Reversible Inactivation and Dissociation of Yeast Hexokinase*

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Recent work on the reactivation and reconstitution of several denatured enzymes has provided increasing understanding of the process of reversible protein denaturation. Studies on ribonuclease by Anfinsen (1) and White (2) have convincingly demonstrated that under favorable conditions a completely denatured protein can resume the active three-dimensional con-

figuration of the native molecule. Essentially similar results have been obtained in the case of lysozyme by Isemura et al. (3, 4) and Goldberger and Epstein (5) and on Taka-amylase by Isemura et al. (3). These examples provide justification for the view that the secondary and tertiary structure of a protein molecule is a result solely of the intrinsic thermodynamic properties of its amino acid sequences (1, 2). These conclusions based on work with single chain proteins have recently been extended to complex, multichain proteins. Thus, reports by Stellwagen and Schachman (6), Deal, Rutter and Van Holde (7), and Hass and Lewis (8) on rabbit muscle aldolase and by Samejima and Yang (9) on catalase illustrate the ability of denatured and dissociated polypeptide chains of a protein molecule to reassemble and refold under appropriate conditions to give the biologically active con-

formation of the native molecule.

Sedimentation and diffusion studies on yeast hexokinase by Kunitz and McDonald (10) indicated that the enzyme has a molecular weight of 96,600 at pH 5.5. These workers, quoting the data obtained by A. Rothen, were the first to report that hexokinase exhibits a second slowly moving peak in the ultra-

centrifuge at pH 6.0, which would now be interpreted to represent the half molecule of the enzyme. They, however, inter-

preteted this as an "effect of ultracentrifugation on the homoge-

neity of the hexokinase protein." More recently Schachman (11) has provided further evidence for the dissociation of hexo-

 kinase into half-molecules without loss of activity, under in-

fluence of pH, phosphate ions, and glucose.†

Ramel, Stellwagen, and Schachman (12) have also reported that treatment of hexokinase with a detergent caused disso-

ciation into approximately quarter-molecules, which were inactive. In this communication we present experiments to show that the inactivation of yeast hexokinase in acid and alkali is accompa-

nied by its dissociation into inactive quarter molecules which reas-

sociate at neutral pH to produce the active enzyme with

the native molecule. This report also includes studies on the factors governing the inactivation and reactivation processes and the kinetics of the physicochemical changes accompanying them. Preliminary accounts of this work have been published (13, 14).

EXPERIMENTAL PROCEDURE

Enzyme—Crystalline yeast hexokinase prepared by the method of Darrow and Colowick (15) was employed for most of the experiments reported in this paper. These preparations had specific activities of 500 to 600 units per mg. For a few experi-

ments, hexokinase purchased from Boehringer and from Sigma was also used after two recrystallizations. The specific activi-

ties of these recrystallized preparations were in the range of 200 to 225 units per mg. A unit here has been defined as that amount of enzyme which can catalyze the phosphorylation of 1 μmole of glucose per min at 30° under specified conditions (15). For all the experiments described in this paper salt free hexo-

 kinase was used. For this purpose, the protein was sedimented from suspension in ammonium sulfate and dissolved in 0.02 m phosphate buffer, pH 7.0. The solution was dialyzed against 1000 volumes of the same buffer for 2 hours followed by 1000 volumes of water distilled three times. Any precipitate formed was removed by centrifugation before use.

Chemicals—ATP was a product of Pabst Laboratories; CMB was obtained from Sigma; β-mercaptoethanol was purchased from Eastman; DTN was obtained from Aldrich.

Enzyme Assays—Hexokinase activity was assayed by the procedure of Darrow and Colowick (15). The pH of measurement was between 8.5 and 8.9. In this range, enzymatic activity is influenced only slightly by pH. The concentration of the en-

zyme usually employed was 1 to 5 μg per ml. The assays were car-

ried out at 30°. A point which deserves emphasis here is that the enzyme inactivated in either acid or alkali did not re-

activate under the conditions of assay. Had the enzyme re-

activated under these conditions, the inactivation in either acid or alkali would not have been observed. This point will be dis-

cussed more fully later.

ATPase activity of hexokinase was determined by measure-

ment of phosphate liberated by the method of Fiske and Subba-

Row (16). A unit of ATPase activity is defined as that amount of enzyme causing the hydrolysis of 1 μmole of ATP per min at

25°.

Protein Concentrations—These were determined from optical density readings at 280 and 260 μμ (17).

Ultracentrifuge Studies—Sedimentation velocity experiments

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‡ In fact, in the original studies of Rothen in 1943, sedimentation values in Veronal buffer, pH 7.4, also indicated complete dissociation into half-molecules (A. Rothen, personal communication).

† The abbreviations used are: CMB, p-chloromercuribenzoate; DTN, 5,5'-dithiobis(2-nitrobenzoic acid).
were performed in a Spinco model E analytical ultracentrifuge equipped with a phase plate as a schlieren diaphragm and with a rotor temperature indicator and a control unit. Photographic plates were analyzed with the aid of a Gaertner microcomparator. All sedimentation experiments were carried out at rotor speeds of 50,780 rpm. Sector cells (12 mm, 2°) with Kel F center pieces were usually employed because of the high alkalinity or acidity of several solutions analyzed. The observed sedimentation constants were corrected to values corresponding to a solvent with the viscosity and density of water at 20° (890.6). For the purpose of calculating molecular weights, sedimentation coefficients were determined at different concentrations and the values obtained by extrapolation to infinite dilution (85) were employed.

Viscosity Measurements—Intrinsic viscosities were measured with a Cannon-Ubbelohde "semi-micro" dilution viscometer with an outflow time of 261.7 sec for water at 20°. An average of five determinations was obtained for each sample. The samples were freed of suspended particles by centrifugation at 105,000 X g for 30 min.

Spectrophotometric Studies—Ultraviolet spectra and ultraviolet difference spectra were recorded with a Cary recording spectrophotometer (model 11) and quartz cuvettes with a path length of 1 cm. Tyrosine titrations and the kinetics of tyrosine ionization were carried out with a Zeiss spectrophotometer (model PMQ II).

