where

$$a = \frac{1}{n_1}\left(n_1 - \frac{B}{P} - \frac{(H^+) - (OH^-)}{P}\right)$$

After determining $1 - \alpha$, and consequently α, from eq 7 and $\Sigma\Delta h_i$ from eq 6, eq 5 may be solved for $\bar{v}$ from which the influence of protein concentration on the results obtained by the ΔpH method may be estimated (Figure 3).

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Structure–Volume Relationships for Proteins. Comparative Dilatometric Study of Acid–Base Reactions of Lysozyme and Ovalbumin in Water and Denaturing Media*

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ABSTRACT: The sign, magnitude, and the time dependence of the volume changes produced by the reaction of proteins with acids and bases are determined by the nature of the reactive species, medium, temperature, composition, and structural organization of the protein. Dilatometric analysis was used to determine the magnitude of the volume changes produced by the reaction of lysozyme and ovalbumin with HCl and NaOH in water and denaturing media. The volume increase which resulted from the reaction of lysozyme with hydroxyl ion in 8 M urea was about 85% of that produced by the same process in water. These data indicate that only minor structural changes were produced by this treatment. The volume changes produced by the protonation of lysozyme in 8 M urea were substantially lower than those predicted from model studies, whereas the volume effects in 6 M guanidine hydrochloride were larger than anticipated. From these data, it is apparent that the degree of denaturation in the two media differ substantially, a conclusion which is in accord with other physical measurements. The time dependence and magnitude of the volume isotherms for ovalbumin reacting with H^+ and OH^- both in water and in denaturants differ substantially from lysozyme. Lysozyme reached steady-state values in all media immediately upon mixing, while ovalbumin, reacting with H^+ or OH^- in 8 M urea, exhibited time-dependent volume effects. The initial rapid volume increases are associated with the ionic reactions involved in neutralization, while the slower volume decreases are related to the formation of molecular aggregates. The singular volume effects produced by ovalbumin at elevated pH in 8 M urea are explicable in terms of the titration of tyrosyl residues which are "masked" in the native protein but which are normalized in this medium.

This study was to determine whether proteins would produce volume changes characteristic of their composition and structural organization upon reaction with acids and bases in water and denaturing media. If so, this could provide another technique for determining the presence of certain types of structural organization in proteins. This approach could be used to study such phenomena as the normalizing of "buried" ionic groups, conformational changes, enzyme–inhibitor interactions, etc. The analysis of the volume changes resulting from the reaction of acids and bases with the appropriate prototropic groups in protein is facilitated by the existence of comparable data for organic acids and bases in water (Weber, 1930; Kauzmann *et al.*, 1962) and in denaturing media (Katz and Miller, 1971).

Lysozyme and ovalbumin, proteins which incorporate 30–35% helical structure and some β structure (Jirgenson, 1969), were selected because of their different response to urea and guanidine hydrochloride. Lysozyme, which contains four disulfide bonds, shows little structural alteration upon exposure to 9 M urea (Steiner, 1964; Warren and Gordon, 1970) but

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undergoes extensive denaturation when subjected to 6 M guanidine hydrochloride (Aune and Tanford, 1969). Ovalbumin, a glycoprotein, is denatured by urea at concentrations ≥ 6 M (Simpson and Kauzmann, 1953; Frensdorff *et al.*, 1953; McKenzie *et al.*, 1963). These dilatometric studies corroborate the differences in the denaturation processes for these two proteins.

Urea was used as the principal denaturing medium because the magnitude of the volume changes of model compounds in this agent was comparable to those produced in water, *i.e.*, about 85% of that produced in water (Katz and Miller, 1971), and under the experimental conditions employed, there were no competitive side reactions (Stark, 1967). Guanidine hydrochloride was less suitable for several reasons: (i) the volume changes for the protonation of carboxylate residues were about one-half that found in water and (ii) it could not be used for alkaline titration since it is a hydroxyl ion acceptor (Nozaki and Tanford, 1967; Katz and Miller, 1971).

