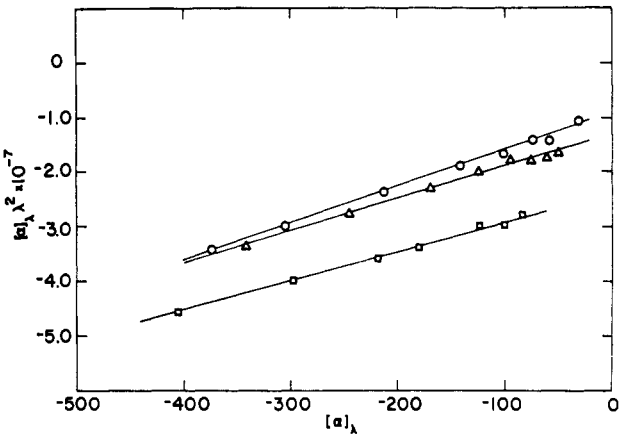

FIG. 2.—Optical rotatory dispersion data of aldolase at various alkaline *p*H values. Aldolase, 1.0 mg/ml, was dissolved in KCl-borate buffer at μ = 0.26. The borate concentration was 0.1 M at *p*H 12.6 and 0.2 M at the lower *p*H values. Specific optical rotations $[\alpha]_\lambda$ were determined as described in the Materials and Methods section. All measurements were made at 23°. O, △, and □ represent data obtained at *p*H 8.2, 10.7, and 12.6, respectively.

oxidation of enzyme sulfhydryl groups promoted by alkaline conditions. At selected intervals, aliquots of the alkaline aldolase solution were rapidly diluted in 0.2 M sodium acetate buffer, *p*H 5.5 (final *p*H, 6.1). The kinetics of enzyme reactivation, measured by standard assay procedures, was followed by comparison with a similarly treated aldolase preparation maintained at *p*H 8.2.

RESULTS

Properties of Alkali-treated Aldolase.—Figure 1 shows that the sedimentation pattern of aldolase is considerably altered as the *p*H of the medium is raised from 9.7 to 10.7. Beyond this *p*H range the formation of a second, slower sedimenting component becomes clearly visible, and by comparing frames C (*p*H 11.4, 25°), D (*p*H 11.8, 25°), and F (*p*H 11.9, 4°) it is evident that the extent of this formation is greatly influenced by both *p*H and temperature. Since an alkali-induced irreversible process would be expected to go to completion at a constant alkaline *p*H, the diffuse boundaries observed in the intermediate *p*H ranges apparently represent an equilibrium mixture of several interacting components. This has been substantiated further by molecular weight studies described later. Above *p*H 12.0 at 25° the slower sedimenting component resolves itself into a species having a single symmetrical boundary.

Figure 2 represents typical optical rotatory dispersion data plotted at selected alkaline *p*H values according to the method of Yang and Doty (1957). From curves such as these, Drude constants λ_c were obtained over the entire alkaline range studied and are illustrated as a function of *p*H in Figure 3. For purposes of comparison, a similar graph of sedimentation coefficients $s^0_{20,w}$ has been incorporated into the same figure.

Upon denaturation the Drude constant for aldolase decreases from 265 mμ at *p*H 8.2 to 222 mμ at *p*H 12.6, with the greatest drop occurring in the *p*H region where two components are observed in the ultracentrifuge. The Drude constants observed at the extreme *p*H values are in fair agreement with those reported by Jirgensons (1961a) for neutral (λ_c = 278 ± 8) and alkaline (λ_c = 220 ± 5) aldolase solutions. Concomitantly, there is also a decrease in the sedimentation coefficient from 7.43 to 1.81 as the *p*H is raised from 8.2 to 12.6. In Figure 3A the solid line resembles a titration curve with a mid-point value of 10.7, while the plot in Figure 3B has an estimated mid-point value of 11.4.

Molecular Weight Determinations.—Figure 4 shows plots of ln *c* vs. x^2 for aldolase at *p*H 8.2 and 12.6. The plots indicate slight or negligible deviations from linearity, demonstrating that at these *p*H values the proteins are essentially homogeneous. No significant trends in the slopes obtained at different protein concentrations are observed, illustrating that there is apparently no dependence of molecular weight on concentration at either *p*H. The molecular weights, therefore, have been calculated from the average values of these slopes. A molecular weight of 140,000 was found at *p*H 8.2, in good agreement with that reported by Stellwagen and Schachman (1962) for native aldolase. At *p*H 12.6 a molecular weight of 22,400 was found. Within the limits of experimental error, this molecular weight shows that under highly alkaline conditions the aldolase molecule disrupts into six apparently homogeneous subunits.