Optical Rotation Measurements—Optical rotation studies were carried out on a Rudolph spectropolarimeter, model 290/658/850/810, equipped with a double monochromator made with synthetic quartz 10° prisms. The light source was a 150-watt xenon lamp powered by alternating current. The readings were obtained in the range 300 to 600 ma. The path length of the polarimeter cell was 1 cm. Corrections were made for the optical rotation of the solvent. Small corrections for the refractive index of the solvent were neglected. The optical rotatory dispersion data were plotted according to the simple Drude equation as [c]X against c where [c]x is the rotation at the wave length X. The dispersion constant Tc was obtained from the slope of this plot with the assumption of a value of 212 r physiology for X0. On the assumption that [c]X and [c]0 are based on the proposals made by Yang and Doty (18). The dispersion data was also treated according to the plot: [c]X/(X2 - X2) against 1/(X2 - X2) by Moffitt and Yang (19). The second term constant b2 in this equation was obtained from the slope of this plot with the assumption of a value of 212 ma for Xb. On the assumption that the b2 value for a completely helical protein would be —630, the per cent helix was calculated as follows: the percentage of helical content = b2 X - 630 X 100 (20).

Light Scattering Studies—All the light scattering experiments were performed at room temperature at a wave length of 436 ma. The measurements were made with a Bric-Phoenix Universal light scattering photometer, series 2000, with a H85A3 General Electric light source and equipped with a Phoenix ratio recorder, model SUC-500-K. Glass cells, 1 cm square, were employed, and the volume of the solutions within the cell was 3 ml. The scattering measurements were made only at 90°. Bovine serum albumin (Armour) dissolved in 0.1 M NaCl was used as the standard. For the purpose of calculating the molecular weights of the unknown samples, the molecular weight of bovine serum albumin was assumed to be 70,000. The refractive index increment for hexokinase was assumed to be 0.1954 (value for bovine serum albumin) where the concentration is expressed in grams per ml. Enzyme solutions and solutions of the standard were clarified by centrifugation at 105,000 X g for 4 hours. Buffers, reagents, and water employed for making dilutions were filtered through fine sintered glass funnels. The turbidity measurements were corrected for small dilutions caused by the addition of reagents and for solvent scatter. They were corrected neither for depolarization nor for dissymmetry. The results were plotted as Hc/9 against 9 where H represents the optical constants, is expressed in grams per ml, and r is the turbidity. The intercept of the plot with the ordinate gave the reciprocal of the molecular weight (21). For following the kinetics of the dissociation process, the value of the parameter Hc/9 for a concentration of 0.0009 g per ml was considered equivalent to the reciprocal of the molecular weight since this concentration is close to infinite dilution.

Sulphydryl Titrations—Titration of the sulphydryl groups were carried out by the spectrophotometric procedure of Boyer (22) and by the method of Ellman (23). The latter method is based on the reduction of DTN by the protein sulphydryl groups giving rise to a colored derivative, the concentration of which can be measured spectrophotometrically.

Inactivation and Reactivation Procedures—Inactivation in alkali was carried out by the addition of NaOH to the required pH or by the addition of 0.5 M phosphate buffer, pH 11.5. Reactivation was brought about by neutralizing with 1 N acetic acid or with 0.5 M phosphate buffer, pH 7.0, or with both.

Inactivation in acid was effected by the addition of 0.2 M HCl to the required pH. Reactivation was brought about by neutralizing with 0.1 N NaOH or with 0.5 M phosphate buffer, pH 7.0, or with both.

The experiments were carried out either at 0° or at room temperature (25°).

RESULTS

I. Reversible Inactivation in Alkali

Inactivation at Alkaline pH Values—During experiments designed to reduce the hexokinase-glucose complex by treatment with sodium borohydride, it was found that the alkalinity imparted by the latter caused an immediate loss of activity, and that activity reappeared as the pH returned to neutrality by the loss of ammonia from the ammonium sulfate present in the enzyme preparations. This led to a systematic study of the inactivation and reactivation processes. As shown in Fig. 1, the enzyme, which retains full activity at pH 9.0, suffers 30% loss at pH 10.5 and is completely inactivated at pH 11.5. When the buffer or salt ions employed in this experiment were replaced by other buffer or salt ions, generally similar results were obtained. Both the extent and rate of inactivation were found to be increased when the experiment was carried out at room temperature. A feature of this inactivation especially noticeable at 0° and around pH 10 to pH 11 was that the inactivation reached a limiting value depending on pH. The curve for pH 10.5, shown in Fig. 1, illustrates this point. The enzyme retained full activity in the range of pH values 4.5 to 0.5.

Effect of Salt—Further investigations showed that the presence of salt was an obligatory requirement for this instantaneous loss of activity. This effect is shown in Fig. 2. When the enzyme solution was adjusted to pH 11.5 by sodium hydroxide in the absence of any salt (Curve A), the enzyme lost activity only
slowly with time; but in the presence of sodium sulfate and phosphate ions (Curve B) this loss in activity was instantaneous.

Regeneration of Enzyme Activity—The alkali-induced inactivation of the enzyme could be reversed by readjustment of the alkaline solution to a pH around neutrality by either acid or phosphate buffer, pH 7.0. As can be seen from Fig. 3, the restoration of activity is very rapid, being about 50% complete in the first 2 min. The enzyme can be reactivated to the extent of about 80 or 90%, provided the enzyme solution is neutralized immediately after addition of alkali.

The extent of restoration of activity was found to be the same in the pH range of 6 to 7.4. Below and above this range the extent of reactivation diminished. At pH 5.0 and pH 8.0, there was no significant reactivation of the enzyme at a concentration below 50 μg per ml. At higher concentrations, however, some reactivation (about 5%) could be observed even at these pH values.

Effect of Mercaptoethanol—The ability of the alkali-treated enzyme to be reactivated was found to be markedly dependent upon the time for which it was exposed to alkali. As shown in Fig. 3B, if the enzyme solution is neutralized immediately after addition of alkali, the recovery of activity is close to 90%. The extent of recovery progressively diminishes on longer exposure to alkali. After exposure to alkali for 24 hours, the enzyme is apparently irreversibly inactivated. The recovery was found to be improved if anaerobic conditions were maintained during long exposures to alkali (not shown). This suggested that oxidation of sulfhydryl groups of the dissociated enzyme (see below) in the alkaline medium probably caused this irreversible inactivation. This idea received support from experiments in which mercaptoethanol was present during addition of alkali. As seen in Fig. 3A, presence of 0.04 M mercaptoethanol results in much higher yields of the reactivated enzyme. The effect is more marked for longer exposures to alkali. As shown in Fig. 3B, even an apparently irreversibly inactivated enzyme can be partially restored to activity when mercaptoethanol is added to the enzyme solution. These results thus strongly indicate that the integrity of —SH groups is essential for the reactivation of the enzyme.

Effect of Protein Concentration—Kinetic studies on this reversible inactivation showed that the extent of inactivation decreases with increasing concentration of the enzyme from 50 μg per ml to 5 mg per ml (Fig. 4). The effect of protein concentration on the extent of inactivation was suggestive of a reversible equilibrium between active molecules and their inactive subunits. The rate of reactivation appeared to be fairly independent of protein concentration and so was the extent, except for the lowest protein concentration used. The decreased extent of reactivation in the case of the lowest protein concentration probably does not reflect an equilibrium state, but rather an oxidation of —SH groups, since no mercaptoethanol was added in this experiment.

