D-Allothreonine Ethyl Ester Hydrochloride. DL-Allothreonin was resolved into the D and L enantiomers according to the procedure of Inui and Kaneko.[15] To an ice-cold suspension of 350 mg of D-allothreonine in 12 ml of absolute ethanol was added 0.6 ml of thionyl chloride dropwise. The solution was refluxed for 4 hr and evaporated to dryness. The last traces of the acid were removed by azeotroping with ethanol–toluene (1:1) several times. The colorless, solid residue was crystallized from ethanol–ether to give 480 mg (80%) of D-allothreonine ethyl ester hydrochloride (**14**) with mp 153–154°. A sample was recrystallized from ethanol–ether for analysis: mp 155°; $[\alpha]^{25}D$ −12.44 (*c* 1.19, H_2O).

Anal. Calcd for $C_6H_{14}NO_3Cl$: C, 39.25; H, 7.68; N, 7.63. Found: C, 39.53; H, 7.77; N, 7.90.

L-Allothreonine Ethyl Ester Hydrochloride. L-Allothreonine ethyl ester hydrochloride was prepared in the same manner as the D enantiomer: mp 151–152°; $[\alpha]^{25}D$ −12.72 (*c* 1.05, H_2O).

D-Allothreoninol Hydrogen Oxalate (**11**). To an ice-cold solution of 400 mg of **14** in 10 ml of water 1 g of sodium borohydride was added in small portions with stirring. The mixture was stirred at 0° for 2 hr and at room temperature overnight. The resulting sodium borohydride complex was decomposed by the addition of Dowex 50-X2 (H^+). Isolation in a manner similar to that described for the degradation of **9** and **6b** furnished 160 mg (70%) of a colorless oil which was subsequently converted into 175 mg (76%) of D-allothreoninol hydrogen oxalate: mp 168–170° dec. One recrystallization from ethanol–water gave an analytically pure sample: mp 175–176° dec; $[\alpha]^{25}D$ −27 (*c* 0.93, H_2O).

L-Allothreoninol Hydrogen Oxalate. L-Allothreoninol hydrogen oxalate was prepared similar to the D enantiomer: mp 175–176° dec; $[\alpha]^{27}D$ +27.26 (*c* 0.73, H_2O). The infrared spectra (KBr) of the D and L enantiomers were superimposable and had strong bands (cm^{-1}) at 3375, 1575, 1300, and 1050.

D- and L-Threoninol Hydrogen Oxalates. D- and L-threoninol hydrogen oxalates were prepared starting from D- and L-threoninol using the same procedure as mentioned above. Their physical constants are given below: (a) D-threoninol hydrogen oxalate, mp 187–188°; $[\alpha]^{25}D$ +7.15 (*c* 0.7, H_2O) [lit.[15] mp 188–189° dec; $[\alpha]^{22.5}D$ +3.2 (*c* 1.078, H_2O)]; (b) L-threoninol hydrogen oxalate, mp 188.5–189° dec; $[\alpha]^{25}D$ −7.84 (*c* 0.9, H_2O).

2(*S*)-α(*R*)-Hydroxyethyl-3(*S*),4(*R*)-dihydroxypyrrolidine (7a). A solution of 100 mg of **6b** in 10 ml of water containing 0.2 ml of 2 *N* hydrochloric acid was hydrogenated in the presence of 50 mg of 5% Pd–C for 6 hr. The catalyst was removed by filtration. The filtrate was concentrated *in vacuo* to give a brown oil. The oil afforded 53 mg (54%) of colorless crystals of **7a** with mp 182–184°. Two more recrystallizations from ethanol–ether gave an analytically pure sample: mp 184–185°; pK_a = 8.15; $[\alpha]^{27}D$ −21.5 (*c* 0.39, MeOH).

Anal. Calcd for $C_6H_{14}NO_3Cl$: C, 39.25; H, 7.68; N, 7.63. Found: C, 39.51; H, 7.67; N, 7.62.