Experimental Section

Dilatometry. The volume changes were determined dilatometrically at 30.0 ± 0.001°; the details of the procedure have been described previously (Katz and Ferris, 1966). The concentration and volumes of reactants were selected to produce volume changes ≥ 2.5 μl at the pH extremes. Standard acid or base was added to one arm of the mixing vessel and an equal volume of protein was added to the other. The concentration of HCl or NaOH in water or 8 M urea ranged from 0.001 to 0.05 M. Guanidine hydrochloride (6 M) was used for the acid titration of lysozyme. Generally 5 ml of 5–6%, w/v, lysozyme was employed for the dilatometric experiments. Ovalbumin, in water, was clarified by centrifugation at 37,000*g* for 20 min. The dissolution of ovalbumin in 8 M urea was incomplete; the precipitate, 20–30%, was removed by centrifugation. For these experiments, 5–8 ml of 3–4% ovalbumin in 8 M urea was used. The dilatometric titration experiments employing water as solvent were performed in the conventional manner (Rasper and Kauzmann, 1962). When denaturing media were used, the protein was in contact with the denaturant 5–6 hr before reaction with either the acid or base. The acids, in 8 M urea, were often prepared 24 hr in advance; however, NaOH solutions were prepared immediately before use.

Titrations. The procedure, calculations, corrections for junction potential, and activity coefficient used to calculate the hydrogen and hydroxyl ion binding isotherms have been described (Katz and Maurer, 1957). Reference curves were prepared for HCl and NaOH in the several media employed. A Radiometer pH meter Model 26 with a semimicro combination electrode, GK 2321C, was used for pH measurement. Several minutes were required before steady-state readings were obtained in the denaturant. The pH meter drift between restandardization intervals in these media was ≤ 0.02 pH unit. Harleco standard buffers were used for standardization. The binding isotherms were determined from the solutions used in dilatometry. To determine the influence of the manometric fluid, *n*-heptane, on the binding isotherm, duplicate titration experiments were performed with heptane-free solutions. The agreement between the two sets was within 3%. The zero point adopted for constructing the isotherms was the pH of the salt-free protein in water.

Cyanate Determination. The rate of cyanate formation in 8 M urea was determined by modification of the Marrier–Rose (1964) version of Werner's (1923) test for cyanate. Two milliliters of 0.4 M sodium cacodylate (pH 6.3) was added to a 60-ml separatory funnel followed by 5 ml of 0.4% $CuSO_4$ and 1 ml of pyridine. The sample (10 ml) was added and the system vigorously mixed; the cyanate complex was extracted with 6 ml of dichloroethane. Concentration was determined spectrophotometrically at 700 nm. The solutions were read 0.5–1.0 hr after extraction; the absorbancy was stable for a 3 hr period after extraction. The sensitivity of analysis was 5×10^{-4} M cyanate.

Acrylamide Gel Electrophoresis. The procedure for the kinetic analysis of protein denaturation by planar acrylamide gel electrophoresis is in print (Katz and Denis, 1969). The methodology simulated the dilatometric experiments; 5–25 μl of 1–2% protein was used for the sample application. Lysozyme was subjected to electrophoresis in 7.5% acrylamide gel with an electrolyte composed of 0.29 M Tris and 0.07 M glycine (pH 9.2). The applied voltage was 250 V; the initial current was 75 mA, run duration was 5 hr. Electrophoresis was also performed with a 10% acrylamide gel employing a pH 4.5 electrolyte composed of 0.35 M β-alanine and 0.128 M glacial acetic acid (Reisfeld *et al.*, 1962). The voltage was 250 V; initial current was 110 mA, and the running time was 4.5 hr. Ovalbumin in denaturants formed material which remained at the point of application necessitating the use of a 4% spacer gel combined with a 7.5% running gel. The electrolyte was a modified Peacock buffer (Peacock *et al.*, 1965) composed of 0.09 M Tris, 0.09 M boric acid, and 0.0025 M Na_2EDTA. The voltage was 250 V, the initial current was 80 mA, and the running time was 4–4.5 hr.