Effect of Proteolysis—An in vitro study of reversibility of inactivation suffered by the enzyme at different pH values, an unexpected result was obtained. Inactivation of the enzyme at pH 10.5 was found to be almost irreversible, although at pH 11.5 it was reversible. The presence of mercaptoethanol did not alter the result. It seemed likely that at pH 10.5 the reversibly inactivated product was further modified to an irreversibly inactivated one by a trace amount of a proteolytic enzyme which was more active at pH 10.5 than at pH 11.5. To test this view, samples of hexokinase, substantially freed from the traces of proteolytic enzyme which regularly occurred in these recrystallized preparations, were obtained by passing the enzyme through a DEAE-cellulose column. This enzyme was then employed for inactivation studies. It was found that the partial removal
The recovery of the enzyme at various time intervals after neutralization is given on the ordinate. The time intervals after neutralization are represented on the abscissa. A, hexokinase at a concentration of 0.250 mg per ml was inactivated in the presence of Na$_2$SO$_4$ (1/2 = 0.3) and 0.04 m mercaptoethanol by the addition of phosphate buffer, pH 11.5 (1/2 = 0.1). of the proteolytic contaminant resulted in a considerable increase in the extent of reactivation of the enzyme at pH 10.5. This result suggests that the irreversible nature of the inactivation at pH 10.5 in the case of the untreated enzyme may be due to an extremely rapid proteolysis of the inactive product even at 0°C. The evidence, however, does not rule out any other explanation of this phenomenon. The extreme sensitivity of the CMB-treated enzyme to trypsin has been noted previously.

Isolation of Crystalline Enzyme from Neutralized Material—The specific activity of the material obtained on neutralization was lower than that of the native material, because of the variable amounts of denatured protein present. To isolate a product free from this denatured protein, the following procedure was adopted. About 150 mg of the reactivated material (specific activity, 220 units per mg) contained in a volume of about 100 ml were dialyzed free of salt and other small molecular weight contaminants. To this dialyzed solution ammonium sulfate was added to full saturation. The precipitated material was taken up in cold 0.1 M potassium phosphate buffer, pH 7.0, containing 0.002 M EDTA; saturated ammonium sulfate was added to it drop by drop until turbidity appeared. The crystalline precipitate which appeared after 24 hours had a specific activity of above 500 units per mg, corresponding to the native enzyme. This represented a recovery of 50% of the starting material.

Comparison of Regenerated and Native Enzyme—Susceptibility to trypsin or heat inactivation can be a subtle indicator to differentiate between apparently similar enzymes. Thus, Allende and Richards (24) have shown that ribonuclease S, which is almost identical with ribonuclease A, is more prone to attack by trypsin than the latter. Schlesinger and Levinthal (25) and Garen and Garen (26) have shown considerable difference in heat sensitivity between mutant and standard alkaline phosphatases from Escherichia coli. In the present case, employment of these methods to detect any fine structural differences

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Figures on the curves refer to the number of hours for which the enzyme was exposed to pH 11.5 buffer before neutralization by acetic acid. B, same experiment as A, but test solution contained no mercaptoethanol. In the case of sample exposed to pH 11.5 buffer for 24 hours before neutralization, 0.04 m mercaptoethanol was added 60 min after neutralization. Temperature was 0°C.

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1 A. Kaji, in preparation.
between native and regenerated hexokinase showed that both of these materials were equally sensitive to heat and trypsin treatment.

The identity of the native and reactivated material was further shown by studies on the intrinsic ATPase activity of hexokinase after inactivation and reactivation. Table I shows that alkali-inactivated enzyme which lacks ATPase activity recovers this activity on neutralization. The ratio between the hexokinase and ATPase activities of the reactivated material is almost the same as the ratio of these activities in the native enzyme.

II. Reversible Inactivation in Acid

Inactivation at Acid pH Values—Studies on the inactivation of hexokinase at acid pH values showed that the enzyme is stable when exposed to acid at pH 4.5 for a period of 15 min at 0°. At pH 3.8, 19% of the activity is instantly lost, but no further loss in activity occurs. At pH 3.4, the loss in activity is continuous until about 62% of the enzyme is inactivated in 15 min. The enzyme is completely inactivated within 90 sec of exposure to acid at pH 2.8. The enzyme concentration employed in these experiments was 1 mg per ml.

**Effect of Salt on Inactivation in Acid**—When the concentration of the enzyme is 100 μg per ml or below, the presence of salt (0.1 M Na2SO4) greatly speeds up inactivation at pH 2.8. As seen in Fig. 5, the time for complete inactivation increases from 5 min to 45 min in the absence of salt. On the other hand, when the concentration is 500 μg per ml or over, the loss in activity at pH 2.8 is instantaneous even in the absence of salt (Fig. 6). The presence of salt in such concentrated solutions leads to the development of turbidity and eventual precipitation of the enzyme.

**Reactivation of Acid-inactivated Hexokinase**—When the acid-inactivated enzyme was readjusted to pH values between 6 and 7.4, rapid restoration of activity ensued. Within this range of pH values, the rate and extent of reactivation varied but little. Above and below this pH range, both extent and rate of reactivation decreased. Presence of EDTA was found to be essential for optimum reactivation. Fig. 5 illustrates this point for low enzyme concentration (50 μg per ml). The restoration of activity in the presence of 10−4 M EDTA is 80% in the absence of salt and 65% in the presence of salt. The corresponding figures in the absence of EDTA are 11% and 16%, respectively. Similar results are obtained when the protein concentration is raised to 5 mg per ml as shown in Fig. 6, but here more EDTA is needed.

The presence of EDTA is necessary only during the process of neutralization. Even if it is absent during inactivation, the recovery is not affected. Fig. 6 shows that once an enzyme is neutralized in the absence of EDTA, later addition of EDTA does not improve recovery. However, addition of 0.04 M mercaptoethanol results in rapid increase in activity until about 55% of the original activity is restored.

The restoration of activity in the absence of EDTA was found to be variable and ranged anywhere between 10 and 50% of that in the presence of EDTA. This variation in results apparently depended upon the heavy metal contamination of the water employed for the experiments. EDTA could be entirely replaced by cysteine or mercaptoethanol. These results point to the conclusion that EDTA functions by binding heavy metal ions which catalyze the oxidation of —SH groups essential for the reassociation of inactive quarter molecules to form the active enzyme (see below). The importance of —SH groups for the reactivation of alkali denatured enzyme has been shown above. It seems that the —SH groups, when exposed by acid treatment, are more readily susceptible to oxidation upon neutralization than after alkali treatment.

