TABLE I

RATE CONSTANT FOR DISSOCIATION OF CdX^- AND RELATED DATA AT IONIC STRENGTH OF 3[a] AT 25°

pH	4.62	5.10	5.35	5.67
$C_{Cd^{++}}$, mmole l.$^{-1}$	1	1	1	0.5
C_{HAc}, mole l.$^{-1}$	0.1	1.0	0.01	0.1
C_{NaAc}, mole l.$^{-1}$	0.1	0.3	0.1	1
$C_{HX^{--}}$, mmole l.$^{-1}$	1.44[b] to 15.7	0.6[b] to 12.0	0.5[c] to 12.4	7
k. 10^6 mole^{-1} l. sec.$^{-1}$ (uncor.)	7.2 ± 0.6	4.1 ± 0.3	8.0 ± 0.5	..
k, 10^6 mole^{-1} l. sec.$^{-1}$	1.4 ± 0.1	1.3 ± 0.2	1.6 ± 0.1	1.7 ± 0.1

[a] Ionic strength adjusted with sodium perchlorate. [b] 5 concentrations. [c] 6 concentrations.

except for only one complex. When one cannot assume that $C_{Cd^{++}} << C_{CdX^-}$ one has

$$k = 1.31 K_{CdX^-} \cdot K_{HX} C_{HX^{--}} a\, t^{-1} \times \frac{h_1 - \frac{(h_1 + h_2)}{K_{CdX^-} K_{HX^{--}} C_{HX^{--}} C_{H^+}^{-1} + 1}}{h_2} \quad (3)$$

Determination of K_{CdX^-} from Oscillographic Current–Potential Curves at High Rates of Potential Change.—In view of the work of Koryta and Kössler[1] discussed in the Introduction, it was thought that the equilibrium constant K_{CdX^-} could be obtained from the ratio of the peak heights h_2/h_1 for current–potential curves obtained at high rates of potential change (oscillographic polarography). This was indeed the case. At sufficiently high rates of potential change the kinetic contribution becomes quite negligible, and the ratio h_2/h_1 is determined by K_{CdX^-}. Results are summarized in Table II. Values of pK_{CdX} of 9.8 (μ = 0.1), 9.9 (μ = 0.3) and 10.3 (μ = 3) which were deduced are in good agreement with the data of Schwarzenbach, *et al.*[3]

TABLE II

DETERMINATION OF K_{CdX^-} FROM OSCILLOGRAPHIC DATA AT 25°

V, v. sec.$^{-1}$	h_2/h_1 (μ = 0.1)	h_2/h_1 (μ = 0.3)	h_2/h_1 (μ = 3)
0.26	1.4	1.4	0.9
1.0	2.6	2.6	1.7
2.5	5.5	..	..
6.1	9.1	2.5	3.9
10.0	9.6	3.2	4.2
25.0	15.2	5.0	4.9
40.0	17.6	6.3	5.1
57.0	19.0	7.6	5.1
100.0	19.7	10.0	5.1

Conditions for 3 μ's: pH 4.13, 4.62 and 5.62, respectively; $C_{Cd^{++}}$ = 1 mmole l.$^{-1}$; C_{HAc} = 0.3 mole l.$^{-1}$; C_{NaAc} = 0.1, 0.3, 3 mole l.$^{-1}$; C_{H_3X} = 5 mmole l.$^{-1}$.

Experimental

Conventional polarographic techniques were applied. No maximum suppressor was needed. Drop times were measured in the plateau range of the second wave. Several capillaries were utilized with drop times varying between 2.9 and 5 sec. Oscillographic recordings were made with an improved version of the instrument described by Favero and Vianello.[7]

Acknowledgment.—This work was begun in Baton Rouge and completed in Padua. I am indebted to Professor Delahay for discussion and to the Center of Polarography of the Italian National Research Council for hospitality and financial support. Many thanks are due to Dr. Vianello for his collaboration in the oscillographic studies. A fellowship from the Italian National Research Council for study in the United States is gratefully acknowledged.

(7) P. Favero and E. Vianello, *Ricerca sci.*, **25**, 1415 (1955); see also paper in course of publication.

ADDED IN PROOF.—Reference is also made to the very recent paper by J. Koryta, *Z. physik. Chem.* (*Leipzig*), Special issue, 157 (1958).

