carboxylate ion bonds, rendering the observed pK of carboxyl groups lower than normal and that of tyrosyl groups higher than normal in the native protein. On the other hand, the abnormality could be due to specific local electrostatic effects[5] or hydrophobic bonding.[6] All three types of interaction might require the specific configuration of the native protein. Since GU disrupts the native configuration,[12] such interactions could be eliminated. There is not yet sufficient experimental evidence to decide between these alternatives. If tyrosyl–carboxylate ion hydrogen bonds do exist in the native protein, they may play a role in the stabilization of the configuration, and their rupture (by binding hydrogen ions to carboxylate ion groups) at low pH might lead to an unfolding of the molecule. While deuterium–hydrogen exchange studies[27] at pH 1 indicate that exposure to low pH leads to instability, this instability arises only after some irreversible change has taken place in the molecule. Similarly, the titration of the 3 inaccessible tyrosyl groups is possible only after an irreversible process has taken place.[6,7] Thus, while some rupture of hydrogen bonding in GU is indicated by the data reported here, the identification of the specific bonds broken will have to await the results of further investigation.

(27) C. L. Schildkraut and H. A. Scheraga, THIS JOURNAL, **82**, 58 (1960).

ITHACA, N. Y.

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, CORNELL UNIVERSITY]

Structural Studies of Ribonuclease. II. Deuterium Exchange Studies of Two Crystalline Forms of Ribonuclease[1,2]

BY CARL L. SCHILDKRAUT AND HAROLD A. SCHERAGA

RECEIVED JUNE 9, 1959

Deuterium exchange studies were carried out on Armour ribonuclease 381-059 and two samples (ribonuclease II and III) prepared by King and associates. Complete deuteration cannot be achieved unless the molecule is above the transition temperature. A significant fraction of the hydrogens exchange slowly, in agreement with the results of Hvidt and Haggis. Below the transition temperature, *e.g.*, at 38°, about 20 hydrogens are sufficiently shielded to prevent exchange. Two groups of 25 hydrogens each exchange more rapidly at 0° but still slowly enough to suggest that they are involved in some folding of the molecule. The remaining 175 exchange essentially instantaneously at 0°, suggesting that they are in random-coil-like parts of the molecule. Ribonuclease II and Armour ribonuclease 381-059 are indistinguishable by the method of deuterium–hydrogen exchange. By this criterion, ribonuclease III appears to have a configuration in solution in which a greater fraction of its hydrogens are shielded.

Introduction

King and associates recently have prepared several crystalline forms of ribonuclease and have characterized them by X-ray diffraction[3] and by their melting points in contact with their mother liquor.[4] In accounting for the properties of these crystals King has suggested the possibility that different molecular configurations of ribonuclease might exist in solution. The possibility that such differences might exist prompted us to look for them by the deuterium–hydrogen exchange method.[5–7] Differences in configuration can be detected by this method only if the various configurations are stable under the conditions of the experiment and if the different types of molecules exhibit a significant difference in the rate or degree of exchange. Two crystalline forms, designated ribonuclease II and III, have been selected for study. Form III has a lower melting point in the mother liquor[4] and form II appears to be the stable form over a wide range of conditions.[3] In conjunction with these experiments, some observations also were made on the relation between the maximum number of exchangeable hydrogens and the temperature of deuteration. These observations are relevant to the question of the transition in ribonuclease observed by Harrington and Schellman.[8]

Experimental

Materials.—Two different crystalline forms were prepared by King[9] from Armour ribonuclease Lot 381-059 by essentially the same procedure outlined by King, *et al.*[3] Modification II was grown from a solution of 50 volume % 2-methyl-2,4-pentanediol and 5 volume % methanol. Modification III was grown from a solution of 50 volume % *n*-propyl alcohol. For purification the crystals were dissolved in water, centrifuged to remove traces of insoluble material and lyophilized.

All other determinations were performed on Armour Ribonuclease Lot 381-059, used without further purification.

Deuterium Oxide, 99.5%, batch No. 7, from the Stuart Oxygen Company, San Francisco, California, was used to prepare the standards for density determinations and for deuteration of the samples.