Analysis. The protein concentrations were determined by dry weights and spectrophotometric analysis. For lysozyme at 280 nm $E^{1\%}_{1\,cm}$ of 26.4 at pH 5.4 was used (Sophianopoulos *et al.*, 1962). For ovalbumin we determined a value of $E^{1\%}_{1\,cm}$ of 7.34 at 280 nm which compared favorably with a value of 7.35 reported by Cunningham and Nuenke (1959).

Materials. Lysozyme, low-salt, was purchased from Worthington (Freehold, N. J.) and chicken egg albumin, five-times crystallized, was obtained from Pentex Biochemicals (Kankakee, Ill.). Standard NaOH (CO_2 free), HCl, and buffers were from Harleco Co. (Philadelphia, Pa.). Tris, glycine, and β-alanine were products of Sigma Chemical Co. (St. Louis, Mo.). Urea, *n*-heptane (Mallinckrodt), and guanidine hydrochloride (Sigma) were purified before use (Katz and Ferris, 1966; Katz and Miller, 1971). The materials for acrylamide gel electrophoresis were from Fisher Co; the remainder of the analytical grade reagents were from Mallinckrodt Co.

Results

The ΔV isotherm[1] produced by the reaction of lysozyme in water with OH^- is a continuous linear function with a slope of 15 ml/mole of OH^- bound at the origin (Figure 1). The slope increases gradually with OH^- concentration reaching a value of about 20 ml/mole of OH^- bound at the pH extreme. The use of 8 M urea as solvent reduces the magnitide of the volume change; the slope increases from 14 ml/mole of OH^- bound near the origin to 16 ml/mole of OH^- bound at the pH extreme. The magnitude of ΔV, at 60 moles of OH^- bound, is 875 and 970 ml in 8 M urea and water, respectively, *i.e.*, the ΔV in 8 M urea is 90% that produced in water.

The ΔV isotherms for the reaction of lysozyme with H^+

[1] The values for ΔV, the volume changes, are calculated on the basis of 10^5 g of protein. All computations in the text are based on 10^5 g of protein unless specific reference is made to the molecular weight.

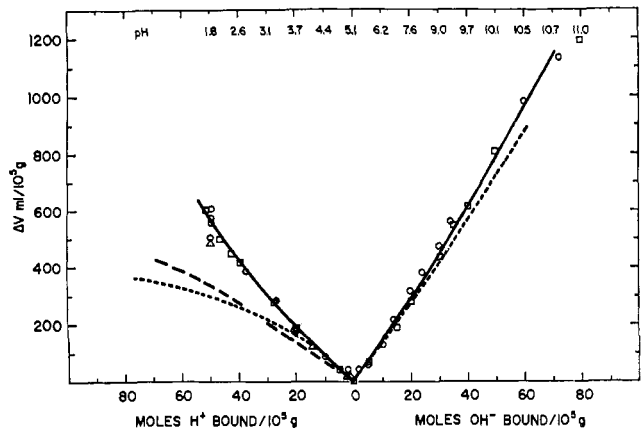

FIGURE 1: Volume changes produced by the reaction of HCl or NaOH with lysozyme at 30.0°. These data are calculated in terms of 10^5 g of protein. —, measurements performed in water; ---, in 8 M urea; — — — in 6 M guanidine hydrochloride. The different symbols indicate different experiments. Symbols were not inserted for systems containing denaturant due to the close juxtaposition of the several curves.

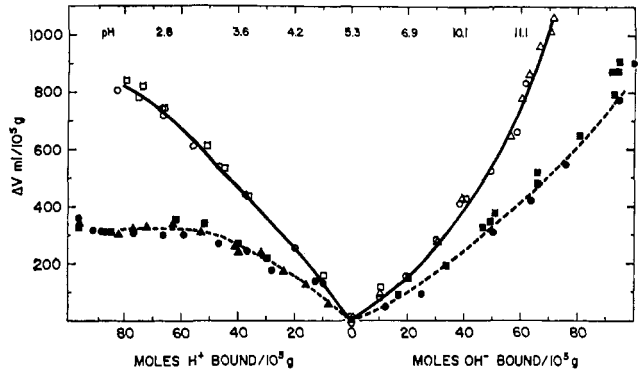

FIGURE 2: Volume change produced by the combination of either HCl or NaOH with chicken egg ovalbumin at 30.0°. For details refer to Figure 1.