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, STATE UNIVERSITY OF IOWA]

The Configuration of Ribonuclease at Low pH in 2-Chloroethanol and in 2-Chloroethanol–Water Mixtures[1]

BY ROBERT E. WEBER[2] AND CHARLES TANFORD

RECEIVED DECEMBER 3, 1958

Imahori, Klemperer and Doty have shown that ribonuclease dissolved in 2-chloroethanol acquires a configuration characterized by a high content of helical polypeptide structure. This paper shows that the transition from native aqueous ribonuclease to the highly helical structure occurs in two stages, through an intermediate with about the same helix content as aqueous ribonuclease but with a high intrinsic viscosity and flexibility, which suggests that its non-helical portions are more or less randomly coiled. The highly helical structure in chloroethanol is presumably the result of intramolecular hydrogen bonding; the compact configuration in aqueous solution is ascribed primarily to hydrophobic forces.

It has been shown recently by Doty, Imahori and Klemperer[3] that 2-chloroethanol is a good solvent for many globular proteins. Both optical rotation and infrared absorption have indicated that protein molecules in this solvent have a strong tendency, much stronger than in water solution, to form intramolecular hydrogen bonds so that the major portion of their polypeptide chains may possess a helical configuration. The purpose of the present paper is to measure the intrinsic viscosity of one globular protein, ribonuclease, in the solvent 2-chloroethanol, and to investigate the transition

(1) Abstracted from the Ph.D. Thesis of Robert E. Weber, State University of Iowa, 1958. Presented at the 134th meeting of the American Chemical Society, Chicago, Ill., September 1958.

(2) Union Carbide (Bakelite Division) Fellow, 1957–1958.

(3) P. Doty, K. Imahori and E. Klemperer, THIS JOURNAL, **81**, in press (1959). We are grateful to these authors for making some of their data available to us in advance of publication.

from the native configuration found in aqueous solution to that found in 2-chloroethanol. The results add little new information concerning the configuration of ribonuclease in 2-chloroethanol, but they provide new evidence concerning the forces which might be responsible for maintenance of the native configuration in aqueous solution.

Experimental

The ribonuclease used was lot 381-059, manufactured by Armour and Co. It was used without further purification. The material was found to be a salt of ribonuclease bearing a net proton charge of about +8. All concentrations, and therefore also all measurements which depend on concentration (*i.e.*, $[\eta]$ and $[\alpha]$), are reported in terms of the dry weight of this salt, and these quantities will therefore differ somewhat from the values they would have if referred to dry isoionic protein. The difference, however, appears to be no greater than the experimental error of our measurements, which was about 2 to 3%. Intrinsic viscosities measured in aqueous solution agreed, within experimental error, with the values (referred to salt-free isoionic protein) which Buzzell[4] determined in this Laboratory.

2-Chloroethanol (ethylene chlorohydrin) was of reagent grade and was further purified by distillation through a fractionating column. The distilled product was kept in a tightly stoppered bottle to avoid unnecessary contact with water vapor in the atmosphere, but no effort was made to keep it perfectly anhydrous. Some HCl present in the commercial reagent was not removed by distillation. RbCl and $N(CH_3)_4Cl$, which were used to adjust ionic strength because of the poor solubility of KCl in chloroethanol, were of reagent grade.

Solutions for measurement were prepared (except for the data of Fig. 4) by weighing out ribonuclease into volumetric flasks and adding chloroethanol, water and electrolyte so as to give the desired solvent composition and ionic strength. A small amount of water present in the ribonuclease preparation was taken into account in computing the solvent composition. In the viscosity studies, where solutions of varying protein concentration, but constant solvent composition and ionic strength, were required, the more dilute solutions were usually obtained by adding solvent of the correct composition to the more concentrated solutions.

The distilled chloroethanol always contained a small amount of HCl. The protein preparation was also acidic. All solutions used therefore contained positively charged ribonuclease molecules. Unless otherwise indicated the proton charge in all experiments is close to the maximum attainable.[5]

Viscosity measurements were made in Ostwald–Fenske viscometers. Flow times t for solutions and t_0 for the corresponding solvents were measured at a series of protein concentrations c, expressed in g./cc. The factor $(t - t_0)/t_0c$ was determined at each concentration and extrapolated to $c = 0$ to yield the intrinsic kinematic viscosity. This was corrected to the true intrinsic viscosity by the expression derived earlier,[6] involving knowledge of the partial specific volume. The latter was found to be close to 0.70 at all solvent compositions, so that the correction applied to the intrinsic kinematic viscosity was always in the range of 0.1 to 0.3 cc./g., depending primarily on variation in solvent density with solvent composition.

Optical rotation measurements were made in 20 cm. tubes, using a photoelectric polarimeter. The polarimeter used for the rotatory dispersion data was a Rudolph Model 80A polarimeter, equipped with a zirconium light source and a quartz monochromator. All other measurements were performed on an older instrument (of considerably lower precision) utilizing the Na-D line.

Sedimentation coefficients were determined with a Spinco Model E ultracentrifuge. The effect of concentration was found to be small. The data reported were extrapolated to zero concentration but are not appreciably different from values which were obtained at a concentration of 0.5 g./100 cc. Kel-F cells were used and were found unaffected by chloroethanol.