<table>
<thead>
<tr>
<th>Hexokinase</th>
<th>Activity</th>
<th>Ratio of ATPase to hexokinase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hexokinase</td>
<td>ATPase</td>
</tr>
<tr>
<td>Native</td>
<td>573</td>
<td>4.4 × 10⁻²</td>
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<tr>
<td>Inactivated at pH 11.5</td>
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<td>0</td>
</tr>
<tr>
<td>Reactivated on neutralization...</td>
<td>840</td>
<td>2.8 × 10⁻²</td>
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</table>

**Fig. 5. Effect of salt and EDTA on the inactivation and reactivation of a dilute solution of hexokinase treated with acid.** Test solutions containing 50 μg of hexokinase per ml, and Na2SO4 (1/2 = 0.3), and 0.0001 M EDTA where indicated, were inactivated by the addition of HCl to pH 2.8. After 45 min in acid, the solutions were neutralized with 0.1 N NaOH, and the recovery of activity was followed with time. Temperature was 0°.

The reaction mixture contained 1.5 mg of native, inactivated, or reactivated hexokinase per ml; 0.01 M ATP; 0.02 M MgCl₂; and 0.06 M Veronal buffer, pH 8.3. The ATPase reaction was carried out for 1 hour at 25°. The recovery of activity was followed with time. Temperature was 0°.

The identity of the native and reactivated material was further neutralized with 0.1 N NaOH, and the recovery of activity was followed with time. Since the enzyme solutions were tested directly without dialysis, small concentrations of salts such as Na2SO4 and CTT&OONa were present in the reaction mixture.

Hexokinase ATPase activity was followed with time. Temperature was 0°. The presence of salt in such concentrated solutions leads to the development of turbidity and eventual precipitation of the enzyme.

Reactivation of Acid-inactivated Hexokinase—When the acid-inactivated enzyme was readjusted to pH values between 6 and 7.4, rapid restoration of activity ensued. Within this range of pH values, the rate and extent of reactivation varied but little. Above and below this pH range, both extent and rate of reactivation decreased. Presence of EDTA was found to be essential for optimum reactivation. Fig. 5 illustrates this point for low enzyme concentration (50 μg per ml). The restoration of activity in the presence of 10⁻⁴ M EDTA is 80% in the absence of salt and 65% in the presence of salt. The corresponding figures in the absence of EDTA are 11% and 16%, respectively. Similar results are obtained when the protein concentration is raised to 5 mg per ml as shown in Fig. 6, but here more EDTA is needed. The presence of EDTA is necessary only during the process of neutralization. Even if it is absent during inactivation, the recovery is not affected. Fig. 6 shows that once an enzyme is neutralized in the absence of EDTA, later addition of EDTA does not improve recovery. However, addition of 0.04 M mercaptoethanol results in rapid increase in activity until about 55% of the original activity is restored.

The restoration of activity in the absence of EDTA was found to be variable and ranged anywhere between 10 and 50% of that in the presence of EDTA. This variation in results apparently depended upon the heavy metal contamination of the water employed for the experiments. EDTA could be entirely replaced by cysteine or mercaptoethanol. These results point to the conclusion that EDTA functions by binding heavy metal ions which catalyze the oxidation of —SH groups essential for the reassociation of inactive quarter molecules to form the active enzyme (see below). The importance of —SH groups for the reactivation of alkali denatured enzyme has been shown above. It seems that the —SH groups, when exposed by acid treatment, are more readily susceptible to oxidation upon neutralization than after alkali treatment.
Fig. 5 shows that with a dilute enzyme solution (50 μg per ml) the presence of salt slightly decreased the recovery of activity after neutralization. This decreased recovery in activity was more pronounced when a more concentrated solution (not shown) was used. Aggregation and precipitation of protein were observed when concentrated solutions of protein were acidified in the presence of salt. Part of this aggregated and precipitated material went back into solution on neutralization and was found to be active.

The remarkable ability of the enzyme to reaggregate after exposure to acid was shown by the results obtained when the enzyme was inactivated at pH 1.7. For a protein concentration of 50 μg per ml, the recovery of activity on neutralization was found to be 62%. Even enzyme precipitated with 4% trichloroacetic acid could be reactivated to the extent of 30% when dissolved in phosphate buffer, pH 7.0.

The reactivated enzyme obtained from different experiments was pooled. As with the reactivated material obtained from alkali, this could be crystallized to give the same specific activity as the original enzyme. One such run carried out on pooled reactivated material showed that about 40% of the recovered activity was associated with the crystals.

### III. Studies on Size and Shape

Studies on the reversible inactivation of hexokinase in acid and alkali were correlated with corresponding investigations on the secondary, tertiary, and quaternary structure of the molecule. The results of these studies are presented below.

#### Partial Specific Volume—Estimation of the size and shape of the hexokinase molecule under different conditions required a knowledge of its partial specific volume. This value was calculated from the amino acid composition by the method of Cohn and Edsall (27) to be 0.74 for each of the two major chromatographic forms of the enzyme (28, 29). The partial specific volumes of several proteins calculated by this method have shown a close agreement with their experimentally determined values (30). Since hexokinase preparations employed in the present studies were a mixture of both chromatographic forms, the partial specific volume of the enzyme in the present case would also be 0.74.

#### Viscosity Studies—The changes occurring in the tertiary structure of hexokinase were followed by viscosity studies. Table II gives the values for intrinsic viscosity of hexokinase under different conditions. The intrinsic viscosity of 4.3 ml per g at pH 5.0 indicates that at this pH hexokinase exists as a fairly compact and rigid molecule. There is only a small increase in intrinsic viscosity either in acid at pH 2.8 or in alkali at pH 11.5. However, a significant rise in intrinsic viscosity occurs below pH 2.8 or above pH 11.5 as shown by values of 8.8 and 10 ml per g observed at pH values 2.2 and 11.8, respectively. The results indicate that hexokinase retains its compact and rigid structure over a wide range of pH values. On the other hand, the high intrinsic viscosity of 17.2 ml per g obtained in 8 M urea solution shows a gross disorganization of the molecule in this solvent. The intrinsic viscosity of hexokinase reactivated by direct neutralization from either acid or alkali is somewhat higher than that of the native molecule. This might perhaps be due to contamination with a few denatured molecules. This explanation is supported by the fact that hexokinase reactivated from alkali shows almost the same intrinsic viscosity as the native molecule. This might perhaps be due to contamination with a few denatured molecules. This explanation is supported by the fact that hexokinase reactivated from alkali shows almost the same intrinsic viscosity as the original enzyme. One such run carried out on pooled reactivated material showed that about 40% of the recovered activity was associated with the crystals.