exhibit a strong medium dependence (Figure 1). In water, the slope near the origin is 10 ml/mole of H^+ bound and this parameter increases to 14 ml/mole of H^+ bound at the pH extreme. The use of 8 M urea or 6 M guanidine hydrochloride reduces the magnitude and slope of the ΔV isotherm considerably. In 8 M urea, the initial slope is 10 ml/mole of H^+ bound but with increasing H^+ concentration there is a reduction of volume rise; the slope at the pH extreme is 2 ml/mole of H^+ bound. The use of 6 M guanidine hydrochloride causes volume effects which differed in degree from 8 M urea. The slope at the origin is 7 ml/mole of H^+ bound and then decreases to 4 ml/mole of H^+ bound at the pH extreme. A comparison of ΔV at the point where 50 moles of H^+ are bound reveals values of 560, 335, and 300 ml in water, 6 M guanidine hydrochloride, and 8 M urea, respectively.

These data are the steady-state values determined 2–3 min after mixing; there are no detectable volume changes in the period from 3 min to 2 hr after mixing. The pH values recorded are a function of the concentrations of acid, alkali, and protein; the values in Figure 1 are for a 5% lysozyme solution in water. These values differ from those measured in the systems containing denaturants since these agents cause pH shift of several tenths of a pH unit (Bates, 1969; Katz and Miller, 1971).

The volume effects produced by the reaction of ovalbumin with H^+ and OH^- ions in water and 8 M urea are substantially different. The ΔV values resulting from the reaction of ovalbumin with H^+ and OH^- in water achieve steady-state values 2–3 min after mixing; however, in 8 M urea the volume varies as a function of time. These volume effects are presumed to consist of (i) the fast reaction, completed 2–3 min after mixing, and (ii) the slow volume effects characterized by volume decreases. The data in Figure 2 pertains to the fast or "3-min reading." The volume rise due to the reaction of ovalbumin with OH^- in water obeys a geometric relationship with respect to OH^- concentration (Figure 2). The slope at the origin is 7.5 ml/mole of OH^- bound and increases to 17 ml/mole of OH^- bound, in the region where 40–50 moles of OH^- are bound, and 40 ml/mole of OH^- bound at the pH extreme. In 8 M urea there is a marked reduction of the magnitude and slopes for these isotherms with the corresponding values for the slopes being 5, 7.5, and 10 ml per mole of OH^- bound. A comparison of ΔV at the point where 70 moles of OH^- are bound gives values of 1100 ml and 515 ml in water and 8 M urea, respectively.

The slope of the isotherm resulting from the reaction of ovalbumin with H^+ in water, in contrast to the OH^- isotherm, tends to decrease with increasing H^+ concentration. The slope at the origin is 12.5 ml/mole of H^+ bound; it decreases to 10.5 ml/mole of H^+ bound in the region where 20–60 moles of H^+ are bound, and then exhibits a further decrease to 7 ml/mole of H^+ bound at the pH extreme. The presence of 8 M urea attenuates the volume effects; the initial slope is 7 ml/mole of H^+ bound and the curve levels asymptotically at the pH where the proton binding ≥ 50 ml/mole of H^+ bound. The diminution of the volume rise by urea is illustrated by comparing ΔV at the point where 50 moles of H^+ are bound; a ΔV of 570 ml is observed in water compared to 310 ml in 8 M urea.

The ΔV produced by the reaction of ovalbumin with HCl or NaOH in water reaches steady-state values 2–3 min after mixing and is time independent in the pH range studied; however, in 8 M urea these processes are time dependent. The volume increase resulting from the reaction with HCl in 8 M urea reaches the maximum values, 2–3 min after mixing. There is a subsequent 30% reduction of peak volume in the next 10-min interval followed by another 10% reduction during the next 30 min; steady-state values are reached about 1 hr after mixing. In alkali–urea a similar time course is observed except that the relative change is smaller. The largest volume increase is found 2–3 min after mixing; this is followed by a 10% reduction in volume 10–15 min after mixing. No further volume effects are observed during the subsequent hour interval. One concludes that the ΔV changes, 2–3 min after mixing, are reduced by these negative time-dependent volume effects.