Measurement of *p*H was required only for the data of Fig. 5, encompassing solvent compositions richer in water than in chloroethanol. The measurements were made in the usual way, with a Beckman Model G *p*H meter standardized with an *aqueous* solution of potassium acid phthalate. Solutions of HCl (0.01 and 0.001 *N*) in water–chloroethanol mixtures over the same range of composition were subjected to *p*H measurement in the same way and found to remain unchanged in *p*H to within about 0.1 *p*H unit. It thus may be concluded that *p*H measured by our procedure has approximately the same relationship to hydrogen ion activity as *p*H measured in purely aqueous systems.

All data reported were obtained at 25°.

Results

Figure 1 shows specific rotations and intrinsic viscosities obtained when crystals of ribonuclease (crystallized from aqueous systems and containing 5 to 10% water) were dissolved in solvents varying in composition from pure water to almost pure chloroethanol. The ionic strength was varied from 0.2 to "no added salt," the latter representing an ionic strength, due to the presence of HCl, of about 0.01. Identical results were obtained when RbCl and $N(CH_3)_4Cl$ were used as indifferent electrolyte.

The figure clearly shows that the configurational change which takes place in going from pure water to pure chloroethanol occurs in two distinct steps. In the first step there is a large increase in intrinsic viscosity, but little change in optical rotation. Furthermore, the intrinsic viscosity of the product (at 75 to 90 mole % water), but not its optical rotation, depends strongly on ionic strength, decreasing as the ionic strength increases. The second step of the reaction, on the other hand, involves a large numerical decrease in the negative specific rotation, with a relatively small decrease in viscosity. The final product has both the specific rotation and the intrinsic viscosity independent of the ionic strength.

The specific rotation data of Fig. 1 may be converted to *effective residue rotations*, $[m']$, by the relation[7]

$$[m']/[\alpha] = 3M_0/100(n^2 + 2) \qquad (1)$$

where n is the refractive index of the solution and M_0 the average molecular weight per residue of the protein molecule, 110 in the case of ribonuclease. We obtain, for aqueous ribonuclease $[m'] = -62°$, for the intermediate form $[m'] = -55°$, for the helical form $[m'] = -19°$. All of these figures are subject to an error of about ±2°, principally because of instability of the polarimeter used.

These properties can be qualitatively interpreted as follows, in terms of theoretical principles which have been fully reviewed elsewhere.[7–10]

(4) J. G. Buzzell and C. Tanford, *J. Phys. Chem.*, **60**, 1204 (1956).

(5) Mixtures of chloroethanol and water are slowly hydrolyzed, with the release of HCl. Initially acidic solutions, however, undergo no further change. In particular, both solvent and solution flow times in the viscometers were found to be reproducible and unaffected by time to within the accuracy of the measurements.

(6) C. Tanford, *J. Phys. Chem.*, **59**, 798 (1955).

(7) W. Moffitt and J. T. Yang, *Proc. Natl. Acad. Sci., U. S.*, **42**, 596 (1956).

(8) The physical theory of optical rotation of polypeptide chains is summarized, with references to earlier papers by the same authors, by W. Moffitt, D. Fitts and J. G. Kirkwood, *Proc. Natl. Acad. Sci., U. S.*, **43**, 723 (1957). Formulation of the equations used in the present paper is given in ref. 7.

(9) For summaries of experimental work using optical rotation see P. Doty and R. D. Lundberg, *ibid.*, **43**, 213 (1957); J. T. Yang and P. Doty, THIS JOURNAL, **79**, 761 (1957); C. Schellman and J. A. Schellman, *Compt. rend. Lab. Carlsberg*, **30**, 463 (1958).

(10) The interpretation of viscosity data is discussed by C. Tanford, "Symposium on Protein Structure" (A. Neuberger, ed.), Methuen and Co., London, 1958.