N-Acetyl-2(*S*)-α(*R*)-acetoxyethyl-3(*S*),4(*R*)-diacetoxypyrrolidine (7b). A solution of 60 mg of **6b** in 8 ml of methanol was hydrogenated in the presence of 30 mg of 10% Pd–C for 24 hr. The catalyst was removed by filtration. The filtrate was concentrated *in vacuo* to give a colorless gum. The gum was acetylated using excess acetic anhydride in pyridine at room temperature overnight. Isolation in the usual manner gave 35 mg (35% overall yield for the two steps) of **7b** with mp 159–161°. Recrystallization from ethanol–ether afforded an analytically pure sample: mp 163–164°; $[\alpha]^{27}D$ +29.03 (*c* 1.04, $CHCl_3$).

Anal. Calcd for $C_{14}H_{21}NO_7$: C, 53.36; H, 6.72; N, 4.44. Found: C, 53.65; H, 6.94; N, 4.51.

For structure proof, **7a** was acetylated in the presence of excess acetic anhydride in pyridine to give the tetraacetyl derivative (**7b**). The infrared spectrum of **7b** was devoid of NH absorptions and had strong bands (cm^{-1}) at 1750, 1645, 1375, 1300, and 1100. On oxidation with sodium metaperiodate, **7a** required 4 mol of the reagent for complete oxidation. The mass spectrum and the nmr spectrum of **7b** were also consistent with the proposed structure.

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Volume Changes Accompanying the Titration of Some Chemically Modified Ribonuclease Preparations[1]

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Abstract: The volume changes produced by the reaction of hydroxide ions with the basic groups of proteins are abnormally low in comparison with those observed with similar functional groups in small model compounds; approximately 16 ml/mol as compared with 25 ml/mol. An explanation of this phenomenon based on dilatometric measurements with highly purified preparations of ribonuclease and two denatured preparations of ribonuclease (reduced-carboxymethylated and oxidized) is offered. The reaction of tyrosine residues was shown to account for the abnormality in the pH range where the lysine residues titrate. Unfolding ribonuclease was found to have only a small, but measurable, effect on the volume change (and therefore the solvation) of the histidine residues and no apparent effect on the lysine residues. Evidence is presented to show that the presence of approximately one phosphate ion per molecule of native ribonuclease could produce the abnormality in the region of the p*K* of histidine. Finally polyvalyl ribonuclease was used to provide a measure of the solvation at particular sites on the protein surface.

Measurements of the volume changes produced by the reactions of the functional groups of proteins can yield information regarding subtle changes in the immediate environment of these groups. Of particular interest is the reaction of amino groups with base. Rasper and Kauzmann[2] made an extensive dilatometric

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study of the ionization reactions of a variety of proteins (ovalbumin, bovine serum albumin, ribonuclease, chymotrypsin, lysozyme, hemoglobin, and pepsin). The results obtained with these proteins were compared with the volume changes produced by the same ionization reactions of small organic compounds.[3] These authors found that all of the proteins measured produced abnormally low volume changes in the pH region where their amino functions titrate. A detailed and comprehensive discussion of the factors influencing the volume changes for the ionization of acids and bases along with the data obtained from studies of numerous model compounds have been presented by Kauzmann, *et al.*[3] Some brief examples of the results of these very thorough studies are presented below.

The expected change in volume for the reaction of a protonated amine with hydroxide ion to yield the uncharged amine plus water is an increase of approximately 23–25 ml/mol. This value varies somewhat and is dependent on several factors, the most important being the presence of other neighboring charged groups. For example, a positive charge in the vicinity of a protonated amine leads to higher values from 26 to 30.5 ml/mol. A negative charge has the opposite effect, yielding values as low as 20.4 ml/mol. For those proteins studied a volume increase of only 16–18 ml/mol was obtained. Pepsin was the only exception, yielding 20 ml/mol in the pH range 7–10. Since pepsin is denatured in this pH range,[4] Rasper and Kauzmann[2] pointed out the possibility that the volume change may, in part, be associated with the unfolding of the protein. None of the model amines produced a volume change as low as the proteins, and no adequate explanation for this phenomenon could be offered.

Carboxyl groups, on the other hand, produce the expected volume change when measured in proteins. The reaction of a negatively charged carboxylate ion with a proton to give the uncharged carboxyl group is accompanied by a volume increase of about 10 ml/mol. Values close to 10 ml/mol are obtained both for small organic acids as well as for proteins.

