JOURNAL OF THE
AMERICAN CHEMICAL SOCIETY

VOLUME 84 JUNE 5, 1962 NUMBER 10

PHYSICAL AND INORGANIC CHEMISTRY

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, FRICK CHEMICAL LABORATORY, PRINCETON UNIVERSITY, PRINCETON, NEW JERSEY]

Volume Changes in Protein Reactions. I. Ionization Reactions of Proteins

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RECEIVED NOVEMBER 13, 1961

Measurements have been made of the changes in the partial specific volumes of proteins on addition of acid and alkali. Below *p*H 5 the reaction $-COO^- + H^+ \rightarrow -COOH$ is accompanied by an increase in volume of about 11 ml. per mole of protons reacting. As has been pointed out by Weber and Nachmansohn, this indicates that the carboxyl groups of proteins are in a state very similar to that obtaining in simple carboxylic acids. There are, however, slight differences in the volume changes observed with different proteins, and in ovalbumin the volume change per proton reacting varies somewhat with *p*H. These variations presumably reflect slight differences in the environments of the carboxyl groups in different proteins. The addition of alkali to proteins gives a volume change of +16 to +18 ml. per mole of hydroxyl reacting in the *p*H range in which imidazole and amino groups are believed to dissociate. This is only about two thirds of the value observed with the same groups in simple molecules and indicates that the environments of these groups in proteins must be somewhat different from that in small molecules. The volume changes in ovalbumin between *p*H 5.3 and 6.8 have very different values from those observed in other proteins, probably reflecting an unusual state for the imidazole side chains in ovalbumin. The optical rotation of ovalbumin has been measured as a function of the *p*H. There is no reason to conclude that the unusual behavior of the imidazole groups in ovalbumin reflects a change in the conformation of the molecule.

The partial molal volume of a protein may be considered to be made up of three increments—a constitutive volume, a conformation increment and a solvation increment. The constitutive volume is the volume determined by the bond lengths and the van der Waals radii of the atoms in the protein, the values being deduced from studies on simple molecules. From these dimensions one can describe an envelope enclosing a region which should be a good first approximation to the volume of the molecule. The constitutive volume, defined in this way, will be a function of the amino acid composition of the protein alone.

When the polypeptide chains and amino acid side chains of proteins are folded into the specific conformations that exist, for instance, in the native protein, the regions included in the constitutive volumes of different portions of the molecule will generally fail to pack perfectly with each other. As a result, voids will occur in some parts of the folded molecule and compressed regions may occur in other parts. The conformation increment is made up of the net contribution of these voids and compressed regions.

The solvation increment exists because protein molecules alter the state of the solvent that lies within their spheres of influence. For instance, the solvent in the vicinity of charged groups on a protein molecule is compressed by the electrostrictive action of the electric fields around the charges.[1] The volume of the solvent adjacent to uncharged groups may also be altered; studies of dilute aqueous solutions of non-polar molecules indicate that the water structure adjacent to these molecules is drastically modified,[2-4] producing a marked decrease in volume.[5] Furthermore, solvent may occupy some of the voids in the conformation increment, reducing this increment to some extent and making it somewhat difficult to separate the conformation increment from the solvent increment by experimental means.

If a protein is modified either by changing its state of ionization or by changing the conformation of the peptide chain, volume changes will be expected which can be ascribed to the conformation and solvation increments defined above. Since the properties of proteins are believed to depend in an important way on the molecular conformation and on the state of solvation of the molecule, one would

(1) P. Drude and W. Nernst, *Z. physik. Chem.*, **15**, 79 (1894).
(2) H. S. Frank and M. W. Evans, *J. Chem. Phys.*, **13**, 507 (1945).
(3) I. M. Klotz, *Science*, **128**, 815 (1958); *Brookhaven Symposia in Biology*, **13**, 25 (1960).
(4) W. Kauzmann, *Advances in Protein Chem.*, **14**, 1 (1959).
(5) W. L. Masterton, *J. Chem. Phys.*, **22**, 1830 (1954).

expect that a study of volume changes accompanying their reactions might be helpful in understanding proteins.

One of the simplest types of protein reactions is that in which acidic and basic groups lose or bind protons. These reactions have been extensively studied,[6-9] predominantly by analysis of titration curves. It is generally felt that, by and large, most of the acidic and basic groups of proteins differ very little in their inherent properties from similar groups in small molecules, though there is much evidence that some groups are abnormal in certain proteins. (For instance, there is evidence of abnormality for 22 histidine groups in hemoglobin,[10] all nine of the tyrosines of ovalbumin,[11] three of the six tyrosines of ribonuclease[12] and three of the carboxyl groups of lysozyme.[13])

It is of interest to learn whether the volume changes accompanying the ionization reactions of proteins have the same values as those expected from small molecules. This question was investigated thirty years ago by Weber and Nachmansohn,[14,15] who showed that ionization of the carboxyl groups of proteins is accompanied by a volume change similar to that observed when acetic acid ionizes. They also noted that the volume change for the ionization of the amino groups of proteins is somewhat smaller than that observed in simple amines. These papers have considerable historical importance because they demonstrated so clearly that proteins are amphoteric ions and that even at the isoelectric point large numbers of charged groups are present in a state not very different from that in simple carboxylic acids and amines.