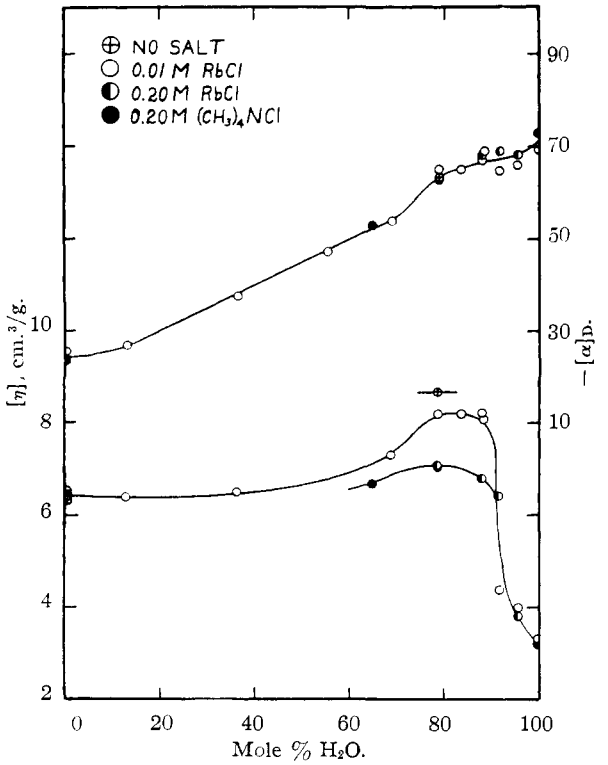

Fig. 1.—Specific rotation (Na-D line) (upper curve) and intrinsic viscosity (lower curve) as a function of solvent composition and ionic strength. It should be noted that the water content expressed as mole % is higher than if it were expressed as weight %. Thus 80 and 90 mole % H_2O correspond, respectively, to 47 and 67% H_2O by weight.

(1) The intrinsic viscosity of 3.3 cc./g. of aqueous ribonuclease indicates that its molecules in water are sparingly hydrated and close to spherical in shape. The specific rotation in water indicates that less than 20% of the polypeptide chain is folded into a hydrogen-bonded, helical configuration.

(2) The much larger intrinsic viscosity of the intermediate reaction product (75–90 mole % water) indicates a large increase in the effective hydrodynamic volume. The influence of ionic strength indicates that the increase is in the direction of a flexible structure, *i.e.*, in the direction of a randomly coiled structure. The relatively small change in specific rotation shows that there has been little change in the helix content of the molecule. The simplest interpretation is that the portion of the polypeptide chain which was helical in the aqueous solution is still helical in the chloroethanol–water mixture.

Schematic diagrams illustrating both the native and the intermediate configuration are shown in Fig. 2.

(3) As was stated in the Introduction, the low negative rotation of the final product is indicative of a high content of hydrogen-bonded helix (about 70%). Presumably a solvent which favors peptide hydrogen bonds will also favor other intramolecular hydrogen bonds, and it is therefore not surprising that the ribonuclease molecule in chloroethanol is rigid. The intrinsic viscosity is compatible (though exact calculations cannot be made) with a model such as is shown in Fig. 2C.[11]

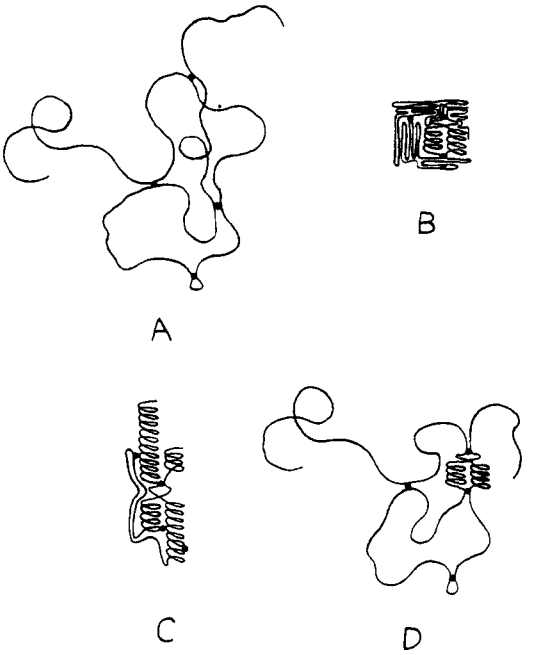

Fig. 2.—Schematic representations of the configuration of ribonuclease in various solvents: (A) 8 M aqueous urea; (B) water at room temperature; (C) almost pure 2-chloroethanol; (D) water–chloroethanol with 80–90 mole % (50–70% by weight) water. The dark circles represent the four disulfide bonds of ribonuclease, located at approximately the correct positions along the polypeptide backbone.