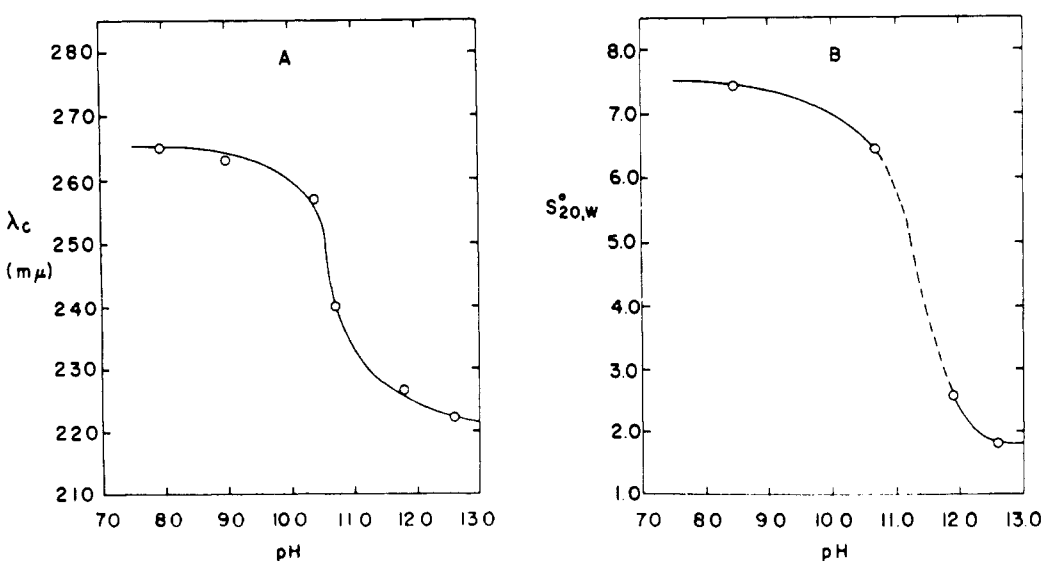

FIG. 3.—(A) Variation in the Drude parameter λ_c of aldolase as a function of *p*H in KCl-borate buffer, μ = 0.26. All other conditions were the same as those described in Fig. 2. (B) Variation in the sedimentation coefficient $s^0_{20,w}$ of aldolase as a function of *p*H in KCl-borate buffer, μ = 0.26.

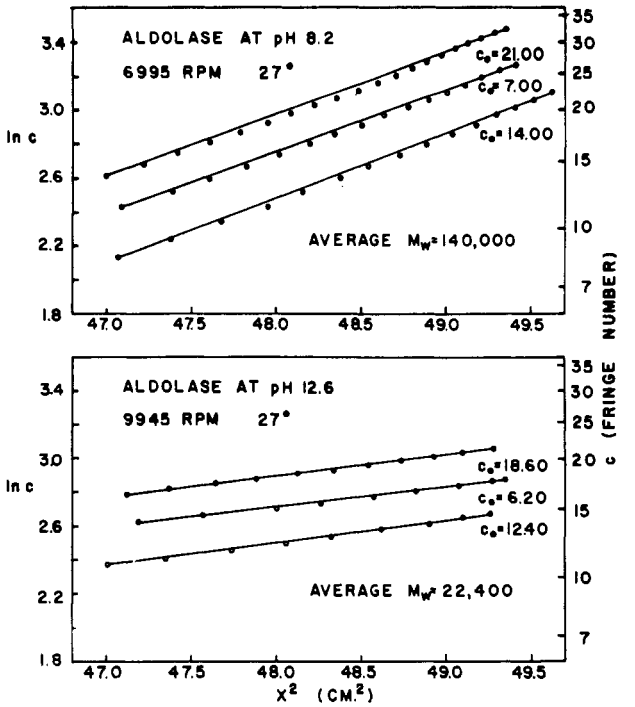

FIG. 4.—Molecular weight determinations from sedimentation equilibrium experiments on aldolase at *p*H 8.2 and 12.6 in KCl-borate buffer, μ = 0.26. The left-hand ordinate gives the logarithm of protein concentration in fringes, the right-hand ordinate gives the protein concentration directly in fringes, and the abcissa represents the square of the distance from the center of rotation.

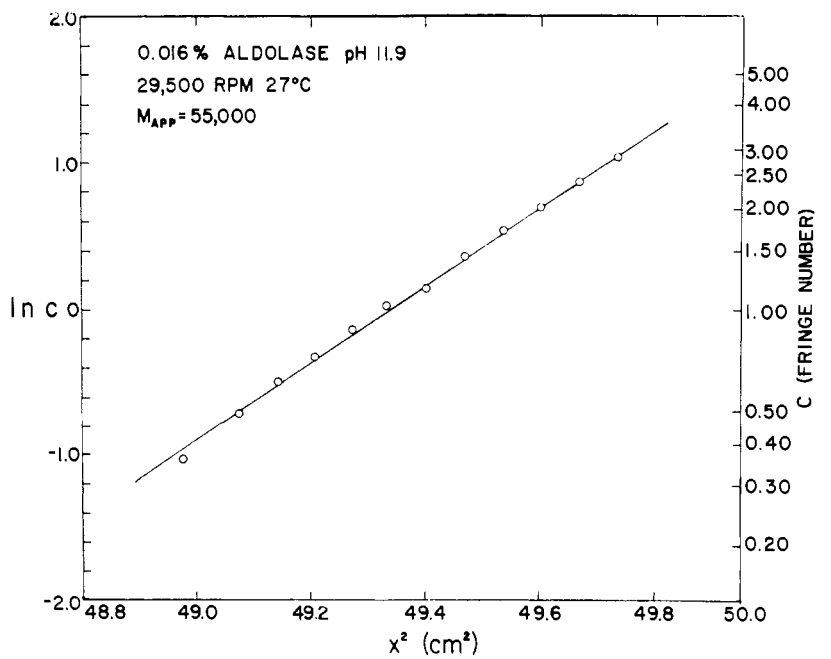

FIG. 5.—Molecular weight determination of a high-speed sedimentation equilibrium experiment on aldolase at *p*H 11.9 in KCl-borate buffer, μ = 0.26. The coordinates have the same designations as those in Fig. 4.