In the present paper these volume changes are investigated more closely, using a greater number of proteins of a greater degree of purity than those available to Weber and Nachmansohn and using improved experimental methods. In order to interpret the observations, the volume changes accompanying the ionization of a number of simple acids and bases have also been studied; these results will be described in the next paper.[16] Later papers will describe the volume changes that accompany ionization reactions in macromolecules[17] and the binding of ions by hair and wool.[18]

(6) J. Steinhardt and E. M. Zaiser, *Advances in Protein Chem.*, **10**, 151 (1955).

(7) J. T. Edsall and J. Wyman, "Biophysical Chemistry," Vol. I, Academic Press, Inc., New York, N. Y., 1958, Chap. 9.

(8) C. Tanford, "Symposium on Protein Structure" (A. Neuberger, ed.), John Wiley and Sons, Inc., New York, N. Y., 1958.

(9) K. Linderstrøm-Lang and S. O. Nielsen, "Electrophoresis. Theory, Methods and Applications" (M. Bier, ed.), Academic Press, Inc., New York, N. Y., 1959.

(10) S. Beychok and J. Steinhardt, *J. Am. Chem. Soc.*, **81**, 5679 (1959).

(11) J. L. Crammer and A. Neuberger, *Biochem. J.*, **37**, 302 (1943).

(12) D. Shugar, *Biochem. J.*, **52**, 142 (1952); C. Tanford, J. D. Hauenstein and D. G. Rands, *J. Am. Chem. Soc.*, **77**, 6409 (1955).

(13) J. W. Donovan, M. Laskowski, Jr., and H. Scheraga, *ibid.*, **82**, 2154 (1960).

(14) H. Weber and D. Nachmansohn, *Biochem. Z.*, **204**, 215 (1929).

(15) H. Weber, *ibid.*, **218**, 1 (1930).

(16) W. Kauzmann, A. Bodanszky and J. Rasper, *J. Am. Chem. Soc.*, **84**, 1777 (1962).

(17) J. Rasper, A. Bodanszky and W. Kauzmann, to be published.

(18) D. D. Kasarda and W. Kauzmann, to be published.

Experimental

Volume changes were measured by means of Carlsberg dilatometers similar to those described by Linderstrøm-Lang and Lanz[19] (see also Linderstrøm-Lang[20] and Kauzmann[21]) with the modifications suggested by Johansen and Thygesen[22] and by Johansen.[23] The dilatometers are in the form of an inverted "V," with one arm containing the protein (usually about 5 ml. of a 5% solution) and the other containing the acid or base. All solutions are evacuated before pipetting into the dilatometers so as to reduce the amount of dissolved air, which otherwise often comes out of solution to form small bubbles when the dilatometers are placed in the thermostat. The space above the solutions is filled with purified kerosene and a graduated capillary (10 μl. full scale, graduated to 0.05 μl. subdivisions; total length of graduations about 25 cm.) is attached to the vertex of the "V" by means of a specially lubricated[23] ground glass joint. After adjusting the level of the kerosene in the capillary, the dilatometers are immersed in a well-controlled thermostat ($\pm 0.001°$), the level of the kerosene is read and the solutions are mixed by pouring back and forth from one arm of the "V" to the other. The change in the kerosene level gives the volume change of the reaction. Volume changes are reproducible to a few hundredths of a microliter. All measurements were made at 30.00°. The volume changes take place, of course, immediately after the acid or base is added to the protein, but since some heat is evolved in the reaction, a short time is required before thermal equilibrium is re-established after mixing. The half time for thermal equilibration is about 0.9 min. if the dilatometers are allowed to stand undisturbed, but if they are agitated by allowing the solution to pour back and forth from one arm to the other, the half time is reduced to 0.3 min. In general, the values reported in this paper are those observed within a few minutes after mixing. In some instances a slow, time-dependent volume change occurred because of gradual denaturation or other modification of the protein. When these changes were not too rapid, the dilatometer readings were extrapolated back to the time of mixing in order to find the volume change due to the reaction with acid or base.

Optical rotations were measured on an O. C. Rudolph and Sons Model 200 photoelectric spectropolarimeter using 2 dm. jacketed tubes through which water at 30.0° ($\pm$ 0.1°) was circulated. Rotation readings could be repeated on a given sample to $\pm 0.002°$, but the reproducibility was less good on a series of identical samples.

Ovalbumin was prepared by the method of Sørensen and Høyrup[24] and was recrystallized four times. Hemoglobin was prepared from ox blood by repeated centrifugation (5 or 6 times) of the red cells suspended in 0.9% NaCl until no protein was detectable in the supernatant (cloudiness with trichloroacetic acid), followed by shaking with toluene to remove the cell membranes, then dialysis. The remaining proteins were purchased: bovine serum albumin from Armour and Co. and from Pentex, Inc. (Lot 12016P; these two materials gave identical results); ribonuclease (Armour and Co. Lots 381-059 and 647-213); lysozyme (Armour and Co. Lot D-638040); and chymotrypsin (Armour and Co. Lot 293).