The qualitative conclusions concerning helix content, discussed above, were based on the values of $[m']$ at a fixed wave length. These conclusions were confirmed by measuring the dispersion of optical rotation. The value of $[m']$ defined by equation 1 obeys a two term expression[7]

$$[m'] = \frac{a_0\lambda_0^2}{\lambda^2 - \lambda_0^2} + \frac{b_0\lambda_0^4}{(\lambda^2 - \lambda_0^2)^2} \qquad (2)$$

where λ_0 is assigned a value of 2120 Å. It follows that $[m'](\lambda^2 - \lambda_0^2)$ or $[\alpha](\lambda^2 - \lambda_0^2)$ is a linear function of $1/(\lambda_2 - \lambda_0^2)$, as shown by the plot of Fig. 3. The value of b_0 may be computed from the slopes of such linear plots by use of equations 1 and 2 and they may be interpreted in terms of what we may call the *apparent helix content*. To do this one assumes that the only kind of structure which contributes to the second term on the right-hand side of equation 2 is a right-handed α-helical structure, and, furthermore, that the value of b_0 for such a structure is the same as that observed in synthetic polypeptides, *i.e.*, −630°. The observed negative value of b_0 divided by 630 is then the fraction of the molecule which must be in such a helical form. These assumptions are, of course, not realistic, and the apparent helix content is not to be literally interpreted. Nevertheless, large changes in

(11) If no disulfide bonds were present, then the molecule could form a continuous helical rod of 34 turns, of length 185 Å. and of diameter 12 to 15 Å., depending on the extent of solvation. The resulting intrinsic viscosity would be 12 to 13 cc./g. Disulfide bonds (*cf.* Fig. 2) prevent formation of a continuous rod. If these bonds effectively reduce the rod length by a factor of 2, increasing the diameter by $\sqrt{2}$, then the calculated intrinsic viscosity becomes 5.0 to 5.5 cc./g.

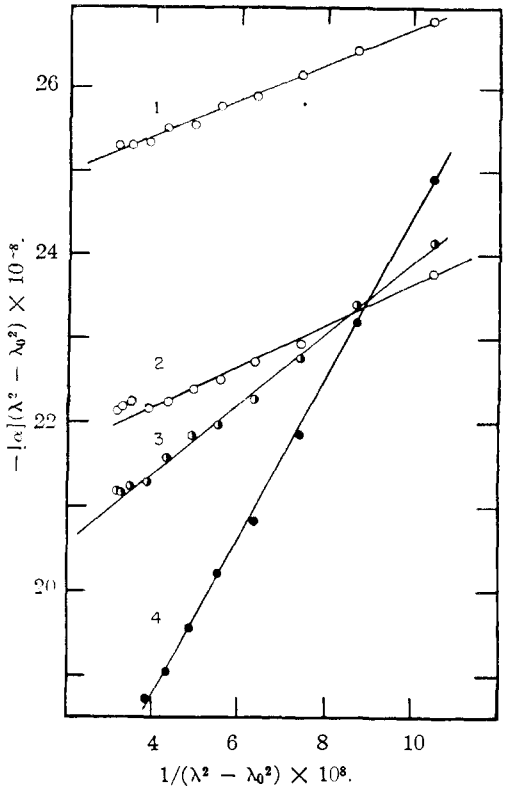

Fig. 3.—Rotatory dispersion data plotted according to equation 2: curve 1, water at *p*H 2 and ionic strength 0.02; curve 2, isoionic protein (*p*H 9.6) in water at ionic strength 0.02, curve 3, intermediate form in chloroethanol-water (78.8 mole % water) at *p*H 2 and ionic strength 0.03; curve 4, helical form in pure chloroethanol at ionic strength 0 03; curve 4, helical form in pure chloroethanol at ionic strength 0.03. The ordinate of curve 4 has been raised by 12×10^8. (The data cover wave lengths from 375 to 600 mμ. The dispersion of refractive index has been neglected in calculating b_0 from these data.)

apparent helix content are currently considered a qualitative reflection of corresponding changes in the organization of the polypeptide backbone.

From the data of Fig. 3 one obtains $b_0 = -380°$ in chloroethanol, $b_0 = -175°$ in the intermediate state and $b_0 = -95°$ in aqueous solution, all of these referring to ribonuclease with maximum positive charge, at ionic strength 0.02. Measurements were also made for isoionic deionized ribonuclease (*p*H 9.6) dissolved in 0.02 *M* salt, the resulting b_0 value being $-105°$. The apparent helix content evaluated from these values is 60, 28, 15 and 17%, respectively. The values for ribonuclease in water and in pure chloroethanol are in good agreement with similar values reported by Doty, *et al.*[3]

Reversibility.—Figure 4 shows that the reactions described above are reversible, or very close to reversible, as far as viscosity is concerned. A relatively concentrated solution of ribonuclease in chloroethanol (protein molecules in the rigid helical configuration) was diluted with water so as to produce a solvent containing 78.8 mole % water. The viscosity, as well as its concentration dependence, is seen to be identical to that obtained when protein solutions in the same solvent are prepared