An interpretation of volume change measurements with proteins in terms of partial molar volumes has been reported.[5] Differences in the electrostriction of solvent by the charged and uncharged species are reflected in these measurements. For this reason dilatometric studies can yield information concerning the solvation of specific charged groups in proteins.

The purpose of this work is to extend the work of Rasper and Kauzmann[2] in order to find an explanation for the abnormally low volume changes in the pH region where the basic groups in proteins titrate. An attempt to explore three of the most likely areas where a solution to this problem might be found is reported. Attention has been focused on ribonuclease, one of the proteins measured by Rasper and Kauzmann.[2] First, there have been many investigations (too numerous to review here) into the exact nature of the solvent associated with molecules of biological importance and the relation of the solvation to the structure and function of these molecules.[6-16] It would therefore be of interest to find out if the abnormally low values may be observed only with native and not with denatured proteins. The low volume changes may be the result of some unique environment of the basic groups in native proteins, such as a degree of hydration not found with model compounds. To determine the validity of this idea, experiments were carried out with two unfolded derivatives of ribonuclease (reduced-carboxymethylated and oxidized). These were found to produce reasonably normal volume changes. Second, basic groups in proteins possessing a similar pK_a (*e.g.*, tyrosine and lysine) cannot be measured independently by this method. When two groups titrate simultaneously it is not possible to determine whether one or both of the groups is abnormal without the aid of additional experiments. Through the use of denatured ribonuclease, the extent to which the reaction of tyrosine residues influences that of the lysine residues has been evaluated. Third, strongly bound ions may also interfere. Through measurements of ribonuclease, with and without phosphate ions, it is shown that phosphate effects the volume change in the histidine region of the titration curve.

In addition, it was of interest to see whether or not the volume changes were, in part, a function of the location of the amino group on the protein surface. Polyvalyl ribonuclease was prepared and used for this purpose. A polypeptidyl protein is one which has amino acids or peptides covalently attached to the available ϵ-amino and α-amino groups in the protein. By observing the volume change for the terminal amino group of the attached peptides, a measure of the solvation at some distance from the original amino functions is obtained. A preliminary report of this method has been presented.[11]

Experimental Section

Materials. Aminonaphtholsulfonic acid was obtained from Fisher and repurified according to Fiske and SubbaRow.[12] Urea was twice recrystallized from water using ethanol. The 8 *M* urea solutions, used for unfolding ribonuclease, were brought to pH 2.3 with HCl, allowed to stand for 1.5–2 hr and then brought to pH 8.6 with 5% methylamine. The solution was used immediately. This treatment minimized the concentration of isocyanate ions.[13] Iodoacetic acid was twice recrystallized out of diethyl ether using petroleum ether (30–60°) as described by White.[14]

Two preparations of ribonuclease were used. The first was crystallized ribonuclease (lot numbers R7HA and R 650) obtained from Worthington. The other was phosphate-free ribonuclease A (lot numbers RAL 6031 and RAF 6061) also obtained from Worthington.

All other chemicals used were analytical grade.

Methods. N-Carboxy-L-valine anhydride was prepared from carbobenzoxy-L-valine by reaction with PCl_5 in a manner similar to that described by Stahmann and Becker[15] for N-carboxyglycine anhydride. Carbobenzoxy-L-valine was synthesized as described by Vaughn and Eichler.[16] The anhydride was crystallized from

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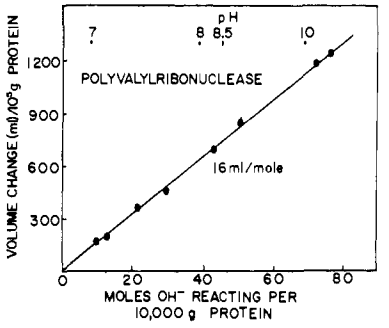

Figure 1. Volume changes produced by the addition of sodium hydroxide to poly-L-valyl ribonuclease in 0.15 *M* NaCl at 30°. The pH scale at the top is approximate.

anhydrous ethyl acetate with petroleum ether (30–60°). The melting point of the final product was 65°.