#### Determination of Molecular Weights—The molecular weight of hexokinase under different conditions of pH and ionic strength was calculated by combination of sedimentation and viscosity data with the use of the β function of Scheraga and Mandelkern (31); the relation of the β function to molecular weight is shown in Equation 1.

\[
\beta = \frac{N s^2_{R,0}}{M^1 (1 - \bar{v}_p)(100)^1}
\]

where the symbols have their usual meanings.

This function, the quantitative interpretation of which is doubtful in the case of flexible coils (32) could be used in the present case in view of the apparent rigidity of the molecule over a wide range of pH. This function is markedly insensitive to configuration. Thus, on the assumption of a reasonable hydrodynamic model and with the use of the corresponding β value in the above equation, values for molecular weights can be obtained (32-34). For this purpose Schachman (33) has suggested a value of 2.16 × 10^5 for the β function. This value has been used for the calculation of molecular weights from sedimentation and viscosity data (Table II).

Values for molecular weight in Table II show that at pH 5.0 hexokinase has a molecular weight of 95,000, a value close to that originally reported by Kunitz and McDonald (10) from December 1965. U. W. Kenkare and S. P. Colowick 4575


Molecular weight of hexokinase from sedimentation and viscosity data: effect of pH and urea

All sedimentation runs were carried out at 5.5° except for the one in urea which was at 23°. Intrinsic viscosity was determined at 0° except for the one in urea, in which case it was determined at 22°. See “Experimental Procedure” for details. Molecular weights were determined with the use of the β function of Scheraga and Mandelkern (31). See the text.

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<td>3.3</td>
<td>4.7</td>
<td>46,000</td>
</tr>
<tr>
<td>Reactivated from alkali by neutralization</td>
<td>7.0</td>
<td>Phosphate buffer, Na₂SO₄</td>
<td>0.4</td>
<td>3.5</td>
<td>4.4</td>
<td>48,000</td>
</tr>
<tr>
<td>Reactivated from acid by neutralization</td>
<td>7.0</td>
<td>Phosphate buffer, Na₂SO₄</td>
<td>0.4</td>
<td>3.3</td>
<td>4.8</td>
<td>46,000</td>
</tr>
</tbody>
</table>

* These sedimentation constants were determined at 8 mg per ml. All others are extrapolated values for infinite dilution.

7 The sedimentation and the diffusion constants reported in this paper as the calculated values for 20° are actually the observed values for 1°. The corrected values for the diffusion and sedimentation constants at 20° are 5.02 X 10⁻¹¹ and 5.0 X 10⁻¹¹, respectively (A. Rothen, personal communication).

centration of enzyme 1 to 5 µg per ml). The molecular weight at pH 11.5 and pH 2.8 indicates a dissociation of the native molecule (initially at pH 5.0) into 4 quarter molecules. Under these conditions, the enzyme is completely inactivated. This is compatible with the results of Ramel, Stellwagen, and Schachman (12), who had earlier reported that hexokinase is cleaved by sodium dodecyl sulfate into inactive chains of molecular weight around 20,000. It can be seen from Table II that the enzyme neutralized from either acid or alkali or crystallized after neutralization from alkali has the same molecular weight as the native enzyme at pH 7.0 and intrinsic strength 0.4. The return of the acid- or alkali-inactivated enzyme to its native shape and size on neutralization is accompanied by the restoration of its biological activity, as shown earlier in this paper.

**Sedimentation Behavior of Hexokinase as Function of Ionic Composition at Different pH Values**—This is shown in Figs. 7, 8, and 9, which illustrate the pH effects described in the previous section as well as the effects of ionic strength and composition. Examination of the sedimentation velocity patterns in order of increasing pH (Fig. 7) shows that, in the presence of high salt concentration (I/2 = 0.4), the sedimentation constant of hexokinase at a concentration of 8 mg per ml drops from 4.6S at pH 5.0 to 3.3S at pH 7.0 and further on to 2.1S at pH 11.5. At pH 7.0, in low salt concentration (I/2 = 0.1), two sedimenting boundaries can be observed (cf. Reference 10), one having a sedimentation constant of 5.3S and the other 3.2S. Increasing the salt concentration to intrinsic strength 0.4 results in the disappearance of the more rapidly sedimenting species in favor of the slower one. At pH 5.0 increasing the intrinsic strength from 0.1 to 0.4 results in a decrease in the sedimentation constant from 5.4S to 4.6S. This suggests that high salt content favors the formation of slowly sedimenting species and that rapid equilibration is maintained throughout the experiment between two sedimenting species of 5.4S and 3.3S. The result is the formation of a single sedimenting boundary with an intermediate sedimentation constant of 4.6S (35). The sedimentation constant at pH 9.0 (not shown) was 3.3S at both high and low salt concentrations. The ultracentrifuge patterns at pH 11.5 also do not show the salt effect although the presence of salt increases the rate of inactivation of the enzyme at this pH. This discrepancy may be explained by the fact that although the effect of salt on inactivation can be studied immediately on addition of alkali, its effect on the size of the enzyme, as measured by a sedimentation run, can be ascertained only after a lapse of several minutes. During this time interval any difference in the size of the molecule in the presence and absence of salt may disappear. The ultracentrifugal patterns for pH 5.0 and 7.0 point to the role of salt in promoting dissociation of the enzyme into half-molecules, assuming, on the basis of the small change in viscosity, that the frictional coefficient of the molecule has not been affected in the presence of salt. The presence of salt under these conditions has no effect on the activity of the enzyme when tested later under the standard conditions. The dissociation of the enzyme into half-molecules that presumably occurs as a result of increasing either pH or salt concentration has thus no effect on the activity exhibited in the subsequent assay.

**Effect of Phosphate Ions**—Sedimentation studies in the pH range 5.0 to 7.0 indicated that phosphate ions as compared to acetate ions were more effective in the dissociation of the enzyme into half-molecules (Fig. 8). It can be seen that at pH 5.5 as well as at pH 6.1 phosphate ions increased the concentration of the slowly sedimenting species. Similar results on the effect of
phosphate ions on the sedimentation patterns of hexokinase were reported earlier by Schachman (11).

Sedimentation Behavior of Hexokinase in Acid—The sedimentation patterns of hexokinase in acid solution are shown in Fig. 9. These runs were carried out in medium at a comparatively low ionic strength of 0.03. Higher ionic strength led to distorted patterns on account of aggregation effects. The patterns illustrate the changes in sedimentation previously summarized in Table II.