Procedure.—The procedure was essentially the same as method 2 developed by Linderstrøm-Lang.[6,10] A 150-μl. sample was placed in a tulip-shaped vessel and the water removed *in vacuo* by cryosublimation into a cold trap at −78°. When the substance was almost dry, it was heated for 4 hr. at 60° while still in the cryosublimation tube. Deuterium oxide (99.5%) then was added to the dried protein

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(2) Presented before the Division of Biological Chemistry at the 136th meeting of the American Chemical Society, Atlantic City, New Jersey, September, 1959.

(3) M. V. King, B. S. Magdoff, M. B. Adelman and D. Harker, *Acta Cryst.*, **9**, 460 (1956).

(4) M. V. King, *Biochim. Biophys. Acta*, **27**, 503 (1958).

(5) A. Hvidt, G. Johansen, K. Linderstrøm-Lang and F. Vaslow, *Compt. rend. trav. Lab. Carlsberg, Sér. chim.*, **29**, 129 (1954).

(6) I. M. Krause and K. Linderstrøm-Lang, *ibid.*, **29**, 367 (1955).

(7) A. Hvidt and K. Linderstrøm-Lang, *ibid.*, **29**, 385 (1955).

(8) W. F. Harrington and J. A. Schellman, *ibid.*, **30**, 21 (1956).

(9) We are indebted to M. V. King for the gift of these samples.

(10) K. Linderstrøm-Lang, "Symposium on Peptide Chemistry," Chemical Society, London, Special Publication No. 2, 1 (1955).

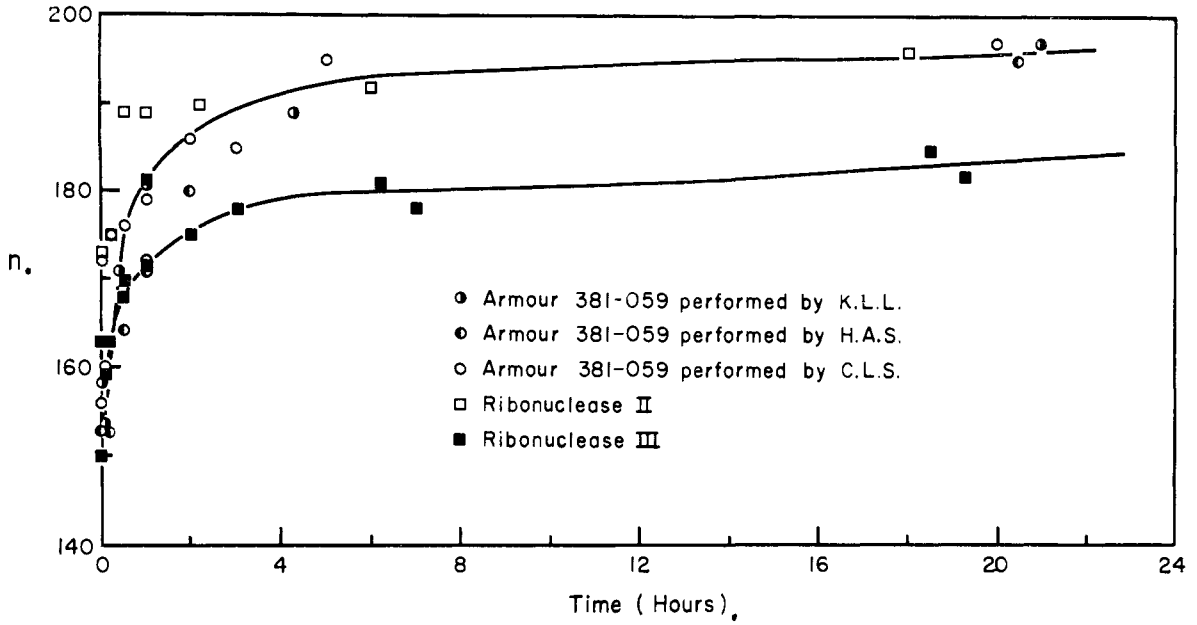

Fig. 1.—Rate of back exchange of ribonuclease at 0° after deuteration at 38° at *p*H 4.8.