At *p*H 12.6 the possible effect of protein charge density upon the apparent molecular weight must be considered. Pederson (1940) has demonstrated that a "primary charge effect" decreases the apparent molecular weight, is more marked at high protein concentrations, and is damped out by increasing the ionic strength of the solution. The absence of any significant dependence of molecular weight on protein concentration, illustrated in Figure 4, strongly argues that the ionic strength of 0.26 is sufficient to damp out the effect of charge on molecular weight; therefore the value reported is believed to be essentially correct.

Short-column equilibrium experiments conducted at *p*H 10.7 indicated the presence of polydisperse material despite the appearance of a single component in the sedimentation pattern shown in Figure 1. When the apparent weight average molecular weights at several protein concentrations were extrapolated to infinite dilution, a value of 129,000 was obtained. This molecular weight indicates that approximately 10–12% dissociation into subunits occurs at *p*H 10.7 while most of the protein remains in the aggregated form.

Studies were made at *p*H 11.9 to determine whether an intermediate with a molecular weight of approxi-

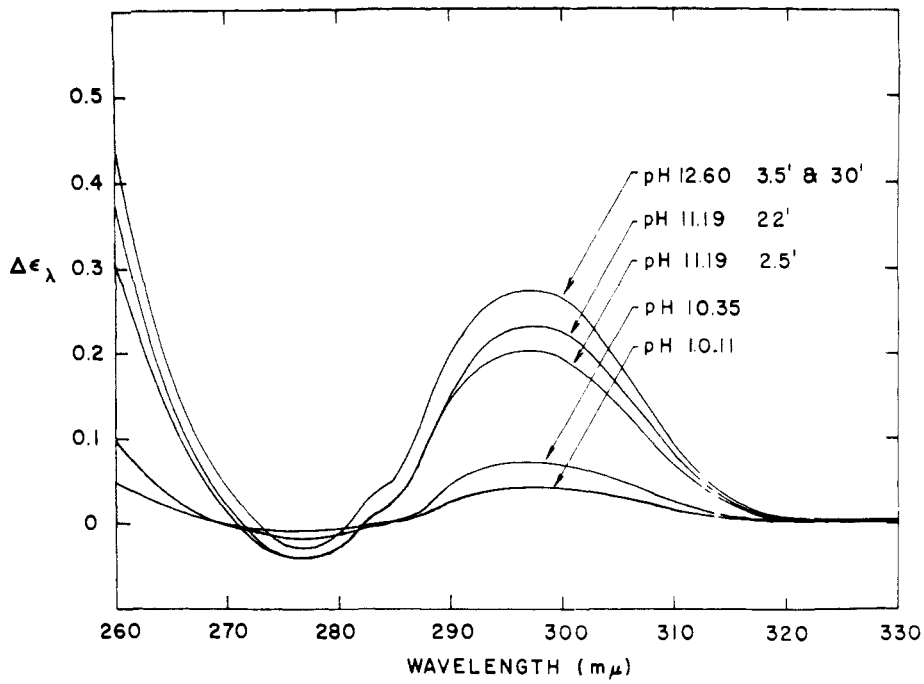

FIG. 6.—Ultraviolet difference spectra of aldolase in 0.2 M KCl and 0.05 M borate as a function of the degree of tyrosine ionization against a blank at *p*H 7.30. Enzyme concentration, 0.5 mg/ml.

mately 47,000 ocould be demonstrated.[2] Preliminary experiments using conventional short-column equilibrium techniques gave several uncorrelated, anomalously high molecular weights along with indications of marked polydispersity. At this time, it was conceived that due to the poor buffering capacity of borate at *p*H 11.9 the medium was altered to lower *p*H values, which upset equilibrium conditions and caused the formation of high molecular weight material. Lowering of the *p*H could be explained by the buffer reacting with the aluminum in the Epon centerpiece during the course of an equilibrium run. Since this effect could be minimized by determining the molecular weight on a very dilute protein solution in an experiment of short duration, the high-speed equilibrium technique was used. Figure 5 shows an ln *c* vs. x^2 plot for a typical experiment at a protein concentration of 0.016%. The molecular weight of 55,000 observed after 150 minutes is very close to the value predicted at *p*H 11.9. The deviation from the expected value of 47,000 could be explained by the normal error in measuring the photographic plate. On the basis of $s^0_{20,w}$ values (2.58 at *p*H 11.9 vs. 1.81 at *p*H 12.6) and sedimentation patterns, there are no indications that lower molecular weight material (22–24,000) is present.