Molecular Weights by Light Scattering Measurements—The conclusions reached above regarding dissociation of hexokinase into half- and quarter-molecules on the basis of sedimentation and viscosity studies were independently confirmed by light scattering measurements. These results are presented in Fig. 10.

It can be seen that at pH 6.3 in the absence of salt hexokinase has a weight average molecular weight of 100,000, which decreases to half this value in the presence of Na₂SO₄ (Γ/2 = 0.3), showing the influence of ionic strength on dissociation of hexo-
Optical rotatory dispersion measurements were carried out at 2°. The concentration of the enzyme employed was 8 mg per ml in all the experiments. For details see "Experimental Procedure."

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

**Fig. 10.** Weight average molecular weight of hexokinase under different conditions of pH and ionic strength, determined by light scattering measurements. All solutions contained 0.04 M mercaptoethanol (see Fig. 11). Conditions of pH and ionic strength as shown on curves. Reciprocal of molecular weight (1/M) were obtained from the intercepts on the ordinate (see "Experimental Procedure"). The values for percentage of helix mentioned in Table III must be regarded with some reservations (36). Subject to this limitation, one may conclude that native hexokinase has a highly ordered structure. This structure is considerably disorganized both in acid at pH 2.8 and in alkali at pH 11.5, but much more so under the latter conditions. On neutralization from either acid or alkali, the helical content rises again to the expected value based on the percentage of reactivation of the molecule. The values for the enzyme crystallized after reactivation from alkali are almost identical with those of the native enzyme, indicating a complete return of the molecule to its native helical structure.

**TABLE III**  
Optical rotation parameters and helical content of hexokinase as function of pH

<table>
<thead>
<tr>
<th>Enzyme preparation</th>
<th>pH</th>
<th>Medium</th>
<th>Ionic strength</th>
<th>[α]_D</th>
<th>λ_0</th>
<th>b_0</th>
<th>Percentage of helix content calculated from:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>7.0</td>
<td>Phosphate buffer, Na_2SO_4</td>
<td>0.4</td>
<td>-35</td>
<td>283</td>
<td>-325</td>
<td>55 50.51</td>
</tr>
<tr>
<td>Native</td>
<td>11.5</td>
<td>Phosphate buffer, Na_2SO_4</td>
<td>0.4</td>
<td>-76</td>
<td>223</td>
<td>0</td>
<td>14 8 0</td>
</tr>
<tr>
<td>Native</td>
<td>2.8</td>
<td>HCl</td>
<td>0.0</td>
<td>-60</td>
<td>257</td>
<td>-280</td>
<td>30 32.44</td>
</tr>
<tr>
<td>Reactivated</td>
<td>7.0</td>
<td>Phosphate buffer, Na_2SO_4</td>
<td>0.4</td>
<td>-54</td>
<td>239</td>
<td>-72</td>
<td>36 36 2011</td>
</tr>
<tr>
<td>Crystallized</td>
<td>7.0</td>
<td>Phosphate buffer, Na_2SO_4</td>
<td>0.4</td>
<td>-37</td>
<td>281</td>
<td>-325</td>
<td>53 49.51</td>
</tr>
</tbody>
</table>

Kinase. The half-molecule is also obtained in glycylglycine buffer (τ/2 = 0.085), pH 9.0. At pH 10.5, the molecular weight of hexokinase in the presence and absence of salt drops down further to 27,000 and 29,000, respectively. At pH 2.8, a value of 20,000 is obtained for the molecular weight of hexokinase in the absence of salt. These values could be interpreted as indicating the dissociation of the molecule to either one-third or one-fourth in both acid and alkali. But the experimental errors involved in the measurement of molecular weights by the light scattering method tend to give higher values for low molecular weight proteins. It can therefore be assumed that these values indicate dissociation of hexokinase into four quarters in both acid and alkali. The negative slopes of the plots representing measurements in acid and alkali indicate interactions between protein molecules.

**Optical Rotatory Dispersion Measurements**—Information obtained from viscosity and sedimentation studies showed that hexokinase and its dissociated quarter molecules remained fairly rigid and compact in the range between pH 2.8 and pH 11.5. The dissociated chains began to unfold only below and above this range of pH values (Table II). These investigations on the tertiary structure of hexokinase were supplemented by optical rotatory dispersion measurements, which gave added information on the secondary or helical structure of the native and dissociated molecules. These results are presented in Table III. In view of the doubts regarding the validity of assumptions made in using parameters λ_0, b_0, and [α]_D for the estimation of helical content of proteins, the values for percentage of helix mentioned in Table III must be regarded with some reservations (36). Subject to this limitation, one may conclude that native hexokinase has a highly ordered structure. This structure is considerably disorganized both in acid at pH 2.8 and in alkali at pH 11.5, but much more so under the latter conditions. On neutralization from either acid or alkali, the helical content rises again to the expected value based on the percentage of reactivation of the molecule. The values for the enzyme crystallized after reactivation from alkali are almost identical with those of the native enzyme, indicating a complete return of the molecule to its native helical structure.

**IV. Correlation of Kinetics of Enzymatic and Physicochemical Changes**

This section describes the kinetics of the physicochemical changes that accompany the inactivation of hexokinase in alkali and acid. These changes have been followed by light scattering measurements in alkali, by spectral studies and by titration of —SH groups.

**A. Light Scattering Studies**

Sedimentation, viscosity, and light scattering studies, described in a previous section, had indicated that hexokinase inactivated in either acid or alkali had a molecular weight about one-fourth that of the native molecule. The object of the light scattering experiments described here was to establish a direct relationship in point of time between biological inactivation and dissociation of the enzyme molecule.

Observation of the changes in the molecular weight of hexokinase occurring in alkali at pH 10.5 was followed by observation of changes in the turbidity of the enzyme solution on addition of alkali. The concentration of the enzyme employed being only 0.9 mg per ml and the plot Hc/r against c being a straight line (see Fig. 10), the changes in turbidity occurring at this concentration were considered as occurring at infinite dilution and thus proportional to changes in molecular weight (see "Experimental Procedure").
Effect of Mercaptoethanol on Molecular Weight Changes in Alkali—In experiments done in the absence of salt, it was observed initially that the molecular weight dropped immediately on addition of alkali but after about 2 min started rising again. This is shown by Fig. 11, Curve A. This result indicated that the dissociation of the enzyme in alkali was accompanied by aggregation of the dissociated molecules. To counteract aggregation reactions, which possibly occurred by oxidation of sulfhydryl groups, mercaptoethanol was added to the reaction mixture. As shown in Curve B, mercaptoethanol effectively prevented the side aggregation reactions, so that the dissociation of the enzyme proceeded nearly to completion. In view of this result, the kinetic experiments were carried out in the presence of mercaptoethanol.