which was allowed to exchange for 20 hr. The deuterated protein then was dried again by cryosublimation as described above. The back exchange was carried out in the same "tulip" by the addition of 150 μl. of 0.1 *M* glycerol with the same pipet used to remove the sample from the ribonuclease stock solution. The exchange was followed by withdrawing 15-μl. samples at suitable intervals, removing their water by cryosublimation and determining its concentration of deuterium by density determinations in the gradient tube.[5]

Before performing experiments on ribonuclease the method was checked on glycine and alanine. It was found that large amounts of the material were carried over into the trap by the strong stream of water vapor during the cryosublimation of the 150-μl. samples. This was found to occur to a much smaller extent in the case of ribonuclease; it was noticed that occasionally as much as 5% of the protein sample would be lost. To eliminate this possible source of uncertainty in the results, the following modifications were found useful.

Two circular pieces of 150 mesh platinum gauze, 6 mm. in diameter, were balanced on top of the ground glass joint of the "tulip" before it was inserted in the Y-tube.[5,10] If small flakes of protein were carried over, they would be visible on the gauze. It was usually possible to tap them back down into the tube without disturbing the cryosublimation. Usually there was no carry-over of ribonuclease at all and the clean platinum acted merely as an indication that low results should not be expected.

The carry-over of protein was eliminated by distillation at 0.17 ± 0.01 mm. (as measured by a McLeod gauge) rather than at the lower pressure of 8×10^{-3} mm. previously used. Since the rate of sublimation at this higher pressure is not fast enough to keep the sample frozen, the donor tulip had to be immersed in a −5° ice–salt bath until it was almost dry. This auxiliary bath could be removed after 1.5 hr. The sample tube then was allowed to come to room temperature over a period of 10 minutes and then slowly up to 60°. After it had been heated at 60° for about 1 hr., the pressure was lowered to 5×10^{-3} mm. and the cryosublimation continued for 3 more hours with the temperature of the donor tulip maintained at 60°. It should be mentioned that this high-pressure distillation was necessary only for the 150-μl. samples and not for the 15-μl. samples.

An additional modification was necessary in the cryosublimation of the 15-μl. samples from the back exchange at *p*H 1. Since a small amount of HCl in the sublimate could alter its density greatly, it was necessary to remove it, using K_3PO_4 as previously described.[5,11] Only the back exchange was carried out at *p*H 1; the samples were deuterated in the usual manner at *p*H 4.8.

Concentration.—The number, n, of deuterium atoms exchanged from one molecule of deuterated protein at any time is related to the density of the sublimate by the equation[5]

$$n = \frac{110.8}{c_s} \frac{U}{U_0 - U}$$

where $U_0 \times 10^{-6}$ is the difference in density (0.106720) between 99.5% D_2O (1.10495) and H_2O (0.99823) at 20°, the temperature of the density gradient tube, and $U \times 10^{-6}$ is the difference in density between the sublimate and water at the same temperature. The quantity c_s is the molar concentration of the protein in the solution which was determined as follows.

Two ml. of an approximately 2% solution of Armour ribonuclease was prepared and at the same time a sample of the powder was taken for a dry weight determination. These were performed on 0.1-g. samples which were heated *in vacuo* for 10–15 hr. at temperatures ranging from 110 to 140°. The molarity of the ribonuclease solutions was calculated from the weight of the dissolved sample and added water after a correction was made for the water content of the protein.

Due to the small amounts of modifications II and III which were available only 5 mg. of these samples could be used for dry weights. Six determinations were made on separate aliquots from the same stock solution. A 100-mg. sample of Armour ribonuclease always was included in the oven for comparison. The fact that deuteration at higher temperatures (see below) yields the correct theoretical value indicates that the dry weights were not significantly in error.