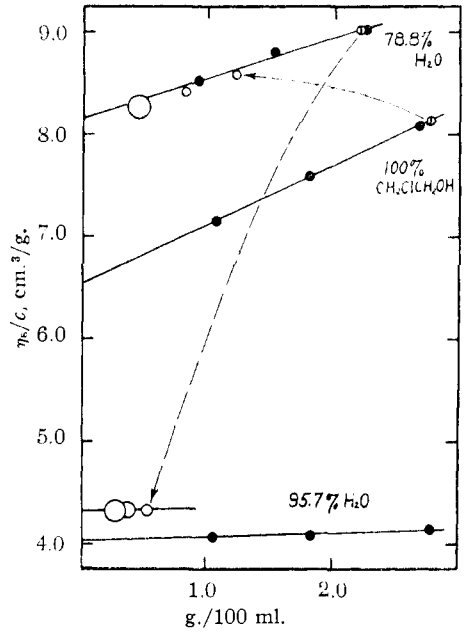

Fig. 4.—Reversibility of the changes in viscosity. Closed circles correspond to the data of Fig. 1, open circles were obtained after exposure to higher chloroethanol concentrations, as shown by dashed lines.

directly from aqueous ribonuclease. Similarly, the dilution of the randomly coiled intermediate with pure water leads to a reversal of the viscosity increase which occurs when chloroethanol is added to a water solution.[12] Optical rotation measurements were made on the same solutions and indicated complete reversibility of that property.

In view of the fact that the solvent action of chloroethanol may involve a chemical combination between protein and solvent, an experiment was conducted to test whether exposure of ribonuclease to chloroethanol leads to an irreversible increase in weight. A known weight of ribonuclease hydrochloride was dissolved in chloroethanol and then diluted with a large volume of water. Most of the solvent was removed by lyophilization and the last traces of solvent were removed by heating at 107°. The residual dry weight in two experiments was 100.5 and 99.9% of the known dry weight of the ribonuclease hydrochloride originally added. The lyophilized product was tested for activity and found to be essentially fully active.[14]

The Effect of *p*H.—Figure 5 shows the effect of *p*H on the first reaction which takes place when chloroethanol is added to an aqueous ribonuclease solution. Since this is an unfolding reaction it should be facilitated by an increase in net charge, *i.e.*, a decrease in *p*H, and the results are in accord with this expectation. The transition requires a higher concentration of chloroethanol at *p*H 4.5 to 5 than it does at *p*H 2.

(12) A small difference is observed in this case which may indicate that reversal is not quite complete. The difference may also be an artifact due to the low protein concentrations which necessarily occur in this experiment. Such artifacts have been observed to occur in viscometers of the type here used, both with ovalbumin[13] and ribonuclease.[4]

(13) H. B. Bull, *J. Biol. Chem.*, **133**, 39 (1940).

(14) Activity measurements were performed through the collaboration of H. Resnick and G. Kalnitsky.

The effect of ionic strength, shown in the same figure, appears to be in the wrong direction for the experiments at pH 4.5 to 5. This result should not be taken too seriously, however, because the pH used is in the steepest region of the titration curve so that the molecular charge at the same pH but different ionic strength will be considerably different.

The Effect of Temperature and Denaturing Agents.—The rigid configuration of ribonuclease in almost pure chloroethanol is remarkably resistant to high temperature. A temperature rise from 25 to 76° alters $[\alpha]_D$ by only 6°, to a value of −32°.

Of greater interest is the effect of temperature and of denaturing agents on the intermediate configuration (Fig. 2D). Since the specific rotation and intrinsic viscosity of this form both indicate that the molecules are not completely unfolded, a further transition would appear to be likely in which the unfolding process is completed, the resulting configuration being the completely unfolded one which is illustrated by Fig. 2A. This configuration is experimentally obtained when ribonuclease is dissolved in 8 M aqueous urea solution, and, perhaps, when an aqueous solution is heated to 70°.[15]

Surprisingly, complete unfolding could not apparently be brought about. Heating to 75° led to a maximum rotation of −81.4°, addition of urea to a concentration of 8 M gave a value of −90.7°, presence of 4 M guanidine hydrochloride a value of −82°. The effect of refractive index (eq. 1) is to make $[\alpha]$ 3 to 4° more negative in the mixed solvent than it would be for an identical configuration in pure water. The observed values are, however, consistently more *positive* than in the control experiments in which water was the solvent.

Sedimentation Coefficients and Molecular Weights.—The results obtained have been interpreted on the assumption that ribonuclease does not undergo association in chloroethanol solutions. To verify this assumption, sedimentation coefficients were determined, and these were combined with the intrinsic viscosities shown in Fig. 1 to obtain molecular weights by use of the Scheraga–Mandelkern equation[16]

$$\beta M^{2/3} = Ns([\eta]/100)^{1/3}\eta/(1 - \bar{v}\rho) \qquad (3)$$

In this equation N is Avogadro's number, s is the measured sedimentation coefficient, $[\eta]$ the intrinsic viscosity in cc./g., η the viscosity of the solvent, $\bar{v}$ the partial specific volume of the protein in the solvent and ρ the density of the solvent (both s and $[\eta]$ being extrapolated to zero concentration).