Kinetics of Dissociation and Inactivation—In this series of experiments, changes in the molecular weight of the enzyme on addition of alkali to pH 10.5 were correlated with changes in its biological activity. In view of the pronounced effect of salt on rate of inactivation in alkali (see Fig. 2), changes in molecular weight in alkali were studied both with and without salt. The results obtained are summarized in Fig. 12. It can be seen that in both cases the inactivation of the enzyme is concomitant with dissociation into quarter-molecules. The slightly lower values for dissociation as compared to inactivation may be due to aggregation reactions that would tend to give higher values for weight average molecular weight and to technical errors involved in light scattering measurements on small molecular weight proteins. The evidence is thus good that the inactivation and dissociation of the molecule into quarters are simultaneous events.

Calculations of the percentage of dissociation into quarter-molecules in Fig. 12 are based on the assumption that, for all experimental values after alkali addition, the protein consists...
of a mixture of half-molecules and quarter-molecules. This assumption seems justified from the data in Fig. 11, which indicate that, even in the absence of salt, there is an essentially instantaneous dissociation of whole molecules into half-molecules upon addition of alkali. The subsequent dissociation of half-molecules to quarter-molecules is relatively slow, and it is this process (accompanied by inactivation) which is speeded in the presence of salt. Whether salt has any effect on the rate of dissociation of whole molecules into half-molecules by alkali cannot be determined from these experiments.

B. Spectral Studies

Treatment of hexokinase with acid and alkali brought about changes in the environment of chromophoric groups in the protein. This was shown by ultraviolet spectra and ultraviolet difference spectra between acid- and alkali-treated enzyme and the native enzyme. The addition of acid produced a blue shift in the absorption spectrum of protein, shifting the absorption maximum from 278 to 275 nm (not shown). In the difference spectrum in acid which is shown in Fig. 13A, major maxima occur at 282, 287, 270, 268, and 265 nm. The maxima at 287 and 270 nm have generally been associated with the changes occurring in the vicinity of tyrosine residues (37, 38). The maximum at 292 nm owes its origin to disturbances in the environment of tryptophan residues and those at 265 and 268 nm are attributable to perturbations around phenylalanine residues (39). The altered environment of these residues in the acid-treated enzyme suggests significant changes in the internal architecture of the molecule brought about by acid. These structural alterations in the protein were also suggested by the optical rotatory dispersion studies described above.

The difference spectrum in alkali is illustrated in Fig. 13B. This difference spectrum, as expected, has a peak at 295 nm which is due to dissociation of phenolic groups of tyrosine residues of the protein.

On readjustment of the pH of the solutions to 7.0, the difference spectra obtained in acid and alkali disappeared, as shown by the lower curves in Figs. 13A and 13B.

Inactivation in Alkali and Phenolate Dissociation—Fisher, McGregor, and Power (40) have shown that the dissociation of glutamic dehydrogenase in alkali is closely paralleled by the appearance of ionized tyrosine phenolic groups. These results indicated to them that the dissociation of glutamic dehydrogenase into subunits in alkali was caused by the cleavage of hydrogen...
for enzyme activity and titratable -SH groups at various time intervals. The maximum number of -SH groups reacting. The maximum number of -SH groups titratable with CMB was determined as follows: 8 × 10^{-6} M hexokinase (0.66 mg per ml) and, where indicated, 0.1 M Na2SO4, were assayed for enzyme activity and titratable -SH groups exposed. They were then adjusted to pH 10.7 with NaOH and were assayed for enzyme activity and titratable -SH groups at various time intervals thereafter. Temperature was 25°C.

**C. Studies on Sulfhydryl Groups**

Fig. 16 shows the correlation between the time course of enzyme inactivation at pH 10.7 with the exposure of its -SH groups at the same pH in the presence and absence of salt. The large effect of salt in speeding inactivation in alkali is reflected in a correspondingly large effect on the rate of appearance of directly titratable -SH groups. Fig. 17 shows that the time course for inactivation in alkali and reactivation on neutralization corresponds closely to the time course for the appearance and disappearance of titratable -SH groups. Fig. 17D shows a similar experiment performed for inactivation in acid. The results obtained are similar to those obtained for alkali inactivation. The -SH groups were estimated by reaction either with CMB or with DTN. It made no significant difference to the results which reagent was used.
Although the above results indicate a close relationship between —SH groups and inactivation, any such idea is not supported by results obtained elsewhere (41) and in our laboratory. These show that about four to six —SH groups in the enzyme can be titrated without any loss of activity at pH 7.0. The close correlation between —SH groups exposed and enzyme inactivated is thus a secondary and not a primary phenomenon.

**DISCUSSION**

**Two Levels of Dissociation of Enzyme**—The hydrodynamic and light scattering data presented here clearly indicate that the inactivation of hexokinase in either acid or alkali is accompanied by its dissociation into four inactive subunits. On neutralization, these subunits reassemble in a specific manner to produce the active enzyme.

The native enzyme, which has a molecular weight of 95,000 (hydrodynamic data) at pH 5.0 in low ionic strength medium, is also subject to a reversible dissociation not accompanied by any inactivation. In this case dissociation results in the formation of two active subunits of about half this molecular weight. This process is favored above pH 5.5 in the presence of high ionic strength and phosphate ions. Schachman (11) had earlier reported that the dissociation of hexokinase is favored in the presence of substrates and phosphate ions. We assume, therefore, that the half-molecule is the catalytically active species and that the whole molecule undergoes dissociation to this form under the conditions of assay. There are several examples known of proteins undergoing this type of monomer polymer equilibrium, among which can be mentioned glutamic dehydrogenase, chymotrypsin, insulin, and hemoglobin (42).

**Number of Polypeptide Chains**—The dissociation of the enzyme into four subunits in the present case indicates that it is made up of four polypeptide chains. Peptide mapping of trypsin digests of the enzyme shows about 26 different peptides, which is the number expected for four identical polypeptide chains, assuming that cleavage at each of about 26 lysine plus arginine residues present per chain in the enzyme gives rise to single distinguishable peptide. However, similar results can be expected if the enzyme is made up of polypeptide chains which are not entirely identical in structure. The solution of this problem must await end group analysis of the native enzyme.