Results

Armour 381-059 Ribonuclease.—Before investigating ribonuclease II and III, some experiments were carried out on Armour 381-059 material, especially in light of the discrepancy between the results of Hvidt[12] and those of Haggis.[13] Hvidt found that all of the theoretically exchangeable hydrogens had exchanged completely at 38°, using the method of Linderstrøm-Lang. Haggis, using the less quantitative method of infrared absorption, found that only 70% exchanged at this temperature. Hvidt's experiments have been

(11) S. J. Leach and H. A. Scheraga, *Compt. rend. trav. Lab. Carlsberg, Sér. chim.*, **30**, 271 (1958).

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(13) G. H. Haggis, *ibid.*, **23**, 494 (1957).

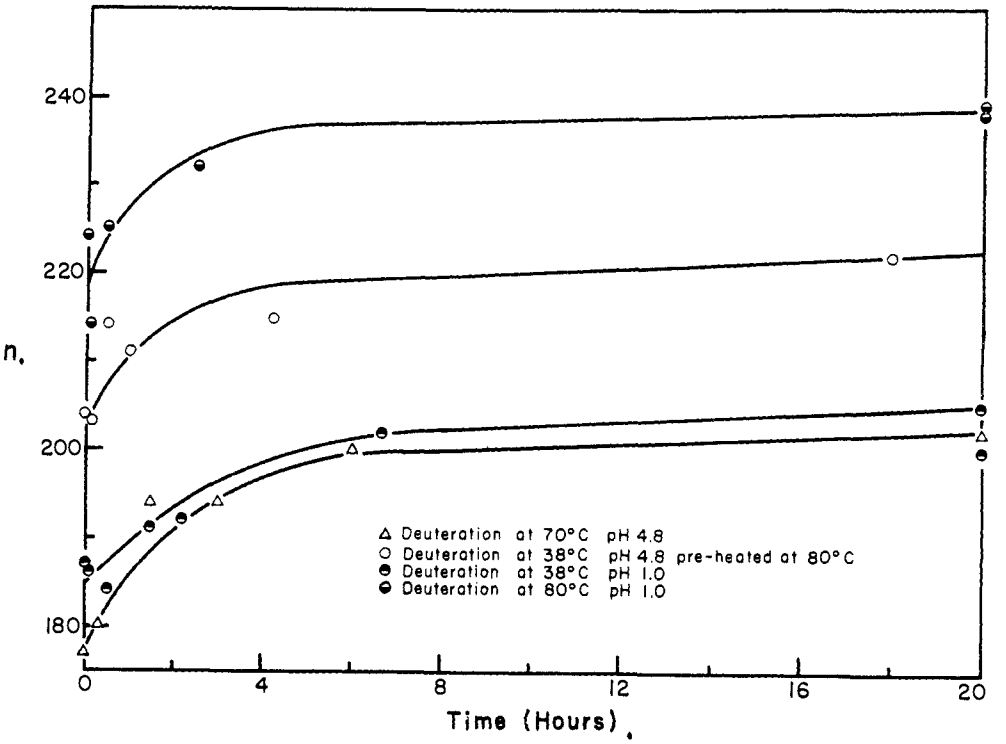

Fig. 2.—Dependence of rate and degree of exchange on temperature of deuteration and *p*H of back exchange at 0°.

repeated by three investigators (Linderstrøm-Lang and Scheraga at the Carlsberg Laboratory, and Schildkraut at Cornell) all of whom agree, within ±2%, that the extent of exchange of Armour 381–059 ribonuclease at 38° is 91%, when deuteration is carried out at 38°. The kinetic curves at 0° are shown in Fig. 1.