The method by which peptides are covalently attached to the available amino terminal and ε-amino groups of a protein to yield polypeptidyl proteins has been described by Stahmann and Becker.[15] Valine was polymerized onto the free amino groups of ribonuclease A to yield polyvalyl ribonuclease by treating a 1% solution of the protein in pH 7.6 phosphate buffer with the Leuchs' anhydride of valine (N-carboxyvaline anhydride). The resulting polyvalyl derivative was chromatographed on Sephadex G-25 according to Krausz and Becker[17] to remove any aggregated material.

Carboxymethylated ribonuclease was prepared by combining the procedures of Anfinsen and Haber[18] and Crestfield, *et al.*[19] Only an outline of the method is given here; details are presented in the above references. Twelve batches of this protein were prepared. In a typical preparation 200 mg of ribonuclease was dissolved in 8 *M* urea which had been adjusted to pH 8.6 with methylamine. The total volume of the solution was between 12 and 15 ml. The solution was flushed with nitrogen by bubbling the gas through it. An atmosphere of nitrogen was maintained above it.

Then 0.2 ml of β-mercaptoethanol was added and the reaction mixture allowed to stand for 5–6 hr. Following this, 604 mg of sodium iodoacetate in approximately 6 ml of Tris-HCl buffer (pH 8.6) was then added to the ribonuclease solution. The pH of the solution was checked routinely. If it was found to be below pH 8.6 a few drops of dilute NaOH solution were added to maintain that pH.

After approximately 20 min 0.8 ml of β-mercaptoethanol was added to the reaction mixture. Fifteen minutes later the mixture was adjusted to pH 4.5 with acetic acid and applied to a Sephadex G-25 column and eluted with 0.1 *N* acetic acid. The column effluent was monitored at 280 mμ. The first peak to come off the column at approximately 240 ml contained the protein. The total volume of the peak was approximately 100 ml. The effluent containing the protein was dialyzed against distilled water and lyophilized. The average yield per 200 mg of starting material was 145 mg. The lyophilized protein was sometimes difficult to dissolve. Occasionally it was necessary first to dissolve it in 4 *M* urea and then to dialyze it exhaustively against 0.15 *N* NaCl. This solution was then directly used in the dilatometric experiments.

Ribonuclease was oxidized with performic acid at −10° as described by Hirs.[20] Instead of using a test tube with a side arm to mix the protein and performic acid solutions, the contents of two separate flasks were rapidly combined. A total of 2 g in four separate batches were prepared for this work.

Proteins were analyzed for amino acid content according to the method of Spackman, Stein, and Moore[21] with a Beckman Model 120 B amino acid analyzer. Proteins in 5.7 *N* HCl (constant boiling mixture) were prepared for hydrolysis by purging with nitrogen and sealing in glass tubes under vacuum. The solutions were hydrolyzed at 110° for times of 24 and 96 hr. The longer hydrolysis time was used to ensure complete liberation of free valine, since polyvalyl proteins were unusually stable to acid hydrolysis.[22] Accuracy was checked by comparing our results with the reported amino acid composition of ribonuclease.[23] The data, expressed as amino acid residues per molecule of protein, were in excellent agreement with published results. The modified proteins possess an internal control in that the analysis for all but the added or modified amino acid should agree with that of unmodified ribonuclease.

The raw data from the analyzer are expressed in micromoles. To convert micromoles into moles of amino acid per molecule of protein, the known value of aspartic acid (15 mol) or leucine (2 mol) per mole of ribonuclease was made proportional to the actual number of micromoles found. The molar quantities of the other amino acids were obtained directly with the use of the proportionality constant. The number of sites allowed to react with anhydride was determined by the use of Sanger's[24] end-group reagent, FDNB.[25] After reaction with FDNB, for each group in the protein modified with anhydride, one NH_2-terminal dinitrophenyl derivative of valine should be formed. DNP-valine was not measured directly. The amount of this derivative was obtained by subtracting the valine (moles per mole of protein) liberated upon hydrolysis of the DNP-polyvalyl protein from that of the polyvalyl protein.

Volume change measurements were made with glass dilatometers (obtained from Jacob Glastechnik, Copenhagen, Denmark) described by Linderstrøm-Lang[26] and incorporating the modifications of Johansen.[27] The use of these dilatometers for protein studies similar to those described here is also reported by Rasper and Kauzmann.[2]

These instruments are capable of measuring volume changes ranging from 0.05 to 10.00 μl.