Difference Spectrophotometry and Tyrosine Titration.—The ultraviolet difference spectra for aldolase at several intermediate *p*H values from 10.1 to 12.6 are illustrated in Figure 6. Prior to determining difference spectra, a constant spectrophotometer base line was established over the entire spectral range studied. The curves illustrate that between *p*H 10.1 and 11.1 there is an apparent slight shift in the absorption maximum from 295 to 297 mμ, with no further change occurring at higher *p*H values. From *p*H 11.1 to 12.6, the optical density changes are obviously time dependent, indicating the presence of anomalously titrating tyrosine groups. A similar phenomenon has been reported by Sage and Singer (1962) for aqueous solutions of ribonuclease and has been attributed to the findings that three of the six tyrosine phenolic groups of ribonuclease titrate at abnormally high *p*H values (Shugar, 1952; Tanford *et al.*, 1955).

Figure 7A shows the forward and reverse spectrophotometric titrations of the tyrosines in aldolase at 297 mμ. Analysis of the forward titration indicates that out of a total of forty-four tyrosyl residues (Velick and Ronzoni, 1948), eleven to thirteen ionize normally, while thirty-one to thirty-three ionize abnormally and show an optical density change with time. Moreover, two to three tyrosyl groups are extremely resistant to ionization and show evidence of titrating only above *p*H 13.5.[3] Although the latter phenomenon is difficult to explain at the present time, it has been observed with several other proteins (e.g., ovalbumin, Crammer and Neuberger, 1943; paramyosin, Riddiford and Scheraga, 1962; ribonuclease, Shugar, 1952, and Tanford *et al.*, 1955).

The obvious irreversibility of the titration of the tyrosines in aldolase is indicated by the optical density values obtained upon reverse titration. Within experimental error, these values follow a normal titration curve (solid line, Fig. 7A) calculated from the equation, $pH = pK + \log \Delta E_{297}/(\Delta E_{max} - \Delta E)_{297}$ (Hermans and Scheraga, 1961), where a pK_a of 10.40 and a molar extinction coefficient ΔE_{max} of 94,000 for forty-two tyrosinate ions has been assumed. The pK_a of 10.40 is slightly higher than the pK_a of 10.15 for unsubstituted tyrosine, but the effect of peptide combination alone is sufficient to increase this value significantly (Beaven and Holiday, 1952). Tanford (1950) also has indicated that the electrostatic effects of charged groups are the most important factors in determining the pK_a values of ionizable residues in proteins.

After 1 hour in 4.0 M urea all the tyrosyl residues in aldolase titrate normally, including those resistant to ionization at *p*H values above 13.5. The experimental curve illustrated in Figure 7B has a pK_a value of approximately 10.45 ± 0.05, which is in good agreement

[2] The value of 47,000 represents one-third of 140,000 which is the molecular weight for native aldolase reported here.

[3] Because of uncertainties in the accuracy of determining *p*H values above 13.0, the estimated *p*H of 13.6 shown in Figure 7A is based on the amount of KOH added to the medium.