Since ribonuclease undergoes a transition[8] above 50°, it was thought that complete exchange could be achieved only at temperatures above 50°. Therefore, several experiments were carried out at 60, 70, 75 and 80°. These involved deuteration at the given temperature, drying of the deuterated protein and back exchange at the temperature of deuteration as well as at lower temperatures. It was found that complete deuteration takes place at temperatures of 60° and higher. If the ribonuclease is deuterated for 20 hr. at 80° and the back exchange carried out for 20 hr. at 38°, complete exchange is achieved, but not immediately (see Fig. 2 and Table I). It has also been found that heating the protein at 80° for 20 hr. before deuteration at 38° gives essentially complete exchange at 38°. At 0°, however, there is still only 91% exchange. These results indicate an irreversible rupture of the structure at 80° at *p*H 4.8 without a complete loss of structure. This observation has been confirmed by optical rotation studies at elevated temperatures.[8,14] However, whereas treatment at 80° produces irreversible changes, a 20 hr. treatment at 70° seems to allow a reversible recovery of the low temperature structure, even though the protein has been maintained above its transition temperature, *e.g.*, the back exchange kinetics results at 0° are similar for samples deuterated at both 38 and 70°, even though the degree of deuteration of both samples differed (91 and 100%, respectively). It should also be mentioned that a sample deuterated at 38° showed no increase in degree of exchange when allowed to exchange back at 70° instead of 38°. This confirms the assumption that the 91% back exchange at 38° is due to incomplete deuteration at 38°.

(14) J. Hermans, Jr., and H. A. Scheraga, unpublished results.

TABLE I

TEMPERATURE AND *p*H DEPENDENCE OF DEGREE OF EXCHANGE

*p*H	Treatment of sample	Temp. of back exchange, °C.	"Equilibrium" per cent of exchange[a] at 20 hr.
4.8	Deuterated at 38° for 20 hr.	0	80
		38	91
4.8	Deuterated at 80° for 20 hr.	0	88
		27	94
		38	100
		50	100
		70	100
		80	100
4.8	Deuterated at 70° for 20 hr.	0	83
		70	100
4.8	Deuterated at 60° for 20 hr.	60	100
4.8	Heated in H_2O solution at 80° for 20 hr. Deuterated at 38° for 20 hr.	0	91
		38	98
1.0	Deuterated at 38° for 20 hr.	0	83
		38	89
1.0	Deuterated at 80° for 20 hr.	0	98

[a] The experimental error is estimated as ±2%.

In light of these results, Haggis' proposed explanations[13] of Hvidt's high value[12] are reasonable, *e.g.*, it is possible that her samples were deuterated while drying at 60°. Presumably the drying rate

may have been different since the more recent similar experiments of Linderstrøm-Lang, Scheraga and Schildkraut did not show this effect.

The results at *p*H 1.0 (see Fig. 2) show that, for a sample deuterated at 38°, the low *p*H increases the rate of exchange slightly (over that at *p*H 4.8) but not the degree. If the sample is deuterated at 80°, however, both the rate and degree of exchange are increased. At 0°, 98% of the deuterium exchanges back. In contrast, at 0° heating alone or acid treatment alone account only for 83 and 88% back exchange, respectively. Since the heating was carried out at *p*H 4.8 (*i.e.*, the *p*H was not lowered until after the heat treatment), it would seem that some irreversible structural change must occur upon heating before the subsequent low *p*H treatment can affect the degree of exchange.

Ribonuclease II and III.—The results of experiments on ribonuclease II and III (deuterated at 38° at *p*H 4.8) at *p*H 4.8 are shown in Fig. 1. Although there is as yet no conclusive proof, experiments of King[15] suggest that modification II is the unaltered protein (*e.g.*, similar to Armour 381-059 ribonuclease) whereas modification III is an altered form. In support of this view, modification II is indistinguishable from Armour 381-059 ribonuclease in the kinetic experiments. Modification III appears to show a slower rate of exchange. The degree of exchange is 5% less both at 0° and at 38° (see Table II). If this were merely due to the effect of an impurity on the experimental determination of the dry weight, the values of the degree of exchange at 80° would also differ by 5%, but they do not.

TABLE II

TEMPERATURE DEPENDENCE OF DEGREE OF EXCHANGE OF TWO CRYSTALLINE FORMS

Crystalline modification	Degree of exchange[a] at 0°+	38°+	80°++
II	80	91	100
III	75	86	100

[a] The experimental error is estimated at ±2%: + deuterated at 38°; ++ deuterated at 80°.