All experiments were performed in the presence of 0.15 *M* NaCl in the protein solution and in all of the added reagents. This makes it possible to add or remove a greater number of protons without going to extreme *p*H values. A Beckman Model G *p*H meter was employed for the *p*H measurements. At low *p*H values the number of protons bound to the protein was calculated from the difference between the amount of acid added to the protein and the amount of free acid. In these measurements the *p*H scale was calibrated using hydrochloric acid solutions of known concentrations. The final *p*H was never so great as to necessitate corrections for free hydroxyl remaining after addition of alkali to the proteins.

(19) K. Linderstrøm-Lang and H. Lanz, *Compt. rend. trav. lab. Carlsberg, Ser. chim.*, **21**, 315 (1938).

(20) K. Linderstrøm-Lang, "Methoden der Enzymforschung" (K. Myrbäck and E. Bauman, eds.), Leipzig, 1940, p. 970.

(21) W. Kauzmann, *Biochim. et Biophys. Acta*, **28**, 87 (1958).

(22) A. Johansen and J. E. Thygesen, *Compt. rend. trav. lab. Carlsberg, Ser. chim.*, **26**, 369 (1948).

(23) G. Johansen, *ibid.*, **26**, 399 (1948).

(24) S. P. L. Sørensen and M. Høyrup, *ibid.*, **12**, 12 (1917-1919).

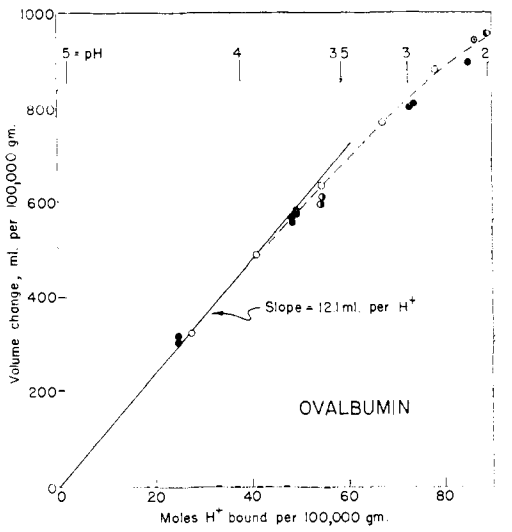

Fig. 1.—Volume changes on the addition of hydrochloric acid to ovalbumin at 30°: ●, measurements of Kauzmann[21] in 0.15 *M* NaCl; ◑, measurements of Kauzmann[21] in absence of salt; ○, present measurements in 0.15 *M* NaCl. The *p*H scale refers to measurements in 0.15 *M* NaCl.

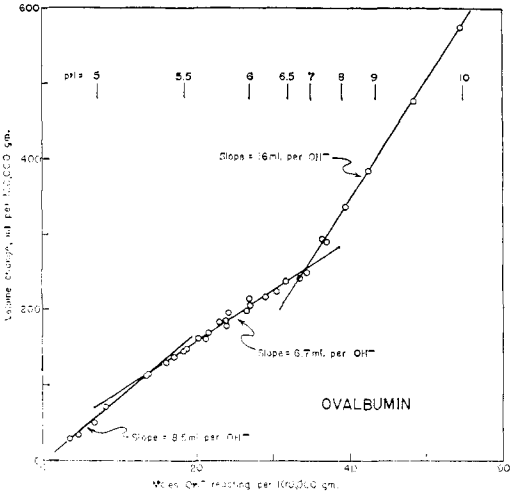

Fig. 2.—Volume changes on the addition of sodium hydroxide to ovalbumin in 0.15*M* NaCl at 30°.

Protein concentrations used were generally 5 to 10%. They were usually determined by weight but were sometimes checked by optical rotation measurements. The principal interest of this paper is concerned with the volume change per proton or hydroxyl group reacting with the protein, and the protein concentration need not be known in order to determine this quantity, which follows directly from the observed volume change and the amount of acid or base reacting.

Results

The volume changes observed after adding alkali or acid to ovalbumin, ribonuclease and lysozyme, initially at *p*H 4 to 6, are shown in Figs. 1–6, and the volume changes produced by adding alkali to bovine serum albumin are shown in Fig. 7. Optical rotation studies and other evidence show that in the *p*H range covered in these figures the volume changes do not include contributions from protein denaturation or other significant conformation changes.

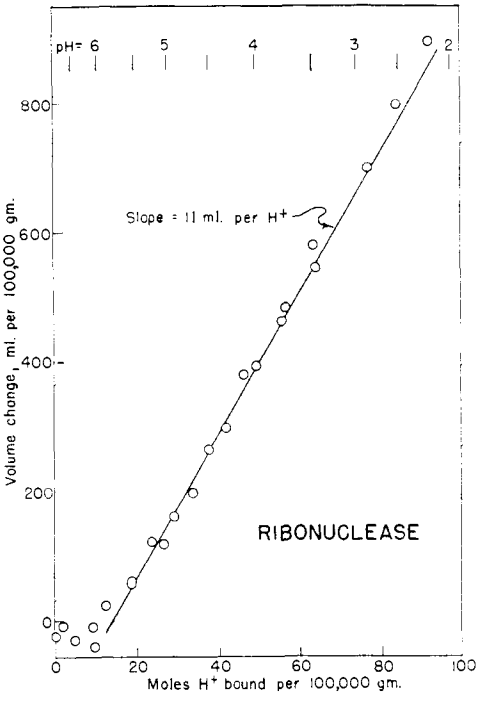

Fig. 3.—Volume changes on the addition of hydrochloric acid to ribonuclease in 0.15 *M* NaCl at 30°.