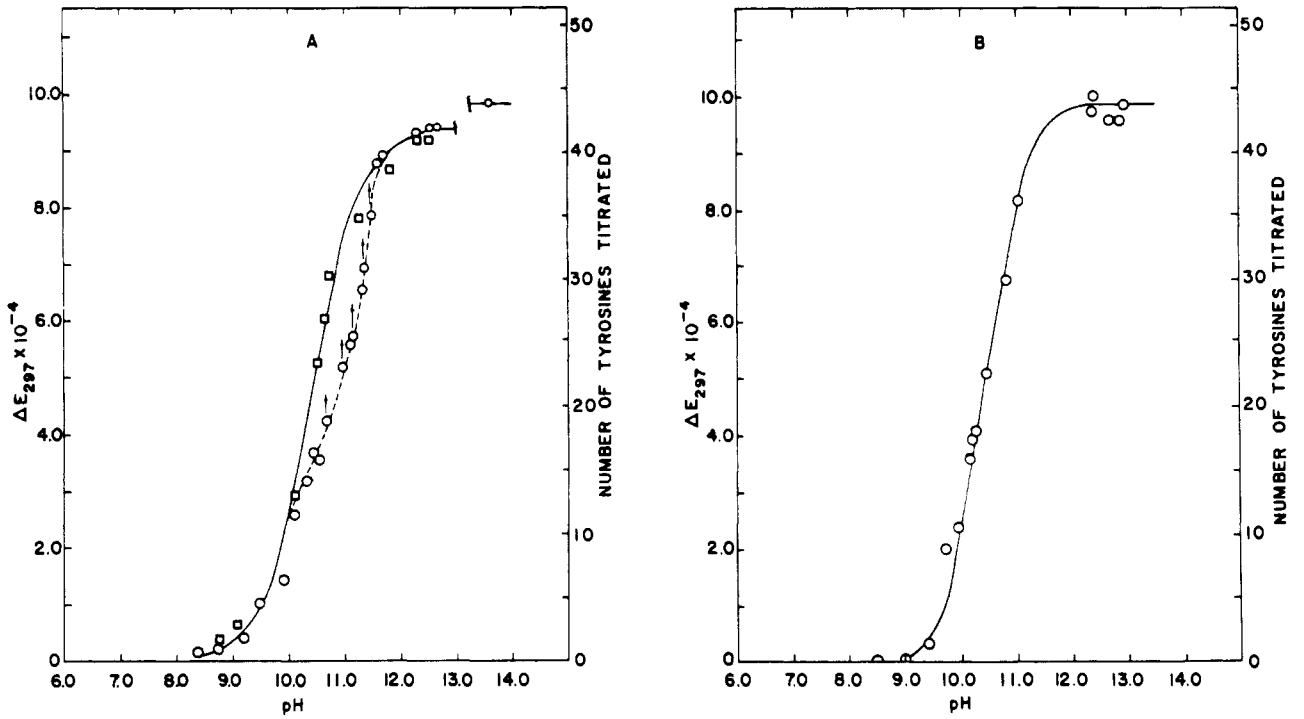

FIG. 7.—(A) Spectrophotometric titration of the tyrosines in aldolase in 0.2 M KCl and 0.05 M borate at 23° and at a wavelength of 297 mμ. Samples were measured against a reference solution at *p*H 7.45. Arrows indicate an optical density change with time. The points in the region of the dotted curve were obtained by extrapolation to zero time. The solid curve is based on the equation derived by Hermans and Scheraga (1961). The squares represent the reverse titration. (B) Spectrophotometric titration of the tyrosines in aldolase in 4.0 M urea containing 0.2 M KCl and 0.05 M borate. Other conditions were the same as in part A. The titration curve is based on experimental values.

with the calculated value mentioned previously. If the protein samples are exposed to urea for shorter periods of time (5 to 10 minutes), anomalous titration curves are obtained similar to the one illustrated in Figure 7A. The action of 4.0 M urea, therefore, appears to be comparable to alkali at intermediate *p*H values in causing unfolding of the native aldolase molecule.

The change in the molar extinction coefficient[4] ΔE at 297 mμ is 98,700 for forty-four tyrosyl residues, or 2,240 per group. The latter value is close to 2,330, which is the molar extinction coefficient reported at 293.5 mμ for free tyrosine dissolved in 0.1 N NaOH (Beaven and Holiday, 1952).

Kinetics of Abnormal Tyrosine Ionization.—Semilog plots of $(\Delta E_{max} - \Delta E)_{297}$ vs. time are illustrated in Figure 8 and show that the rates of abnormal tyrosinate anion formation obey the first-order rate law. At *p*H 10.85 (Figure 8A), where ionization occurs relatively slowly, the rate of tyrosinate formation is biphasic. The initial rate (k = 0.041 min^{-1}) is approximately three times faster than the final rate (k = 0.014 min^{-1}), indicating that certain tyrosine groups are more susceptible to OH^- attack than others. Since the initial rate of abnormal tyrosine ionization apparently increases rapidly as a function of small changes in *p*H, the data in Figure 8B represent the second phase of tyrosinate formation only. Even in this figure, the rate of tyrosine ionization increases 2.5-fold (k increases from 0.39 to 1.06 min^{-1}) as the *p*H is raised approximately 0.1 unit. At *p*H values higher than those indicated, optical density changes due to tyrosinate formation are extremely rapid and become immeasurable by the methods used.

[4] The molar extinction coefficient is based on a molecular weight of 149,000 for aldolase (Velick and Ronzoni, 1948). ΔE has not been corrected for the insignificant optical density contributions due to tryptophan and cysteine.

Reactivation of Alkali-treated Aldolase.—In order to prevent random aggregation of polypeptide chains by the direct neutralization of concentrated aldolase solutions, reactivation experiments were performed by the rapid dilution technique described in the Materials and Methods section.

Figure 9A illustrates that the per cent aldolase reactivation is a function of two parameters: the length of time the denatured enzyme remains in the renaturing medium and the length of time the native enzyme is exposed to alkaline conditions. If aldolase is limited to a 1-minute exposure at *p*H 12.6 and 23°, a maximum of 70–75% of its initial activity is regained; however, if the enzyme remains at this *p*H value for longer periods of time the total per cent reactivation diminishes. After 20 minutes at *p*H 12.6, the maximum activity recovered is only about 19%.

The irreversible inactivation of aldolase at *p*H 12.6 is best illustrated in Figure 9B. Linear semilog plots of the fraction of total aldolase activity at several reactivation times vs. time at *p*H 12.6 indicate inactivation follows first-order kinetics. The rate constant 0.077 min^{-1} is low compared with the values obtained for tyrosine ionization at intermediate alkaline *p*H values.