Discussion

The theoretical number of exchangeable hydrogen atoms in ribonuclease, based on the amino acid analysis of Hirs, Moore and Stein[16] has been listed in Table III. There are 256 exchangeable hydrogens in the completely protonated enzyme at low *p*H. This must be corrected to take account of the state of ionization of the dissociable groups at the *p*H of the solution in D_2O (which is assumed to be the same as the *p*H in the original stock solution before lyophilization). At *p*H 4.8 we must subtract 11 leaving 245 as the value we should expect to find when deuteration proceeds at temperatures of 60° or above.

Despite the discrepancies in total number of exchangeable hydrogens between our results and those of Hvidt, we confirm her findings of a slow rate of exchange for some of the hydrogens at 0° in solution at *p*H 4.8. Therefore, we confirm her conclusion that there is "some kind of hydrogen-bonded, folded structure in the molecule, shielding part of the hydrogen atoms from instantaneous exchange." In addition, from our result that *complete* deuteration is achieved only if the temperature is raised above the transition temperature, we conclude that about 8 to 10% of the hydrogens are more strongly shielded than the remaining ones, their exchange being too slow to detect below the transition temperature. Considering the data for the curve represented by the triangles of Fig. 2, we can divide the hydrogens of ribonuclease into several groups: about 175 rapidly exchangeable hydrogens, about 25 more slowly exchangeable ones (requiring up to 6 hr.), and the remaining 45 which exchange very slowly at 0°. Of these 45, about 20 (*i.e.*, 8% of the original 245) do not exchange below the transition temperature.

Some information is now available to enable postulates to be made on possible foldings of the ribonuclease chain (see, *e.g.*, Anfinsen),[17] and further studies are in progress at present in this Laboratory on the denaturation of ribonuclease[14,18] in order to obtain more experimental data on the secondary structure. It seems to be premature at the moment to try to reconcile the various groups of exchangeable hydrogens with the proposed structures. Among the pieces of evidence that must be fitted into the picture are the inaccessibility of 3 tyrosyl groups to hydrogen ions,[19] the abnormally low *pK*'s of the carboxyl groups,[20]

TABLE III

THEORETICAL NUMBER[a] OF EXCHANGEABLE HYDROGEN ATOMS, H_e, IN RIBONUCLEASE AT *p*H 1.0

Amino acid	No. of residues	Backbone	Side chain	End group
Glycine	3	3		
Alanine	12	12		
Valine	9	9		1
Leucine	2	2		
Isoleucine	3	3		
Histidine	4	4	8	
Lysine	10	9	30	3
Arginine	4	4	20	
Aspartic acid	4	4	4	
Asparagine	11	11	22	
Glutamic acid	6	6	6	
Glutamine	6	6	12	
Serine	15	15	15	
Threonine	10	10	10	
Proline	4	0		
Phenylalanine	3	3		
Tyrosine	6	6	6	
Cystine (1/2)	8	8		
Methionine	4	4		
Total	124	119	133	4

Total H_e at *p*H 1.0 = 256
−11
Total H_e at *p*H 4.8 = 245 Mol. wt. = 13,683

[a] Amino acid composition from Hirs, Moore, and Stein.[16] We are indebted to Dr. Hirs (private communication) for the most recent data on aspartic acid, asparagine, glutamic acid and glutamine.

(15) Private communication.

(16) C. H. W. Hirs, S. Moore and W. H. Stein, *J. Biol. Chem.*, **219**, 623 (1956).

(17) C. B. Anfinsen, Jr., *Federation Proc.*, **16**, 783 (1957).

(18) C. Y. Cha and H. A. Scheraga, THIS JOURNAL, **82**, 54 (1960).

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the normality of all the tyrosyl and carboxyl groups in denaturing solvents,[18,21] the possible existence of tyrosyl–carboxylate ion hydrogen bonds,[22] the results of Richards[23] on the relationship between the N-terminal tail and biological function, etc. A summary of current structural information on ribonuclease has been presented by Linderstrøm-Lang and Schellman.[24]

If tyrosyl–carboxylate ion hydrogen bonds play any significant role in stabilizing the folded configuration, the loss of this stabilization should be evident at *p*H 1.0. However, our results indicate that exposure to *p*H 1.0 produces a significant loss of folding only after an irreversible change has taken place in the molecule.