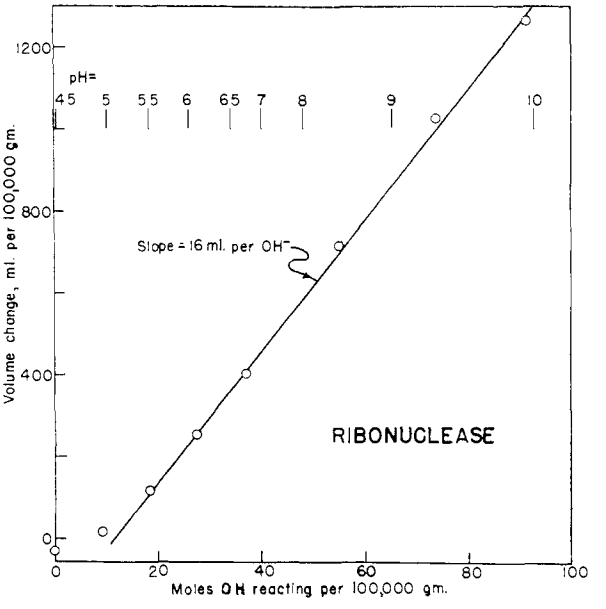

Fig. 4.—Volume changes on the addition of sodium hydroxide to ribonuclease in 0.15 *M* NaCl at 30°.

In addition to the proteins represented in Figs. 1–7, several other proteins were studied.

Hemoglobin.—Addition of alkali to hemoglobin initially at *p*H 7.5 gave a volume change of 18.6 ml. per mole of hydroxyl reacting in the *p*H range 7.5 to 11. At *p*H 11 there were small and slow changes in volume with time. Addition of acid to hemoglobin initially at *p*H 7.5 gave complex changes in volume with time when the final *p*H was below 5: there was an initial instantaneous increase in volume (caused by the immediate reaction of the carboxyl groups with the hydrogen ions) followed

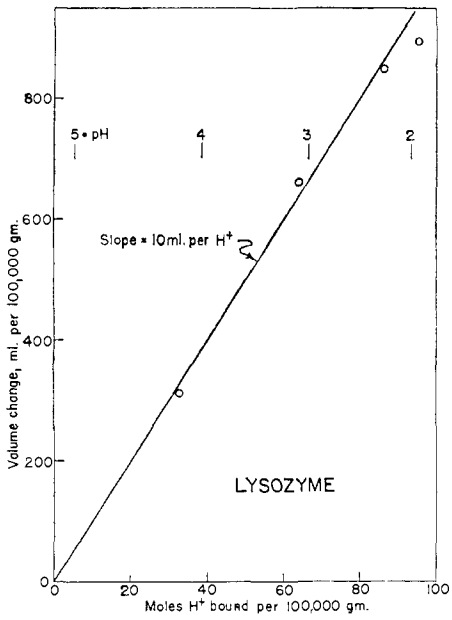

Fig. 5.—Volume changes on the addition of hydrochloric acid to lysozyme in 0.15 *M* NaCl at 30°.

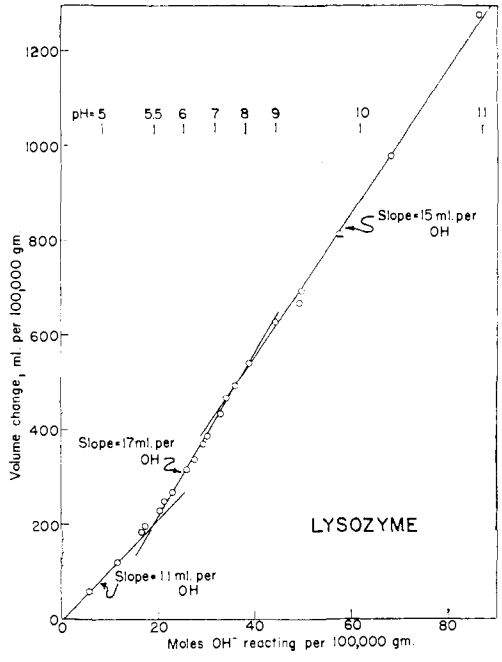

Fig. 6.—Volume changes on the addition of sodium hydroxide to lysozyme in 0.15 *M* NaCl at 30°.

by a rapid decrease in volume and followed in turn by a slow increase in volume. These changes are undoubtedly associated with the complex acid denaturation reactions recently described by Beychock and Steinhardt,[25] and the dilatometer would appear to be an excellent means of studying them.

Ovalbumin.—If the *p*H is brought below about 2, ovalbumin denatures at a rate that increases rapidly as the hydrogen ion concentration is increased. The solution becomes milky and eventually sets to a gel. After the initial instantaneous

(25) S. Beychock and J. Steinhardt, *J. Am. Chem. Soc.*, **82**, 2756 (1960).