Acrylamide Gel Electrophoresis. The gel electrophoretic studies corroborated the different response of these proteins to acids and bases in water and in 8 M urea Lysozyme exposed to 0.05 M HCl in water and 8 M urea and to 0.05 M NaOH in water for times ≤ 18 hr shows no alteration of electrophoretic patterns. However, exposure to 8 M urea for 18 hr before contact with 0.02 M NaOH for 1 hr produces a normal electrophoretic pattern except for the appearance of a fraction, 5–10%, with essentially zero mobility.

Ovalbumin dissolved in 0.05 M HCl or 0.05 M NaOH for times ≤ 1 hr produces patterns similar to native ovalbumin (compare columns 1, 2, and 3, Figure 3). Ovalbumin exposed for 5 hr to 8 M urea at 30° produces material which remains at

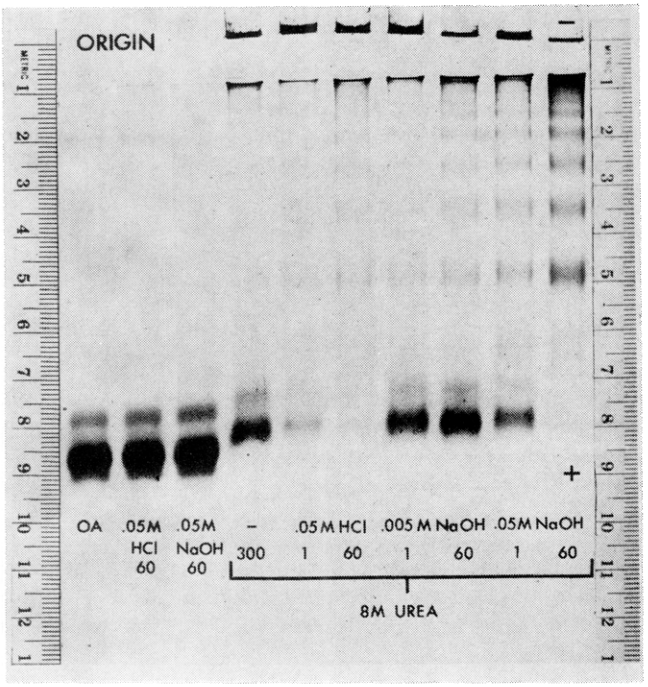

FIGURE 3: Kinetics of the reaction of ovalbumin with HCl and NaOH in water and 8 M urea at 30.0°. The pattern on the extreme left is the control; ovalbumin, OA, in water. The two adjacent patterns are OA in 0.05 M HCl and 0.05 M NaOH, exposure time 60 min. The fourth pattern represents OA exposed to 8 M urea for 300 min. The other patterns represent OA exposed to 8 M urea for 5 hr before contact with HCl or NaOH in 8 M urea for varying periods of time expressed in minutes. Details of the electrophoretic procedure are described in the text; a 4% spacer gel was used in conjunction with a 7.5% running gel.

the origin and layers at the interface between the spacer and running gel. The remainder of the denatured protein can be resolved as 6–10 discrete bands (column 4, Figure 3). The addition of acid to ovalbumin pretreated with 8 M urea to produce a 0.05 M HCl–8 M urea system causes an increase of aggregated material and a corresponding decrease of fractions with intermediate mobility. The results of 1- and 60-min exposure to this system are depicted in columns 5 and 6 (Figure 3). NaOH–8 M urea promotes the formation of fractions with intermediate mobility; increasing the concentration of NaOH from 0.005 to 0.05 M causes an increase of material with intermediate electrophoretic mobility (see columns 7–10, Figure 3).

Cyanate formed by the isomerization of urea at alkaline pH could introduce a source of error by competitive reaction with the amino and sulfhydryl residues in proteins (Stark, 1967). The rate of formation of cyanate in 8 M urea as a function of pH was determined in order to establish the magnitude of this factor. Urea (8 M) was exposed to NaOH for 6 hr at 25° at pH ranging from 8 to 12; the maximum cyanate formation was at pH 9 and its concentration was approximately 5×10^{-4} M. Thus cyanate formation under these experimental conditions does not introduce an appreciable source of error.