**Changes in Secondary and Tertiary Structure**—Although optical rotation and spectral studies indicated that significant structural changes were occurring in the molecule in either acid or alkali, the increase in intrinsic viscosity obtained under the same conditions was relatively small. Thus, on the assumption that optical rotation parameters truly indicate the helicity of the molecule, we conclude that changes in the secondary structure of the enzyme occurred without changes in the tertiary structure, at least up to pH 2.8 and 11.5. The results indicate that, in spite of the loss of a considerable portion of its ordered structure, the quarter-molecule can remain relatively stable even in the absence of stabilizing disulfide bonds. One would, therefore, conclude that in the case of hexokinase the elements of stability do not entirely reside in the helical portion of the molecule. That the helix is not the only source of stability in a globular protein is obvious since many proteins are known which are rigid and almost spherical but which have no significant helicity (36, 43). This lack of correlation between changes in viscosity and changes in optical rotation noticed in the case of hexokinase finds a parallel in the conformational changes observed in the conversion of chymotrypsinogen to chymotrypsin (44). These two parameters of structure and conformation are often studied in protein denaturation may not always change synchronously.

**Forces Involved in Maintenance of Quaternary Structure**—The effect of salt or pH on the dissociation of the enzyme into half or quarter molecules points to electrostatic attractions between polypeptide chains as probably being important in holding the subunits together (42, 45). It may be pointed out here that, although the role of salt in the dissociation of the enzyme in the neutral pH range is readily understandable in terms of shielding against electrostatic interactions, its role at extremes of pH in the inactivation and dissociation of the enzyme into quarter-molecules is not so obvious. If one assumes that dissociation due to high pH or salt results from the partial neutralization of the electrostatic attractions between positively and negatively charged side chains holding the subunits together, then it is necessary to conclude that, even at the high pH used (10.5 at room temperature; 11.5 at 0°C), electrostatic interactions are only slowly abolished unless salt is present. In acid, excess of salt beyond a certain concentration causes aggregation, indicating that the repulsive forces generated by the high positive charge have in part been neutralized by salt. It is difficult to evaluate the influence of other forces in the association of subunits. The results of Ramel, Stellwagen, and Schachman (12) on the dissociation of hexokinase by sodium dodecyl sulfate indicate that hydrophobic interactions might play a role in the binding together of the subunits (46). On the other hand, no clear evidence could be obtained for the involvement of tyrosine hydrogen bonds or hydrogen or hydrophobic bonds involving —SH groups (47) in the association of subunits. On the basis of the effect of mercaptoethanol on the reactivation process, one can only say that the —SH groups of the quarter-molecule must be in the reduced state for reassociation to occur.

**Molecular Weight Measurements in Acid**—The determination of the molecular weight of hexokinase in acid solution on the basis of sedimentation velocity measurements requires some comment. Since the presence of salt caused aggregation of the enzyme in acid, the sedimentation constants had to be determined at very low ionic strength, probably insufficient to damp the charge due to acid. In spite of the fact that sedimentation constants obtained at infinite dilution were employed for molecular weight determination in acid, the value of 26,000 obtained therefrom could be in error. However, light scattering measurements under the same conditions gave a value of 29,000 for the molecular weight. This indicated that, in spite of low concentration of salt employed in the determination of sedimentation constants in acid, the value of 26,000 for the molecular weight obtained therefrom was correct enough to conclude that acid caused the dissociation of the enzyme into quarter-molecules.

**Failure of Inactivated Enzyme to Reactivate under Assay Conditions**—It is rather surprising that the acid- or alkali-inactivated enzyme failed to reactivate under the conditions of assay (pH 8.5 to 8.0; enzyme concentration 1 to 5 µg per ml). It is possible
that high dilutions of the enzyme employed in the assay system prevented the reassociation of dissociated chains. However, high concentrations of the inactivated enzyme preadjusted to pH 8.5 to 8.9 also failed to show any appreciable activity when tested under conditions of assay. Moreover, alkali-treated hexokinase also failed to show any intrinsic ATPase activity at pH 8.3, even when tested under high enzyme concentrations. The reason for failure of the inactivated enzyme to show any activity under assay conditions must therefore be sought elsewhere. It seems most likely that the rate of reactivation is extremely pH-dependent, being rapid at pH 7.0 and undetectable above pH 8.0. Another interesting point that arises in this context is whether dilution of the native enzyme in the assay system produces an active quarter-molecule, as in the case of glutamic dehydrogenase (48, 49). On the basis of the light scattering data for the native enzyme (Fig. 10), we feel this to be unlikely; a process which takes place at extremes of pH may not necessarily be brought about by mere dilution. However, molecular weight data at extremely low protein concentrations are needed to decide this point.

Reversibility of Dissociation—The reconstituted enzyme obtained after neutralization and recrystallization of the alkali-inactivated enzyme had physical, chemical, and biological properties almost identical with those of the native molecule. In the case of the acid-inactivated enzyme, no detailed studies were carried out on the crystallized material. The properties of the material obtained by direct neutralization of acid (without recrystallization) suggested, however, that the native secondary, tertiary, and quaternary structure of the molecule had been largely restored. The over-all results strongly favor the current view that a protein molecule can assume its secondary, tertiary, and quaternary structures as a direct result merely of the primary amino acid sequences of its polypeptide chains.

SUMMARY

Crystalline yeast hexokinase, an enzyme with a molecular weight of 95,000 as measured by hydrodynamic and light scattering methods, is inactivated in solutions of high and low pH with a concomitant dissociation into quarter-molecules of molecular weight 24,000. The rate of inactivation as well as dissociation as measured by light scattering is increased in the presence of salt. The enzyme, which exists in solution as essentially globular, compact, and highly ordered particles, loses a large part of its helical conformation as measured by optical rotatory dispersion when it dissociates in alkali or acid but very little of its tertiary structure, as measured by changes in viscosity. A distinct expansion of the quarter-molecule occurs only at pH values above or below those required for complete dissociation.

On neutralization, the alkali- or acid-inactivated enzyme rapidly regains its lost activity. In the case of the alkali-treated enzyme, this ability to regain activity is gradually lost in air with time of exposure to alkali but is restored by the addition of mercuric captoethanol. The reversibility of the inactivation in acid is not appreciably lost with time; it is optimal in the presence of EDTA or mercaptoethanol. Presence of salt or high protein concentration decreases this reversibility due to aggregation of the protein. Under optimal conditions, the neutralization of the acid- or alkali-inactivated enzyme results in a recovery of at least 80% of the original activity. The enzyme recrystallized after neutralization has the same specific activity as the native enzyme and has other physical, chemical, and biological properties identical with those of the native molecule.

In its native state the enzyme exists as a reversible mixture of whole and half-molecules. Dissociation into the active half-molecules is favored at pH values above 5.5 in the presence of salt, especially phosphate ions, so that this would presumably be the form present under assay conditions.

Acknowledgment—We express our appreciation to Mr. Clark Simmons, who prepared the hexokinase used in this study through the first crystallization step.

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