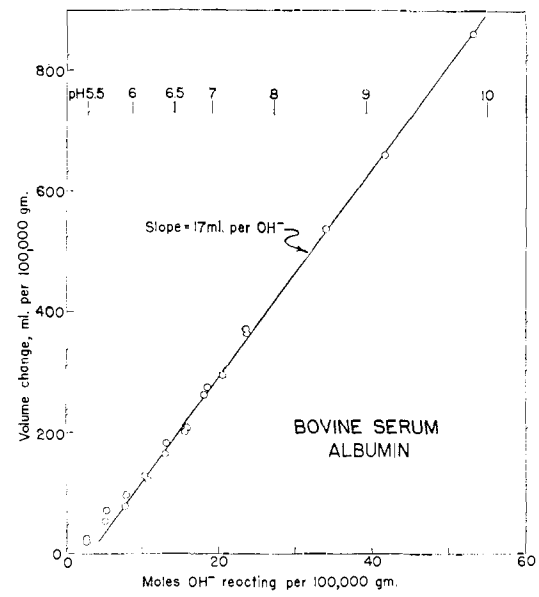

Fig. 7.—Volume changes on the addition of sodium hydroxide to bovine serum albumin in 0.15 *M* NaCl at 30°.

increase associated with the reaction of protons with the carboxylate ions, the volume under these circumstances increases with time. Denaturation also occurs gradually when the *p*H is greater than about 12, but here the volume decreases with time. These changes will be discussed further in a later paper.

Bovine Serum Albumin.—When bovine serum albumin was brought to a *p*H greater than 10.5, a time-dependent decrease in volume was observed. The volume changes that take place when serum albumin combines with protons below *p*H 4 have been described elsewhere.[21] They reflect the unfolding of the molecule that is known to take place under these conditions.

Chymotrypsin.—Addition of alkali to chymotrypsin, originally at *p*H 3.5, gave large, time-dependent volume changes whose rate increased as the *p*H increased (*e.g.*, at *p*H 6.7 there was a contraction of 1300 ml. per 10^5 g. of protein over a period of 24 hr.) By extrapolating the time-dependent volume change back to the time of mixing, it was found that in the *p*H range 3.5 to 5 there was an initial expansion of 8 ml. per mole of hydroxyl ions reacting, and in the *p*H range 6 to 8 there was an initial expansion of 17 ml. per mole of hydroxyl ions reacting. These volume changes are similar to the values found with other proteins in the same ranges of *p*H. The time-dependent volume change undoubtedly is caused by self-digestion of the enzyme (or by proteolysis brought about by traces of trypsin usually present in chymotrypsin preparations), since the enzyme concentration was quite high—about 5% (*cf.* Linderstrøm-Lang and Jacobsen[26]).

Pepsin.—Addition of alkali to pepsin initially at *p*H 4.4 gave an abnormally small increase in volume immediately after the *p*H was brought into the range 5 to 7. The initial increase was followed

(26) K. Linderstrøm-Lang and C. F. Jacobsen, *Compt. rend. trav. lab. Carlsberg, Ser. chim.*, **24**, 1 (1941).

by a decrease with time. In this pH range pepsin is known to denature at a rate that is very sensitive to the pH. Between pH 7 and 10 the volume change per mole of hydroxyl ion reacting is about 20 ml. At these pH's the protein is immediately denatured.

Discussion

When acid is added to a protein initially at pH 5 to 6 the principal reaction is undoubtedly

$$R\text{-}COO^- + H^+ \longrightarrow R\text{-}COOH \qquad (1)$$

Studies on simple molecules[15,16,26,27] show that for this reaction we should expect a volume increase of between about 10 and 14 ml., depending on the nature of the group, R, attached to the carboxyl group. If positively charged groups exist close to the carboxyl group (as in glycine and other amino acids), the volume increase is only 6 or 7 ml. If another negatively charged ion is next to the carboxyl group (as in doubly charged dicarboxylic acid ions such as oxalate and phthalate), the volume change may be 15 or 20 ml. or even more.

According to our results (Figs. 1, 3 and 5), the volume change associated with reaction 1 in proteins is generally about +11 ml., which agrees with the observations of Weber and Nachmansohn.[14,15] This value is well within the range expected for the carboxyl group and indicates that there is nothing unusual about the state of this group in these proteins. There are, however, slight differences in the values of this volume change for ovalbumin at different pH values, the volume change being appreciably smaller at low pH than it is between pH 4 and 5. (Between pH 2 and 3 the volume change per proton is only about 7 ml.) This dependence of the volume change on the pH does not seem to be present in ribonuclease below pH 5. The data are not sufficiently accurate to show whether the effect exists in lysozyme. Between pH 4 and 5 the slope for ovalbumin is distinctly greater than the slopes for the other proteins (12 ml. per proton for ovalbumin as compared with 11 ml. for ribonuclease and less than 11 ml. for lysozyme). These differferences are believed to be real and must reflect some subtle differences between the carboxyl groups in the different proteins, and, for ovalbumin, in the pH regions above and below pH 4. The low values of the volume change may result from the presence of positively charged histidine, lysine or arginine residues close to the carboxyls, and the high values may indicate that some of the carboxyl groups are clustered together in one region of the molecule's surface. (This explanation would be in accord with the observation that the groups with the smaller volume change act as though they have lower pK's than the groups with the larger volume change, since a positive charge located next to a carboxyl group should make the carboxyl a stronger acid.)