Since most of the data presented indicate that aldolase reactivation probably involves the reassociation of several subunits, the effect of enzyme concentration has been investigated. Figure 10 shows the kinetics obtained over a 10-fold concentration range in the reconstitution medium. As indicated, there is no obvious distinction between the initial rate and extent (70–75%) of reactivation over a 3-fold difference in enzyme concentration (100 μg to 33 μg/ml). This observation is opposed to the report that the total recovery of enzymic activity is substantially decreased below 40 μg/ml for acid-dissociated aldolase (Stellwagen and Schachman, 1962). Even at an enzyme concentration of 10 μg/ml, Figure 10 shows that 50% activity

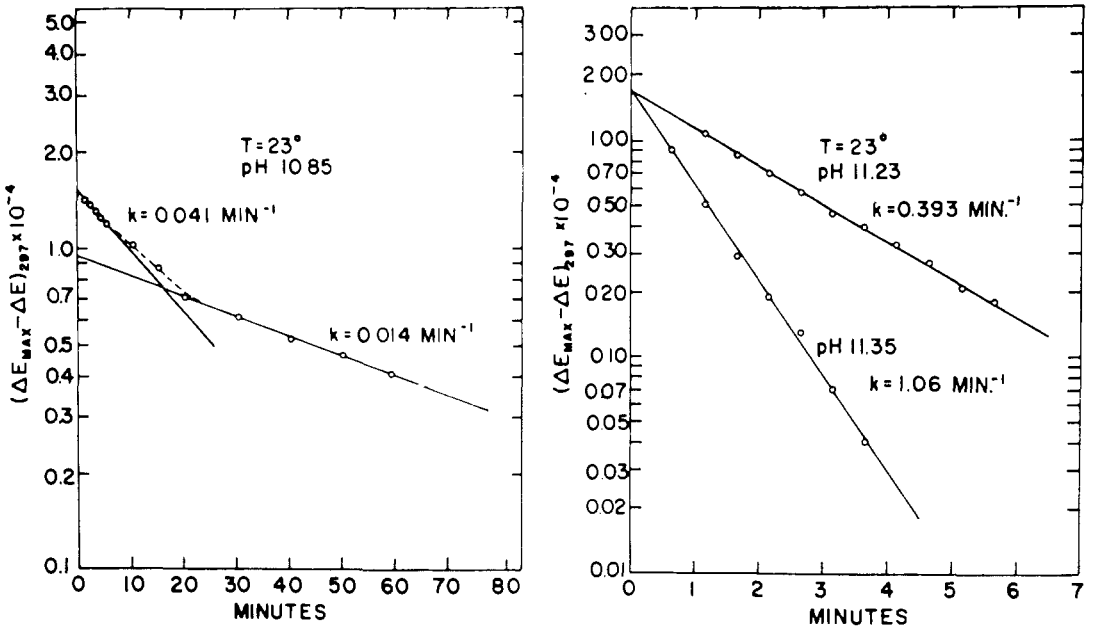

FIG. 8.—Kinetics of the ionization of the abnormal tyrosines in aldolase at several *p*H values. ΔE_{max} represents the change in the molar extinction coefficient at infinite time and ΔE represents the change in the molar extinction coefficient at time t. Conditions are the same as in Fig. 7.