The presence of phosphate ions was quantitatively determined by a modification of the Fiske and SubbaRow[12] method described by Meun and Smith.[28] Instead of using the prescribed 150 g of sodium bisulfite (probably an error in printing) 1.5 g was used. Digestion of the protein samples for 2 hr in sulfuric acid and 2 drops of 30% hydrogen peroxide was found satisfactory. The method is easily capable of detecting between 1 and 10 μg of phosphorus in a given protein sample. For each determination from 5 to 20 mg of protein was weighed directly into a Kjeldahl flask. All proteins were stored over anhydrous $CaSO_4$ and assumed to contain 7.5% water.

Results

Polyvalyl Ribonuclease. Analysis of amino acid composition and amino terminal end groups showed that this preparation had eight valine residues attached to seven sites, giving an average chain length of added valine of 1.1 residues.

The results of dilatometric measurements in the pH range 7–10 are shown in Figure 1. The volume change at any point is the slope of the curve at that point. In the pH region studied the volume change is a constant 16 ml/mol of hydroxide ion reacting with the protein. The pH scale at the top of the figure is approximate. The purified kerosene used in the course of the dilatometric measurements floats as a separate layer above the protein solutions. This kerosene through which the electrode must pass appears to prevent rapid equilibration of the electrode. As much as 20 min was often required before a constant pH value was obtained. The pH scale is, however, sufficiently accurate to show that for this protein the type of group being titrated is predominately one having a p*K* characteristic of the α-amino group and is obviously that of the

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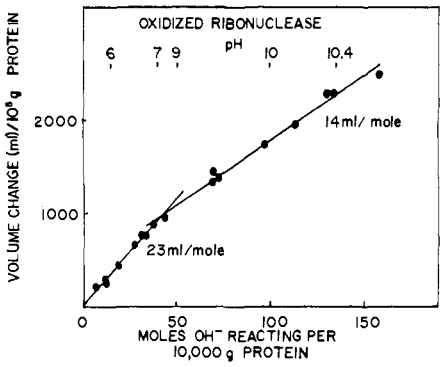

Figure 2. Volume changes produced by the addition of sodium hydroxide to oxidized ribonuclease in 0.15 *M* NaCl at 30°. The pH scale at the top is approximate.

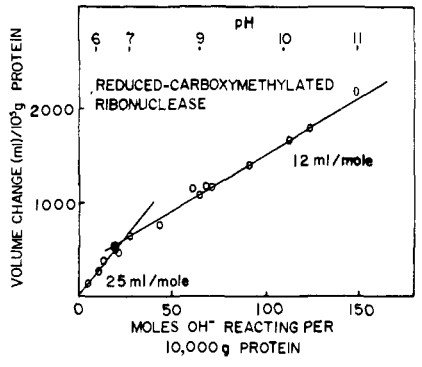

Figure 3. Volume changes produced by the addition of sodium hydroxide to reduced-carboxymethylated ribonuclease in 0.15 *M* NaCl at 30°. The pH scale at the top is approximate.

terminal valine of the attached peptides. The unmodified, native protein used in earlier work produced the same volume change of 16 ml/mol for all the functional groups in the pH range 5–11 including the histidine residues.[2]

Oxidized Ribonuclease. All of the cystine in this protein was oxidized as judged by the total absence of its hydrolysis product on the amino acid analyzer.

A plot of volume change *vs.* moles of base added to the protein solution is shown in Figure 2. The data for this denatured (or unfolded) preparation show clearly that two distinct types of volume change occur. There are two regions through which straight lines can be drawn. The slope of line in the pH range 5.5–7.0, where the histidine residues react, is 23 ml/mol. In the more alkaline region where the lysyl and tyrosyl residues react the volume change is 14 ml/mol.

Some phosphate was present in this sample. The equivalent of 9.5 g of phosphorus/14,000 g of protein was found. This is approximately 0.3 mol of phosphorus/mol of protein.

Carboxymethylated Ribonuclease. Amino acid analysis showed a total loss of cystine for each of the twelve preparations of this protein, indicating the conversion of this amino acid to the carboxymethyl derivative. There was no significant loss of any other amino acid which might have occurred as a result of carboxymethylation.