The volume changes that occur on adding alkali to proteins initially at pH 4–5 are more interesting. The reactions that may take place are

$$R\text{-}COOH + OH^- \longrightarrow R\text{-}COO^- + H_2O \qquad (2)$$

$$R\text{-Imidazole}\cdot H^+ + OH^- \longrightarrow R\text{-Imidazole} + H_2O \qquad (3)$$

$$R\text{-}NH_3^+ + OH^- \longrightarrow R\text{-}NH_2 + H_2O \qquad (4)$$

Studies on small molecules[15,16,26,27] indicate that the volume changes to be expected here are +7 to +11 ml. per mole of OH^- for reaction 2 and +23 to +25 ml. per mole of OH^- for reactions 3 and 4. Although there is some variation depending on the nature of the groups R, this variation is less for amines than it is for the carboxyl group. Even the introduction of a negative charge next to the amino group (as in an amino acid) reduced the volume change in reaction 4 only to +21 or +22 ml. per mole of OH^-. It is therefore quite surprising that in proteins reactions 3 and 4 appear to involve volume changes that are only about two thirds of the expected values (17 ml. per OH^- for bovine serum albumin between pH 6 and 10, 16 ml. per OH^- for ribonuclease in the same pH range, 16 ml. per OH^- for ovalbumin between pH 7 and 10, 15 to 17 ml. per OH^- for lysozyme between pH 5.5 and 11, 17 ml. per OH^- for chymotrypsin between pH 6 and 8, 18.6 ml. per OH^- for hemoglobin between pH 7.5 and 11 and about 20 ml. per OH^- for denatured pepsin between pH 7 and 10). Evidently the amino and imidazole groups in proteins are in a somewhat different environment from that of the same groups in small molecules.

In ribonuclease, lysozyme and bovine serum albumin below about pH 5.5, the volume changes per OH^- are considerably smaller than they are above pH 5.5 and are consistent with the values expected for reaction 2. Therefore, in these three proteins there is no indication that carboxyl groups are titrated in appreciable quantities above about pH 5.5; if they do titrate above this pH, their volume changes must be abnormally large.

For lysozyme between pH 5.5 and about 7.5 the volume change is 17 ml. per OH^-, which is slightly greater than the value of 15 ml. observed in more alkaline solutions. The measurements on bovine serum albumin indicate that the volume change per OH^- is constant between pH 6 and 10. These slight differences between the volume changes at alkaline pH's are believed to be real and again indicate that the ionizable groups of these proteins have slightly different environments.

The results for ovalbumin are especially interesting. Below pH 5.3 the volume change is about 8.5 ml. per OH^- reacting, which is roughly the expected value for reaction 2. Between pH 5.3 and 6.8, however, the volume change per OH^- decreases to only 6.7 ml. About 20 hydroxyl ions per 10^5 g. of protein are involved in this unusually small volume change, which is not observed in the same pH range for any of the other proteins studied.

A part of this anomalous behavior of ovalbumin may be explained by the presence of phosphate groups in this protein. Ovalbumin is known to be a mixture of three components containing, respectively, 0, 1 and 2 phosphate groups per 45,000 grams of protein—the average number per molecule in a typical preparation being 1.7 to 1.8 (corresponding to 3.8 to 4.0 groups per 10^5 g.).[28,29] Judging from the dissociation constants reported for glycerol-1-phosphoric acid and glycerol-2-

(27) S. D. Hamann and S. C. Lim, *Australian J. Chem.*, **7**, 329 (1954).

(28) L. G. Longsworth, R. K. Cannan and D. A. MacInnes, *J. Am. Chem. Soc.*, **62**, 2580 (1940).

(29) G. E. Perlmann, *J. Gen. Physiol.*, **35**, 711 (1952).