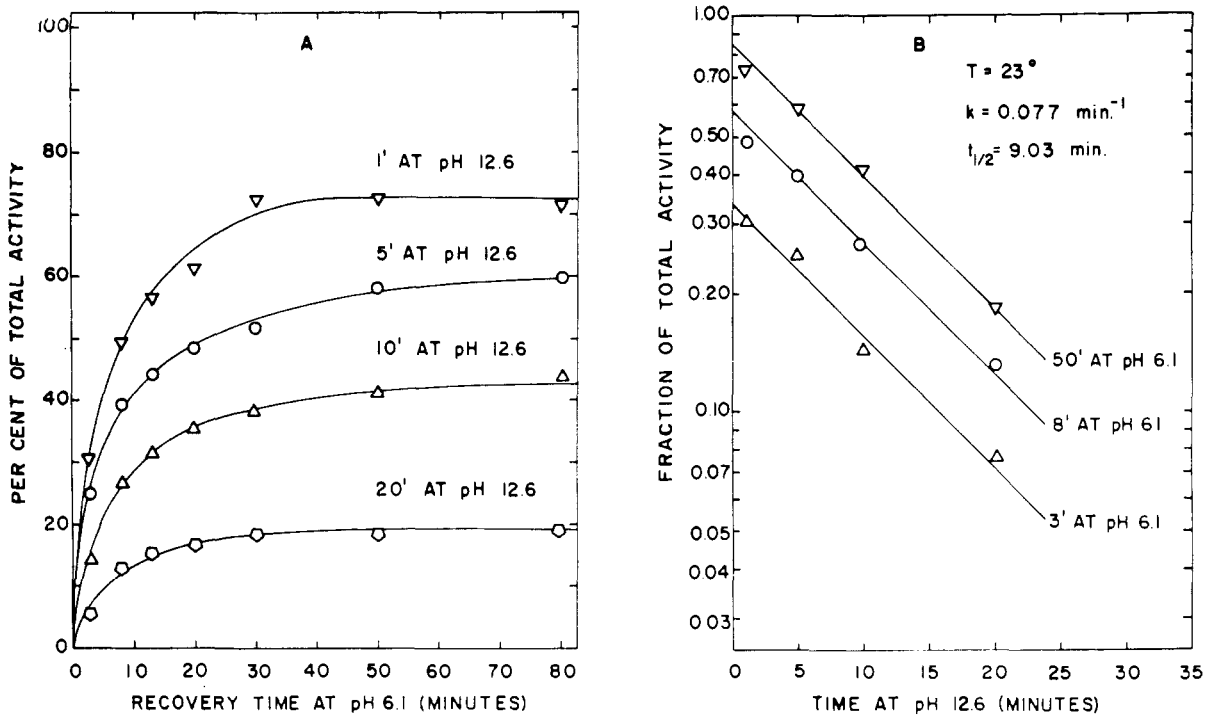

FIG. 9.—The kinetics of aldolase reactivation and irreversible dissociation as a function of time exposed to *p*H 12.6 buffer at 23°. (A) Aldolase, 1.0 mg/ml, was exposed to *p*H 12.6 KCl-borate buffer (μ = 0.26) for different intervals and was rapidly diluted to 100 μg/ml in the reconstitution medium as described in the Materials and Methods section. The per cent activity regained was followed as a function of time in the reconstitution medium at *p*H 6.1 and 23°. (B) Semilog plots of the fraction of total activity regained vs. time exposed to *p*H 12.6 buffer. The experimental points at different reactivation times are the same as those in part A.

can be regained with no essential difference in the initial reactivation rate. Lack of comparable extents of reactivation over the entire concentration range investigated can be explained by the fact that native aldolase itself becomes inactivated at 10 μg/ml or less.

DISCUSSION

The experimental observations reported in this paper indicate that when muscle aldolase is exposed to a highly alkaline environment the molecule undergoes an initial rapid expansion which is followed by dissociation into six randomly coiled subunits. Undoubtedly the electrostatic repulsive forces generated by a high negative charge density play an important role in the denaturation process (Kauzmann, 1954).

The conclusion that molecular expansion precedes dissociation is supported by comparing several physical parameters obtained in the intermediate and highly alkaline *p*H ranges with similar data at *p*H 8.2 where

the enzyme is considered to be in the native state. At *p*H 10.7, for example, there is a concurrent decrease in the Drude constant (from 265 to 240 mμ) and the sedimentation coefficient (from 7.43 to 6.47), indicating a loss in compact structure. Within this *p*H region the weight average molecular weight is approximately 129,000, illustrating that the aldolase molecule remains largely undissociated. In the alkaline range between *p*H 11.0 and 13.0, however, there is a pronounced decrease in the weight average molecular weight (55,000 to 22,400). This is accompanied by a large increase in intrinsic viscosity from 0.057 dl/g at *p*H 7.0 to 0.185 dl/g at *p*H 11.2 (Jirgensons, 1961a) and a further decrease in λ_c and $s^0_{20,w}$. Also, in part of this *p*H region, two components are observed in the ultracentrifuge (Figure 1).

Another observation concerning the influence of *p*H on structure stems from the fact that half the conformational optically active centers have been destroyed at *p*H 10.7; it is approximately in this region that half the anomalous tyrosines are titrated. Thus, in keeping with current concepts, the data may be interpreted as showing a close relationship between tyrosine-hydrogen bonding and the maintenance of secondary or helical structure.

The observation that alkali causes aldolase to dissociate irreversibly into six subunits does not agree with the findings that the native molecule reversibly disssociates into three subunits when treated with either acid or urea (Stellwagen and Schachman, 1962; Deal *et al.*, 1963b). The data, however, do not preclude the possibility of primary bond cleavage which could account for the production of low molecular weight material. In fact, the relatively slow rate of irreversible denaturation at *p*H 12.6 (Figure 9) seems to support covalent bond hydrolysis. Since peptide bonds ordinarily are insensitive to hydrolysis under the experimental conditions reported here, the possible existence and cleavage of alkali-labile ester bonds must be considered. Thus, ester linkages of seryl or threonyl residues could be involved in the maintenance of aldolase structure, but cysteinyl (Stellwagen and Schachman, 1962) and tyrosyl residues could not be involved since all these groups titrate normally when aldolase is exposed to 4.0 M urea.

Mention should be made of observations on crystalline beef liver glutamic dehydrogenase (Frieden, 1962; Fisher *et al.*, 1962; Jirgensons, 1961b). Results show that the glutamic dehydrogenase molecule undergoes two distinct types of dissociation which have been classified as reversible and irreversible. Reversible dissociation involves the production of four units of 250,000 mw from 1,000,000 mw material. At high enzyme concentrations this type of dissociation is caused by 3 M urea, *p*H values between 4 and 5, and numerous other reagents. Irreversible dissociation results in the production of eighteen to twenty-five subunits and is caused by sodium dodecyl sulfate, 6 M urea, and exposure to either highly acid or highly alkaline conditions. It is conceivable that aldolase also might undergo reversible and irreversible dissociation processes depending on the denaturing conditions employed.