Discussion

One objective of this work was to determine whether proteins with differing compositions and structural organizations, such as lysozyme and ovalbumin, would produce characteristic volume changes upon dilatometric titration with acids and bases in water and denaturing media. The volume isotherms for these two proteins differ considerably, thus substantiating this hypothesis. An insight of the molecular processes involved is provided by comparing the experimental with the calculated ΔV. The latter factor is computed from the protein's composition and the mean volume change produced by the appropriate prototropic group titrated (Rasper and Kauzmann, 1962). Differences between the "calculated" and experimental ΔV may reflect the protein's structural contribution to these volume effects. The ΔV for the reaction of lysozyme with OH^- in water, pH region between 8 and 10.5, is calculated as follows: the mean ΔV values for the neutralization of ammonium radicals is 23 ml/mole of OH^- (Weber, 1930; Kauzmann *et al.*, 1962) and about 3 ml/mole for tyrosyls (Weber, 1930; Krausz, 1970); since there are 48 titratable ammonium and 14 tyrosyl residues[2] the mean slope per mole of OH^- bound is 18.8 ml/mole. This is larger than the experimental value of 17 ml/mole; if all the tyrosyls reacted with OH^-, the calculated value would be reduced 17 ml/mole of OH^- bound. One cannot select between these alternatives since the mean values are derived from simple organic compounds; the actual values can vary considerably as a function of neighboring groups, charge, salt effects, etc. (Weber, 1930; Kauzmann *et al.*, 1962). This analysis is applicable to the corresponding 8 M urea system; the value for the neutralization of protonated nitrogenous bases is taken as 19/ml mole of OH^- bound (Katz and Miller, 1971). The ΔV for neutralization of tyrosyls in 8 M urea is assumed to be similar to that in water and all tyrosyls are considered to be titratable. The calculated value of 14 ml/mole of OH^- bound is in good agreement with the experimental value of 15 ml/mole of OH^- bound.

Lysozyme dimerizes at pH values ≥ 5 at the concentrations employed in this study (Sophianopoulos and Van Holde, 1964). The volume effects were not demonstrably affected by this phenomenon; apparently the physical juxtaposition of the protomers does not interfere with titration and/or the rate of dimerization is considerably slower than the neutralization processes.

The reaction of OH^- with ovalbumin in water and 8 M urea produces volume changes which differ substantially from lysozyme. The low value for the slope, 8 ml/mole of OH^- bound between pH 5.3 and 7 in water, is due to the neutralization of carboxyl and phosphate groups. The ΔV for the neutralization of carboxyl groups is about 8 ml/mole and about 5 ml/mole for phosphate esters (Rasper and Kauzmann, 1962). In the region where 40–50 moles of OH^- are bound, the slope increases to 17 ml/mole of OH^- bound which is lower than the calculated value for the neutralization of nitrogenous bases. Since the "masked" tyrosyls are not titrated (Crammer and Neuberger, 1943) this must represent the contribution of the 11 cysteinyl sulfhydryl residues. We assume that the volume changes produced by these residues is small; however, the lack of data for sulfhydryls precludes a more definitive statement. At higher OH^- concentrations, the slope increases to 40 ml/mole of OH^- bound, a value almost double the ΔV for neutralization of protonated nitrogenous bases. This additional volume increment must be due to the structural destabilization of ovalbumin which occurs at elevated pH (Crammer and Neuberger, 1943). Volume increases have been demonstrated for certain types of denaturation, *e.g.*, urea de-

[2] Only two of the three tyrosyl residues in native lysozyme are titratable according to Sakakibara and Hamaguchi (1968).

naturation of albumin (Katz and Ferris, 1966; Olesen and Pederson, 1968), and myoglobin (Atanasov, 1970; Katz and Denis, 1970), and sodium dodecyl sulfate denaturation of albumin and myoglobin (S. Katz and J. E. Miller, unpublished results). Charge effects provide no basis for predicting a volume rise; on the contrary, one might anticipate a reduced effect since a localized increase of negatively charged carboxylate residues attenuates the ΔV of neutralization of ammonium groups (Kauzmann *et al.*, 1962).