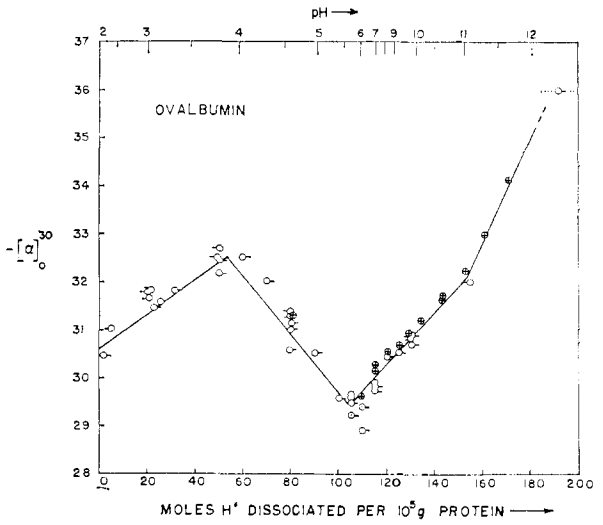

Fig. 8.—Dependence of the specific rotation of ovalbumin on the number of protons dissociated and on the *p*H. All measurements in 0.15 *M* NaCl at 30°: ⊕, run 1; ⊙, run 2. All specific rotations in run 1 have been made less levo by 1.60° in order to bring them into conformity with the results of run 2. Protein concentrations: run 1, 6.67 g./100 ml. run 2, points with tabs on left, 6.62 g./100 ml.; points with tabs on right, 3.31 g./100 ml.; point with tab below 1.655 g./100 ml. The point at the extreme upper right was obtained with a solution containing 3.31 g. of ovalbumin per 100 ml., initially at *p*H 4.60, to which 200 moles of NaOH per 10^5 g. protein had been added. The calculation of the number of protons dissociated is only approximate for this solution.

phosphoric acid,[30,31] the phosphate ester group has a pK_a of about 6.5. Therefore, the reaction

$$R\text{-}O\text{-}PO_2^{-}\text{-}OH + OH^- \longrightarrow R\text{-}O\text{-}PO_3^{=} + H_2O \quad (5)$$

undoubtedly takes place at least to some extent in the *p*H range under consideration. The data of Smith[32] on the volume change of the reaction

$$H_2PO_4^- \longrightarrow HPO_4^{=} + H^+$$

indicate that reaction 5 has a volume change of about +5 ml. per OH^- reacting. Let us assume that, of the 20 groups reacting with the hydroxyl ion between *p*H 5.3 and 6.8, four groups are phosphates with a volume change of +5 ml. per OH^-. The total volume change between *p*H 5.3 and 6.8 is 134 ml. Of this, 20 ml. may be ascribed to the four phosphate groups. The remaining 114 ml. can be ascribed to the 16 groups other than phosphate that titrate in this range—a volume change of 7.1 ml. per group. This is still far from what one would expect if imidazole groups were titrating in this range, and it is somewhat small even for carboxyl groups.

In order to gain further information on the identity of the groups titrating in this range, it is necessary to consider the stoichiometry of the ovalbumin molecule in relation to its titration curve. Our titration data on ovalbumin in 0.15 *M* NaCl (which agree closely with the data of Cannan, Kibrick and Palmer[33] in KCl solutions) indicate that between *p*H 2 and *p*H 5.3 about 98 groups react with protons per 10^5 g. of ovalbumin. The amino acid analyses tabulated by Tristram[34] and Warner[35] show that there are 109 ± 2 carboxyl groups (uncombined with ammonia), 15.5 histidine residues, 44.4 lysine residues and 33 arginine residues per 10^5 g. of protein. Thus, no more than 109 minus 98 or 11 carboxyl groups remain to be titrated in going to *p*H values above 5.3; if some carboxyl groups are already dissociated at *p*H 2, then correspondingly fewer carboxyl groups remain to be titrated above *p*H 5.3. Since a total of 20 groups are titrated between *p*H 5.3 and 6.8, no more than four of which can be phosphate groups, at least five groups other than phosphate and carboxyl must be titrated in this range. If these groups are imidazolium ions, they must react with hydroxyl ions to give much smaller volume changes than those observed for imidazolium ions in small molecules or in other proteins. If the groups are not imidazolium ions, then the pK's of the imidazoles of ovalbumin must be considerably greater than the values generally assumed for this residue (6.4 to 7.0 according to Edsall and Wyman[7,p.534]). That is, the 15.5 imidazolium ions of ovalbumin must only begin to dissociate appreciably at a *p*H above 6.8. It is clear that, regardless of how these results are interpreted, the imidazole residues of ovalbumin have properties quite different from those in other proteins. Either their pK's are well above 6.8, or some of them have volume changes much smaller than those observed in small molecules and in other proteins.

It is possible that small conformation changes are responsible for the peculiar volume changes in ovalbumin. In order to investigate this possibility, measurements were made of the dependence of the optical rotation on *p*H.

Great care was taken that the protein concentrations were accurately known in all measurements. A large amount of stock solution of ovalbumin was prepared for each of the two runs that were made. A fresh sample of this solution was mixed with HCl or NaOH for each rotation measurement. In the more acid solutions cloudiness gradually developed, so the readings could be made only immediately after mixing. In the most alkaline solutions the optical rotation changed with time, probably because of denaturation; here the optical rotation readings were extrapolated back to the time of mixing. Thus all of the points measured refer to rotations before any time-dependent denaturation has taken place.

Several protein concentrations were employed. There is no clear indication that the protein concentration had any effect on the specific rotation, but the more dilute solutions tended to have

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(31) J. H. Ashby, H. B. Clarke and S. P. Datta, *ibid.*, **59**, 203 (1955).

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(34) G. R. Tristram, "The Proteins" (H. Neurath and K. Bailey, eds.), Vol. I, Academic Press, Inc., New York, N. Y., 1953, p. 219.

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slightly smaller specific rotations, especially at the lower *p*H values.