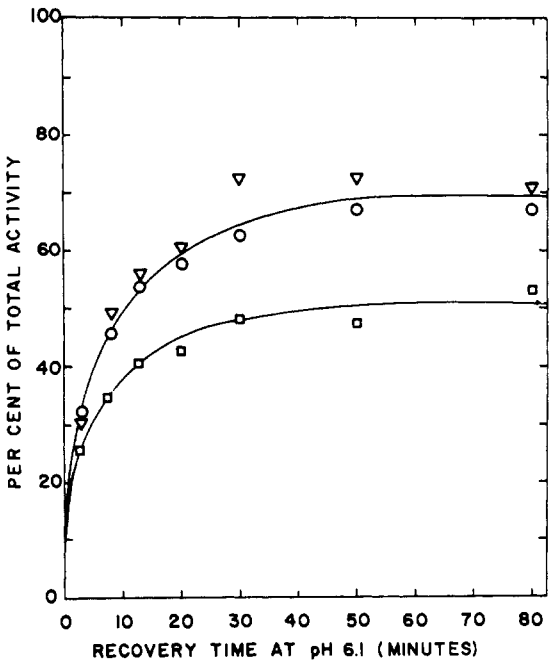

FIG. 10.—The recovery of aldolase activity as a function of enzyme concentration in the reconstitution medium at 23°. Aldolase, 1.0 mg/ml, was exposed to *p*H 12.6 KCl-borate buffer (μ = 0.26) for 1.0 minute at 23° and was rapidly diluted in appropriate volumes of the reconstitution medium. ▽, ○, and □, represent 100, 33, and 10 μg/ml in the reconstitution medium, respectively. In all cases an equivalent amount of aldolase was added to the assay mixture used for determining activity.

Arguments favoring a six-chained aldolase molecule are as follows: (1) It seems fortuitous that under alkaline conditions a subunit should be obtained having a molecular weight equivalent to half that observed in either acid or urea. (2) Despite the large number of protein-bound nucleophilic groups, the 22,400 mw material remains stable at *p*H 12.6 for periods at least as long as 4 days at temperatures varying between 4° and 23°. The latter observation strongly argues against random protein breakdown by inter- or intramolecular nucleophilic attack. Moreover, due to the possibility of buried C-terminal amino acid residues and the limitations of carboxypeptidase substrate specificity (Neurath, 1960), the three C-terminal tyrosine groups found by Kowalsky and Boyer (1960) must be considered minimal. In view of the findings reported here, the C-terminal amino acid analyses cannot be interpreted as indicating the total number of aldolase subunits until substantiated by further experimentation.

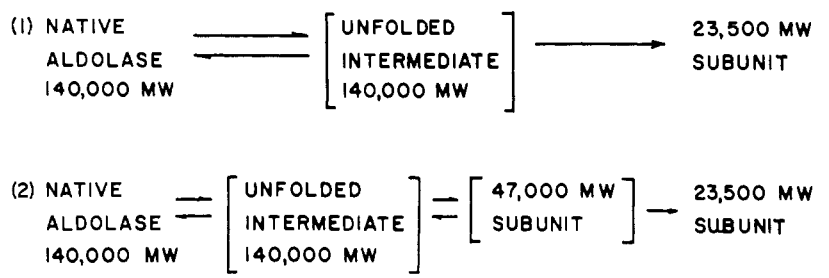

FIG. 11.—Proposed mechanisms for the alkaline denaturation of aldolase.

Renaturation of alkali-treated aldolase is based on activity measurements assuming that the renatured enzyme attains the same molecular conformation as the native enzyme. Justification for the above assumption is based on the observation that aldolase, dissociated in either acid or urea, resembles native aldolase upon renaturation. The kinetic data illustrated in Figure 9 show that, after brief exposure to alkali at *p*H 12.6, 70 to 75% of the initial activity is restored; whereas longer periods of exposure at *p*H 12.6 result in eventual irreversible inactivation which obeys the first-order rate law. Possible explanations for this phenomenon are as follows: (1) slow oxidation of —SH groups to —S—S— groups, (2) hydrolysis of specifically labile covalent bonds, and (3) slow irreversible dissociation into monomeric subunits. The first explanation is unlikely since anaerobic conditions were maintained throughout the course of the reactivation experiments; however, no distinction can be made between the latter two explanations until more data become available. When the time course of the reactivation of alkali-denatured aldolase is followed at *p*H 6.1 and different enzyme concentrations (Figure 10), it is seen that the reactivation process also follows first-order kinetics. Similar results were recently reported by Deal *et al.* (1963b) for acid-dissociated aldolase.

In order to account for the kinetics of aldolase denaturation and reactivation after exposure to alkali, the schemes illustrated in Figure 11 have been considered. Both mechanisms contain an irreversible step which is associated with the formation of 23,500 mw material from a single intermediate. Each scheme also accounts for first-order reactivation kinetics if the rate-limiting step is considered to be associated with refolding of the 140,000 mw intermediate. Scheme (I) does not take into consideration the strong evidence that 47,000 mw intermediates may be involved in the denaturation process as indicated by the sedimentation velocity data and the high speed sedimentation equilibrium experiment illustrated in Figure 5. For this reason, scheme (II) is favored; however, if this scheme is to comply with the experimental kinetic data, it must be postulated that the 47,000 mw subunits rapidly aggregate in a specific manner to form the 140,000 mw unfolded intermediate. Aside from the fact that alkali causes aldolase to dissociate into six subunits, a close correlation between acid- and alkali-induced denaturation can be visualized.

Acknowledgment

The authors would like to thank Dr. A. H. Mehler for his stimulating discussions during the course of this investigation.

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