The ΔV for the neutralization of ovalbumin in 8 M urea is substantially reduced relative to water (Figure 2). This is due to the titration of the previously "masked" tyrosyl residues which produce volume changes about 15–20% that produced by nitrogenous bases (Krausz, 1970). We determined 23 additional titratable goups in 8 M urea (Figure 2), a value in accord with the 21 ± 1 tyrosyl residues determined by chemical analysis (Lewis *et al.*, 1950; Habeeb, 1961). The time dependence of the volume changes produced in 8 M urea is undoubtedly related to the kinetic effects observed by spectral, optical rotation, and viscosity measurements (Simpson and Kauzmann, 1953; McKenzie *et al.*, 1963). These have been interpreted as being the summation of slow and fast reactions with the slow reaction being associated with aggregate formation; this has been substantiated by our electrophoretic studies (Figure 3). The slow-volume decrease specifically reflects the structural changes associated with the intra- and intermolecular disulfide exchange processes which occur under these conditions (Simpson and Kauzmann, 1953; McKenzie *et al.*, 1967; Katz and Denis, 1969).

The isotherm resulting from the protonation of lysozyme in water exhibits a slope which increases gradually with increasing pH (Figure 1). This is explicable in terms of the titration of carboxylate groups with different pK values and different electrostriction propensities. Even though 10 ml/mole was adopted as the mean value for the protonation of carboxylate residues, values for protonation range from 7.8 for formate to 22 ml/mole for maleate ion (Kauzmann *et al.*, 1962). The titration curve for lysozyme in this region has been analyzed as consisting of one carboxylate with pK = 6.0, six with pK = 4.3, one with pK = 3.5, and three "masked" residues (Donovan *et al.*, 1960; Sakakibara and Hamaguchi, 1968).

The corresponding isotherm for ovalbumin exhibits a curvilinear relationship characterized by a progressively decreasing slope in contrast to the increasing slope for lysozyme. Obviously, the carboxylates in these two proteins exist in different molecular environments to produce such diametrically different volume effects. The reduced value in ovalbumin may reflect their interaction with the 21 "masked" tyrosyls. Harrington (1955) found an increase of acid and base binding groups upon denaturation of ovalbumin and postulated tyrosyl–carboxylate bonding in native protein.

The ΔV isotherms resulting from the reaction of HCl with both of these proteins in 8 M urea are not only similar in gross detail, but also the volume changes are substantially lower than that predicted. Another similarity is the tendency for the slopes to level asymptotically at the point where 60 moles of H^+ are bound. The basis for the reduction of volume effect is a subject for continuing investigation. A contrasting feature is the time dependence for the volume changes; lysozyme reaches a steady state immediately upon mixing while the volume for ovalbumin decreases with time. The time effects noted for ovalbumin are due to the formation of aggregates produced from disulfide interchange reactions (Frensdorff *et al.*, 1963; McKenzie *et al.*, 1963; Katz and Denis, 1969).

The anomalous volume effects produced in HCl–8 M urea prompted a similar study employing 6 M guanidine hydrochloride. The resultant volume rise for lysozyme was larger than calculated. The isotherm was characterized by a linear slope, 7 ml/mole in the region where 0–40 moles of H^+ was bound and then the slope decreased to 4 ml/mole at the pH extreme. The value for the initial slope is larger than the mean value of 5 ml/mole of H^+ bound determined for carboxylate-containing compounds in 6 M guanidine hydrochloride (Katz and Miller, 1971; S. Katz and J. E. Miller, unpublished data). The elevated values found for lysozyme may be due to a concomitant volume increase produced by the acidification of the protein in this denaturing medium. Ovalbumin was not studied because the rapid rate of aggregation complicated the data analysis.