The two stock solutions were prepared on separate occasions by dialysis of the same batch of ovalbumin (stored in the refrigerator as a paste in half-saturated ammonium sulfate). The results from one run were parallel to those from the other run, but the specific rotations differed by 1.60°. This difference may have been caused by the incomplete removal of the last traces of sulfate ions, since the *p*H values of the two stock solutions differed by 0.18 unit, and the *p*H values (4.62 and 4.80 in 0.15 *M* NaCl) were appreciably below the value for the isoionic protein reported by Cannan, Kibrick and Palmer[33] (4.95 and 4.97 in 0.133 *M* and 0.267 *M* KCl, respectively).

The results are plotted in Fig. 8 as specific rotation *vs.* number of protons dissociated. The observations appear to fall on a series of straight lines with markedly different slopes. The largest changes in slope occur at *p*H 4.0 and 5.8, but there is also an indication of a small change in slope at *p*H 11. The change in slope at *p*H 4.0 may be related to the change in slope that occurs in Fig. 1 at this *p*H. The change in slope at *p*H 5.8 does not, however, correspond to any break in the volume curve (Fig. 2). It takes place at almost exactly the point corresponding to the dissociation of the last of the 109 carboxyl groups of ovalbumin. If the changes in rotation between *p*H 5.3 and 6.8 reflect continuous changes in conformation with *p*H, then these same changes in conformation continue to take place above *p*H 6.8 and below 5.3, where the volume changes have more nearly their normal values. Therefore, if the abnormal volume changes observed with ovalbumin between *p*H 5.3 and 6.8 are caused by alterations in the conformation, these alterations are not reflected in the optical rotation.

According to Charlwood and Ens,[36] ovalbumin undergoes a small decrease in sedimentation constant below *p*H 4. The character of this change is, however, not the same as that observed with the optical rotation, since the sedimentation constant does not vary with *p*H except in the immediate vicinity of *p*H 4, whereas the optical rotation varies continuously with *p*H from *p*H 2 to about 12.

NOTE ADDED IN PROOF.—Dr. I. Klotz has made the pertinent suggestion that our volume changes may be influenced by changes in anion binding, especially in acid solutions. As Fig. 1 shows, however, the volume change per proton bound by ovalbumin is practically the same in 0.15 *M* NaCl as in the absence of salt. Furthermore, we have repeated some of the measurements shown in Fig. 2 without adding NaCl to any of the solutions. The observed points in these experiments fall on the same lines as those shown in Fig. 2. (Of course, the pH scale is not the same under these conditions.) Therefore it seems very unlikely that either anion or cation binding is a factor in these experiments. Kasarda[18] also has studied the effects of added electrolytes on the volume changes accompanying the titration of wool. He has found that 0.2 *M* NaCl does not have appreciable effects on these volume changes.

Dr. Klotz also has asked whether kerosene hydrocarbons might not be bound by our proteins. If such binding took place, and if it produced a volume change, time-dependent volume changes would have been observed both before and after mixing the solutions in the dilatometers because of the finite rate of diffusion of the hydrocarbons into the protein solutions. Furthermore our volume changes would have been much less reproducible than we have found them to be, since they certainly would have depended on the manner in which the acid and base solutions were mixed with the protein during a run. We have not seen any such effects in the experiments reported here for proteins, and we have, in fact, looked for them.

Acknowledgment.—This work was supported by a grant from the National Science Foundation.

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Volume Changes in Protein Reactions. II. Comparison of Ionization Reactions in Proteins and Small Molecules

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RECEIVED NOVEMBER 13, 1961

The volume changes that accompany the reactions $R\text{-}COO^- + H^+ \rightarrow R\text{-}COOH$ (1) and $R_1R_2R_3NH^+ + OH^- \rightarrow R_1R_2R_3N + H_2O$ (2) have been measured dilatometrically for a number of acids and bases. As has been pointed out by Hammann and Lim, the variations in ΔV for reaction (1) when the group R is changed are considerably larger than the variations in ΔV for reaction 2 when R_1, R_2, and R_3 are changed. If the group R in reaction 1 bears a negative charge which is close to the carboxylate ion that binds the proton (as in oxalate$^=$, malonate$^=$ and maleate$^=$) the volume change of reaction 1 is considerably increased. If the group R contains a positive charge (as in glycine) the volume change of reaction 1 is decreased. Analogous effects of charges in the groups R_1, R_2 and R_3 are observed for reaction 2. These effects are qualitatively in agreement with the Drude–Nernst theory of ionic electrostriction, but the theory fails to give the observed dependence of volume change on ionic size. The relationships between the value of ΔV and the values of ΔF, ΔH, ΔS and ΔC_p for reactions 1 and 2 are discussed. They appear to be complex and suggest that ΔV provides a useful means of gaining insight into the structures of the hydration layers that surround organic anions and cations. The significance of the abnormally small volume changes observed for reaction 2 in proteins, as reported in Part I, is not clear. In none of the amines studied were values of ΔV observed that are as small as those found in proteins.

Part I[1] describes the volume changes that accompany the reactions of some typical native proteins with hydrogen and hydroxyl ions. It was found that in these proteins the reaction

$$-COO^- + H^+ \longrightarrow -COOH \qquad (1)$$

is accompanied by a volume change of about 11 ml., which (as has been noted by Weber and Nachmansohn[2,3]) is similar to the values observed in

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