The volume changes produced by adding alkali to this derivative are shown in Figure 3. The results paralleled those of oxidized ribonuclease. The two volume changes are 25 ml/mol and 12 ml/mol at the lower and higher pH values, respectively.

The dilatometric procedure used here is readily capable of detecting differences greater than 1 ml so that the small differences between the two denatured forms of ribonuclease are real. While the numbers obtained with the two unfolded proteins are not identical they are very close and show the same trend at each pH.

No phosphate could be detected in this preparation.

Ribonuclease. A commercial preparation of phosphate-free ribonuclease A was studied dilatometrically. Two different volume changes were recorded in the basic region. From pH 5.5 to 8.0 a value of 21.5 ml/mol was produced and from pH 8.0 to 10.0 the volume change was 18 ml/mol.

The volume change in the histidine region is considerably higher than the 16 ml/mol found previously with commercial preparations available at that time. In the range of lysine and tyrosine the values are similar to but still 2 ml higher than those found earlier.

Monosodium phosphate was added to ribonuclease in increasing molar ratios of phosphate to protein. In the pH range 5.5–8.0 (histidine region), at a 1:1 ratio, the volume change was 18 ml/mol. A 2:1 ratio of phosphate to ribonuclease gave 14 ml/mol and a 3:1 ratio gave 13.5 ml/mol. To double check the phosphate content, each sample was analyzed for the ion.

Discussion

The results presented here extend the work of Rasper and Kauzmann[2] on ribonuclease and are a further demonstration of the use of dilatometry to detect differences in the immediate environment of the charged groups of proteins. The data show that the histidine residues of ribonuclease are more normal (*i.e.*, similar to model compounds) with respect to volume change when either phosphate-free or denatured preparations are used. A comparison of denatured ribonuclease with phosphate-free ribonuclease A shows that unfolding has only a small, but measurable, effect on the volume change associated with the histidine residues. The two unfolded derivatives produce 23–25 ml/mol and phosphate-free ribonuclease A produces 21.5 ml/mol. These values are considerably higher than the 16 ml/mol found with polyvalyl ribonuclease and with ribonuclease used in earlier work.[2]

The expected change in volume for the removal of a proton from the positively charged imidazole ring of histidine in ribonuclease can be estimated by using the results of dilatometric studies with model compounds. The data obtained from these studies are summarized in Table I. The presence of a positive charge near the nitrogen reacting, as with histidine methyl ester (having a protonated α-amino group), causes the

Table I. Volume Changes for the Reaction of the Protonated Nitrogen in the Imidazole Ring with Hydroxide Ion[a]

Compd reacted	pH range	ΔV (ml/mol)
Imidazole	7.5–8.5	22.4
Histidine	5.0–7.0	23.1
Histidine methyl ester	7.5–8.5	24.5
Acetylhistidine	7.0–8.0	19.8

[a] See ref 3.

volume change to be higher than for imidazole alone. A negative charge produces the opposite effect, lowering the volume change, as with acetylhistidine (having a negatively charged α-carboxyl group). Histidine having both positively and negatively charged groups is intermediate in volume change. In general, the effect of a like charge near the group being measured is to increase the volume change and an oppositely charged group lowers the volume change.[3] Depending on the model selected from Table I, the expected value varies from 19.8 to 24.5 ml/mol. The two denatured proteins and phosphate-free ribonuclease A give results within this range. It appears that the best model for histidine in reduced-carboxymethylated ribonuclease is histidine methyl ester; for histidine in the native protein, imidazole is best.

The volume changes produced when denatured ribonuclease is allowed to react with base in the pH range 9–11 can be used as evidence to show that the lysine residues are only slightly affected by the unfolding of the protein. In the pH range 9–11 both the ϵ-amino and phenolic groups react with base. The contribution of the phenolic portion of tyrosine must be estimated since the amino acid itself is too insoluble to measure. The expected volume change for the tyrosyl residues can be obtained from the studies of Weber[29] on the ionization of phenol and from values for the ionization of water.[30-32] The data for these reactions are summarized in Table II and the volume change for the