The previous study of these proteins by Rasper and Kauzmann (1962) provides a basis for determining variations due to differences of proteins' preparation, technique, materials, etc. They employed 0.15 M NaCl to reduce variation of ionic strength upon titration, whereas low-salt systems were used here to minimize competitive ion binding and to reduce the number of variables. The agreement between the two groups of investigators is within 60 ml/10^5 g of protein, calculated at the pH extremes. The comparison was complicated by the difference of pH of the initial protein solutions; values of 5.3 and 5.1 were determined for ovalbumin and lysozyme in water as compared to 4.9 and 5.0 in 0.15 M NaCl. Another source of uncertainty was the difficulty inherent in extrapolation from graphs reduced upon publication. Therefore, the agreement between these studies can be considered as being satisfactory.

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A New Type of Peptide Subunit in the Murein of *Arthrobacter* Strain J39*

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ABSTRACT: The murein (peptidoglycan) of *Arthrobacter* strain J39 was found to contain D-alanine, L-lysine, glycine, and D- and L-glutamic acid in the molar ratio of 1:1:3:2. Approximately half of the D-glutamic acid was hydroxylated. The UDP-activated precursors of the murein were isolated. The molar ratios Mur:Gly:Glu = 1:1:2 and Mur:Gly:Glu:Ala = 1:1:2:2 were found for the tripeptide and the pentapeptide, respectively. Partial acid hydrolysis of the purified precursors and analysis of the obtained peptides proved the sequence *N*-acetylmuramylglycyl-γ-D-glutamyl-L-glutamic acid for the UDP-*N*-acetylmuramyl tripeptide, and the sequence *N*-acetylmuramylglycyl-γ-D-glutamyl-L-glutamyl-D-alanyl-D-alanine for the UDP-*N*-acetylmuramyl pentapeptide. Analysis of the peptides isolated from partial acid hydrolysates of cell walls confirmed the amino acid sequence of the peptide subunit as found in the murein precursors. The cross-linkage of the peptide subunits was shown to be performed by the peptide glycylglycyl-L-lysine. The *N*-terminal glycine is attached to the α-carboxyl group of the D-glutamic acid, while the ε-amino group of the lysine is bound to the D-alanine of the adjacent peptide subunit.

Most mureins (peptidoglycans) contain only 1 mole of glutamic acid/mole of peptide subunit. When additional glutamic acid is found, it is localized in the interpeptide bridge (Niebler *et al.*, 1969; Kandler *et al.*, 1970; Schleifer and Kandler, 1970; D. Bogdanovsky *et al.*, 1971, in preparation). The cell wall of an *Arthrobacter* isolated by Keddie *et al.* (1966) was found to contain 2 moles of glutamic acid/mole of peptide subunit. The present paper describes the amino acid sequence of this murein. Unlike the other mureins which are rich in glutamic acid, in this case the second mole of glutamic acid is not a constituent of the interpeptide bridge, but of the peptide subunit.

Material and Methods

The *Arthrobacter* strain J39 was kindly supplied by Dr. Keddie, Department of Microbiology, The University, Reading, England. The bacteria were grown aerobically in yeast extract glucose broth (Schleifer *et al.*, 1967a) with 0.5% NaCl at 28°. Cell walls were prepared by the usual technique (Schleifer and Kandler, 1967).

Total hydrolysis of cell walls, peptides, and murein precursors was carried out in 4 N HCl at 100° for 16 hr or in 5 N HCl at 100° for 5 hr. Partial acid hydrolysis was performed as described previously (Schleifer and Kandler, 1967).

The following solvent systems were used for separation of amino acids, amino sugars, and peptides by paper chromatography: (I) isopropyl alcohol–acetic acid–water (75:10:15, v/v) and (II) α-picoline–25% NH_4OH–water (70:2:28, v/v).

Peptides containing hydroxyglutamic acid and glutamic acid were separated by high-voltage electrophoresis under the following conditions: 58 V/cm, 2–4 hr, formic acid–acetic acid–water (5:15:80, v/v), pH 1.9, Whatman paper No. 3MM (Schleifer *et al.*, 1967b).

Quantitative determination of amino acids was performed with an amino acid analyzer.

Dinitrophenylation of cell walls and peptides was carried out according to Primosigh *et al.* (1961). The dinitrophenylated amino acids were identified by paper chromatography

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