The parameter β of equation 3 depends on the hydrodynamic characteristics of the solvated protein molecule. Its value is 2.12×10^6 for rigid spheres, 2.28×10^6 for a rigid ellipsoid of axial ratio 6:1 and approximately 2.5×10^6 for random coils in good solvents. Experimental values as low as 2.0×10^6 have been observed. By choosing $\beta = 2.25 \times 10^6$, $M^{2/3}$ can clearly be determined with an accuracy of about 10%, and M therefore with an accuracy of about 15%, regardless of the characteristics of the solvated particle. (β would be larger for a very long rod, but this possibility does not apply to ribonuclease.)

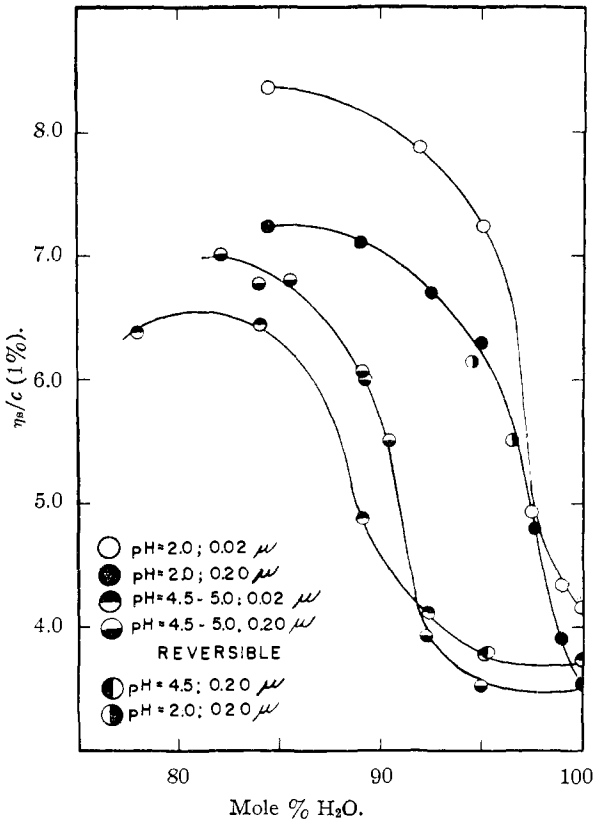

Fig. 5.—The effect of pH on the unfolding reaction produced by addition of chloroethanol to aqueous ribonuclease.

Table I shows the result of such a determination for ribonuclease in pure chloroethanol at pH 2 and ionic strength 0.01. The result is within the allowed error of 15% of the true molecular weight (13,700) of the protein.

TABLE I

MOLECULAR WEIGHT DETERMINATION BY EQUATION 3

	Helical form 100% chloroethanol	Intermediate form dioxane–H_2O (1:1 by vol.)
$s \times 10^{13}$	0.23	0.64
$[\eta]$. cc./g.	6.5	7.6
$\bar{v}$, cc./g.	0.72	0.72
ρ. g./cc.	1.196	1.035
$\eta \times 100$	3.00	1.85
Mol. wt.	12,400	13,800

A similar determination could not be made in the chloroethanol–water mixture which gives rise to the intermediate configuration of the protein because the large difference in density between chloroethanol and water leads to sedimentation of the chloroethanol in the solvent mixture, with the result that a refractive index gradient is set up which masks the gradient at the sedimentation boundary of the protein.

It has been found, however, that the behavior of

(15) W. F. Harrington and J. A. Schellman, *Compt. rend. Lab. Carlsberg*, **30**, 21 (1956). Ribonuclease in 8 M urea was found to have $[\alpha]_D = -108°$ and $[\eta] = 8.9$ cc./g. The same authors found that heating an aqueous solution to 70° leads to a product with $[\alpha]_D = -85°$.

(16) H. A. Scheraga and L. Mandelkern, THIS JOURNAL, **75**, 179 (1953).

ribonuclease in the solvent system dioxane–water is quite similar to that in the system chloroethanol–water, except that only partial conversion to the helical form can be observed because ribonuclease becomes relatively insoluble as the dioxane-rich end of the composition scale is approached. The intermediate form in this solvent system occurs in a 1:1 mixture by volume, and it behaves well in the ultracentrifuge because dioxane and water differ only little in density. The molecular weight determined for this form is also shown in Table I. Again no deviation from the accepted molecular weight is observed.