Table II. Dilatometric Data for the Ionization of Phenol, $H_2PO_4^-$, and Water

	Reaction	Method[a]	ΔV (ml/mol)
1.	Phenol $\longrightarrow$ phenolate$^-$ + H^+	A	−18.7[b]
2.	$H^+ + OH^- \longrightarrow H_2O$	A	21.3[c]
		B	23.4[d]
		A	20.7[e]
3.	Phenol + $OH^- \longrightarrow$ phenolate$^-$ + H_2O	C	2.6
4.	$H_2PO_4^- \longrightarrow HPO_4^{2-} + H^+$	B	−28.1[f]
5.	$H_2PO_4^- + OH^- \longrightarrow HPO_4^{2-} + H_2O$	C	−6.8

[a] Method A, measured directly in dilatometer; B, from apparent molar volumes of reactants and products; C, calculated as described in text. [b] Reference 29. [c] Reference 30; this most recently determined value is used in calculations discussed in the text. [d] Reference 31. [e] Reference 32. [f] J. S. Smith, Dissertation, Yale University, 1943, as quoted by H. S. Harned and B. B. Owen, "The Physical Chemistry of Electrolyte Solutions," 3rd ed, Reinhold Publishing Corp., New York, N. Y., 1958, p 406.

desired reaction (reaction 3) is calculated by adding reactions 1 and 2. The resulting volume change is 2.6 ml/mol. Since native ribonuclease A in the pH range 9–11 produces 18 ml/mol (16 ml/mol for less pure preparations) and the two unfolded protein derivatives produce only from 12 to 14 ml/mol, there is reflected in these measurements the contribution of some factor possessing an intrinsically small volume increment. The influence of the smaller volume increment increases when the protein is unfolded. This factor is probably the phenolic portion of tyrosine. Using the free amino acid lysine as a model, the volume change for the ϵ-amino group is 22 ml/mol.[3] This value was also obtained with a lysine-rich fraction of histone from calf thymus[33] and therefore seems a reasonable value to use for the following calculations. The ten lysine and three tyrosine residues of native ribonuclease react with base from about pH 9–11.[34,35] Assuming the volume change for the ϵ-amino function to be 22 ml/mol, then the average volume change for ten lysine and three tyrosine residues is 17.5 ml/mol. This is close to the 18 ml actually observed. Now, when the protein unfolds it is well known that three additional tyrosine residues are exposed.[34] The average of ten lysine residues producing 22 ml/mol and six tyrosine residues producing 2.6 ml/mol is 14.7 ml/mol, a value near what is actually observed with oxidized ribonuclease. Reduced-carboxymethylated ribonuclease yields only 12 ml/mol. These calculations strongly support the idea that the low-volume increment associated with the ionization of tyrosine enters into the measurements at the higher pH range. To be consistent with the results only minor variations in the value for lysine in the folded or unfolded protein would be allowed.

That ribonuclease may bind phosphate ions is well known. It was shown here that phosphate-free ribonuclease, in the pH range where both histidine and phosphate react, is unlike the preparation used in earlier work, the volume changes being 21.5 ml/mol and 16 ml/mol, respectively. (The difference is much smaller in the lysine and tyrosine region; 18 ml/mol compared with 16 ml/mol.) The effect of phosphate ions in ribonuclease can be estimated by using the data of Smith[36] for the ionization of $H_2PO_4^-$ as given in Table II.

The sum of reactions 2 and 4 (Table II) gives the reaction (reaction 5) taking place during the course of the dilatometric measurements in the pH range where histidine residues titrate. The volume change for phosphate is calculated to be −6.8 ml/mol.

The addition of known amounts of monosodium phosphate to ribonuclease significantly reduced the volume change in the histidine region. Adding 1, 2, and 3 mol produced 18 ml/mol, 14.5 ml/mol and 13 ml/mol, respectively. It can be seen from the values for the protein plus monosodium phosphate that to produce the 16 ml/mol actually observed, more than 1 mole, but less than 2 mol, would be required. This small amount of phosphate may have been present.

The values observed here are not a simple average of phosphate and histidine as would be the situation if these groups reacted independently. As a first approximation, if 0.0 ml/mol is taken as the volume change for phosphate, rather than −6.8 ml/mol, total volume changes can be calculated that are consistent with all the measured values. For example, the average of four histidine residues and one phosphate group is (22.4 ml/mol × 4 − 0.0 ml/mol × 1) /5 = 17.9 ml/mol. This compares well with the 18 ml/mol measured. It should be noted that because 1 mol of phosphate was added it does not *necessarily* mean that 1 mol was bound by ribonuclease.

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The two denatured preparations of ribonuclease give similar, but not identical, volume changes in the histidine and in the lysine region. The differences in either region are small but still measurably beyond experimental error, and may reflect minor dissimilarities between the two proteins. The proteins are not the same chemically, one having extra carboxyl groups and the other sulfonic acid groups. (Methionine and tryptophane may also be affected by oxidation.) The starting material for both denatured derivatives contained some phosphate. The ribonuclease obtained commercially contained from 0.02 to 0.07% phosphorus (except, of course for the phosphate-free protein which was used to make polyvalyl ribonuclease). As a result of preparation, the carboxymethylated derivative contains no phosphate ions. While it is difficult to remove the last traces of this ion from native ribonuclease by dialysis against distilled water, the dialysis step in the preparation of the carboxymethylated protein does remove phosphate. The oxidized protein, on the other hand, was never dialyzed because most of the undesirable reaction products are volatile and are removed during lyophilization. This does not remove phosphate so that the original phosphate remains with the oxidized protein. Assuming that the ion is not specifically bound when the protein is denatured, this small amount of phosphate may explain the 2-ml lower volume change in the histidine region with this protein as compared to the carboxymethylated protein.

Rasper and Kauzmann[2] considered the effect of the presence of phosphate groups in another protein, ovalbumin. These authors concluded that for ovalbumin, in the region where the imidazole groups titrate (pH 5.3–6.8), the very low volume change of 6.7 ml/mol was not seriously affected by the phosphate present. The ratio of histidine to phosphate was too large to allow the few phosphate groups to enter significantly into the average value for both groups.

The polyvalyl ribonuclease preparation used for this work had an average of 1.1 valine residues attached to seven amino groups in the protein. Only the free ϵ-amino group of the lysine residues and the terminal amino group are sites for the addition of valine.[37] In ribonuclease there are eleven such sites, seven of which did react. None of the original material was available for analysis, but polyvalyl ribonuclease probably contained phosphate ions since it was prepared in a phosphate buffer and only dialyzed against distilled water. Polyvalyl ribonuclease generally retains enzymatic activity.[38] It has been shown that a lysine residue at the active site, lysine 41, in polyalanyl ribonuclease appears to be protected by phosphate ions from reaction with anhydride since this residue remains unmodified.[39] The same is most likely true for polyvalyl ribonuclease, so that a phosphate ion may be bound to lysine 41 in both the native and the modified proteins.

The volume increase of 16 ml/mol for the removal of protons from the terminal α-amino groups of the attached valine peptides is identical with that observed with older preparations of unmodified ribonuclease. Although the new group being titrated must be some distance from the ϵ-amino group to which the peptide is attached, this new position in the protein is not reflected in the volume change.

One might expect to see at least two different volume changes in the pH range measured because the ionizations of some groups, such as the α-amino groups and of the tyrosine residues, should not overlap significantly. Although attention was focused mainly on the α-amino groups in order to demonstrate how attached peptides could serve as probes of the surface environment of the protein, the data indicate a uniform volume change of 16 ml/mol for all ionizable groups between pH 7 and 10. The presence of the proper number of phosphate ions could account for the value of 16 ml/mol for titration in the region of the α-amino groups as was the case for unmodified ribonuclease. In the total pH range where measurements of polyvalyl ribonuclease were made, histidine, phosphate, terminal glycine residues, unmodified lysine residues, and tyrosine all react with base. Measurements made over smaller pH intervals between pH 9 and 11 should yield more information about the tyrosine and the remaining unmodified lysine residues. Work along these lines using myoglobin and polypeptidyl derivatives of myoglobin is in progress.

The conclusions drawn from these dilatometric studies apply only to the enzyme ribonuclease. Some of the factors which enter into the total volume change when ribonuclease is measured have been evaluated. These factors may also contribute to the volume change for other proteins. It is very likely that each protein requires a particular set of circumstances to bring about its characteristic volume change in a given pH region.

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