Molecular weights were also determined by Archibald's method, following the procedure of Klainer and Kegeles.[17] A synthetic boundary cell is required in this method in order to relate observed displacements of the Schlieren bar image to concentration gradients. Unfortunately, synthetic boundary cells made of Kel-F are not available, and the materials used for existing cells are rapidly consumed by the acidic chloroethanol solutions. The relation between the observed displacement and the true dc/dx had therefore to be obtained from the sedimentary boundary in the sedimentation velocity experiment. This boundary was very diffuse, and the accuracy with which the area under the boundary could be determined was poor. Molecular weights of 17,000 and 15,000, respectively, were obtained in pure chloroethanol and in the 1:1 dioxane–water mixture.

Discussion

Perhaps the most interesting aspect of the results here reported is the insight they provide into the forces which lead to the compact native configuration which ribonuclease, in common with most other globular proteins, possesses in aqueous solution. It is generally recognized[10,18] that six distinct factors may be operative: (1) intramolecular peptide hydrogen bonds, (2) intramolecular side chain hydrogen bonds, (3) intramolecular ion-pair formation between oppositely charged side chains, (4) long distance electrostatic effects depending on the existence of large numbers of both positive and negative charges, (5) solvent–solvent hydrogen bonds leading to formation of intramolecular hydrophobic regions (*i.e.*, intramolecular micelle formation),[19] (6) disulfide bonds. In general the coöperation of all of these forces may be expected to be necessary. In the case of ribonuclease, however, the hydrophobic forces appear to be of special importance. We are looking in Fig. 1 at a transition from what is almost certainly the maximally hydrogen-bonded configuration of ribonuclease to the native aqueous configuration. Disulfide bonds cannot be the prime factor in this configurational change, for no change in these bonds takes place. The two types of electrostatic forces cannot be involved, for the molecules bear close to their maximum possible positive charge, and essentially no negative charges are present. Thus only hydrogen bonding and hydrophobic bonding can be responsible for the observed results.

Examining Fig. 1 (or the schematic model, Fig. 2C → Fig. 2D → Fig. 2B) with these forces in mind it is clear that water tends to rupture intramolecular hydrogen bonds. The rigid configuration in chloroethanol, with its high content of peptide and presumably side chain hydrogen bonds, is destroyed as the water content is increased, until, at 75 mole % water, most of the molecule is randomly coiled. It is difficult to see how a further increase in water content can suddenly reverse the role which water plays with respect to intramolecular hydrogen bonds and much more reasonable to suppose that the collapse of the molecule which occurs on going from 90 to 100 mole % water is due to hydrophobic bonding or to a similar phenomenon which involves the specific structure of the solvent.[21]

It is possible that the special importance here ascribed to hydrophobic bonding applies quite generally to all globular proteins in water solution. There are two reasons for believing that this might be so. (1) Almost all globular proteins have intrinsic viscosities between 3.0 and 4.0 cc./g. in water solution. Such low viscosities have never been reported for any solvent other than water. (2) Only one type of synthetic polymer is known to have any tendency for collapsing into compact unimolecular particles in aqueous solution, these being poly-soaps in which hydrophobic forces are unquestionably the cause of the collapse.[23]

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(17) S. M. Klainer and C. Kegeles, *J. Phys. Chem.*, **59**, 952 (1955).

(18) W. Kauzmann, "The Mechanism of Enzyme Action" (W. D. McElroy and B. Glass, ed.), Johns Hopkins Press, Baltimore, Md., 1954.

(19) Or similar effects reflecting the ease with which portions of the protein molecule can be accommodated by the structure of the solvent. Klotz and co-workers,[20] for instance, have suggested that surface structure is an important consideration.

(20) I. M. Klotz, *et al.*, THIS JOURNAL, **79**, 4078 (1957); **80**, 2132 (1958).

(21) It is of great interest in this connection that three of the six phenolic groups of ribonuclease are inaccessible to titration. The most likely explanation, suggested earlier,[22] is that these groups are dissolved in a non-polar region of the protein molecule.

(22) C. Tanford, J. D. Hauenstein and D. G. Rands, THIS JOURNAL, **77**, 6409 (1955).

(23) U. P. Strauss and N. L. Gershfeld, *J. Phys. Chem.*, **58**, 747 (1954); U. P. Strauss, N. L. Gershfeld and E. H. Crook, *ibid.*, **60**, 577 (1956). The lowest intrinsic viscosity observed was 4.2 cc./g. for a polyvinyl-N-alkylpyridinium bromide in which 28.5% of the alkyl side chains were dodecyl, the remainder being ethyl. This value was obtained when the polymer was dissolved in aqueous potassium bromide solution, the corresponding value in water without added electrolyte being 10 cc./g. The intrinsic viscosity of the same polymer containing 100% ethyl side chains was 2500 cc./g.