Finally, the data of Fig. 1 indicate that ribonuclease III has a greater degree of hydrogen-shielding since a larger fraction of its hydrogens do not exchange in 20 hr. at 0° than was observed for the other forms of ribonuclease. Whether or not ribonuclease III has a greater degree of helical character remains to be seen. Aside from kinetic factors affecting the rate of transformation of one configuration into another, the solvent also influences the equilibrium configuration. Therefore, it is not at all surprising that variation of solvent (*i.e.*, crystallizing medium) can change the equilibrium configuration of the molecule from one form to another.

ITHACA, NEW YORK

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(24) K. U. Linderstrøm-Lang and J. A. Schellman, "The Enzymes," Ed. P. D. Boyer, H. Laidy and K. Myrbäck, Vol. I, Academic Press, Inc., New York, N. Y., 1959, p. 443.

[CONTRIBUTION FROM THE LOW TEMPERATURE LABORATORY, DEPARTMENT OF CHEMISTRY AND CHEMICAL ENGINEERING, UNIVERSITY OF CALIFORNIA, BERKELEY]

The Thermodynamic Properties of Aqueous Sulfuric Acid Solutions and Hydrates from 15 to 300°K.[1]

BY W. F. GIAUQUE, E. W. HORNUNG, J. E. KUNZLER AND T. R. RUBIN

RECEIVED JUNE 1, 1959

The heat capacities, entropies, free energies and heat contents of all stable phases in the H_2SO_4–H_2O system are correlated and tabulated from 15 to 300°K. Ice is known to have frozen-in disorder at low temperatures and thus is unstable. Sulfuric acid and all of its hydrates approach perfectly ordered crystalline states at low temperatures as is indicated by the complete entropy agreements. The stable phases are H_2SO_4, $H_2SO_4 \cdot H_2O$, $H_2SO_4 \cdot 2H_2O$, $H_2SO_4 \cdot 3H_2O$, $H_2SO_4 \cdot 4H_2O$ and $H_2SO_4 \cdot 6.5H_2O$. The various phases are connected by means of thermodynamic properties of the liquid solutions at 298.15°K. (25°). At this temperature values of the partial molal free energies, heat contents, heat capacities and some values for the change of heat capacity with temperature are given. The activity of water is tabulated at concentrations up to pure H_2SO_4, where $a_{H_2O} = 1.56 \times 10^{-9}$. Pure H_2SO_4 is used as a reference zero for most of the properties; however, values of the activity coefficient in the ordinary hypothetically molal reference system are given up to 1000 molal. The activity of pure H_2SO_4 in this system is 3.41×10^9 ($m = \infty$). It is concluded that no intermediate hydrate becomes unstable with respect to a higher and lower hydrate. This statement also includes ice and pure sulfuric acid. It is pointed out that such functions as free energy/temperature, heat content/temperature, heat capacity and entropy need a common and short name for their common unit, defined cal. deg.$^{-1}$. The name should not be one which has applied particularly to any one of these properties. The use of the short name Gibbs is suggested, thus 1 gibbs (g. mole)$^{-1}$ = 1 gbs. (g. mole)$^{-1}$ = 1 defined cal. (°K.)$^{-1}$ (g. mole)$^{-1}$ = 1 defined B.t.u. (°R)$^{-1}$ (lb. mole)$^{-1}$.

This paper correlates an extensive series of researches on aqueous sulfuric acid. Throughout the publication of the numerous papers in this series we have omitted tables of smoothed data because it was felt desirable to have all of the thermodynamic properties correlated and in one place. This also adds considerably to the efficiency of the presentation. Much of the original data has been recalculated in order to eliminate errors as completely as possible before the data were used to derive the new results presented here, such as the partial molal free energies and absolute partial molal entropies of the components.

The measurements have been particularly designed to give information in the concentration range from several molal to the anhydrous acid and over the entire temperature region below 300°K. In addition to the anhydrous acid and ice, the solid phases known at low temperatures are the mono-, di-, tri-, tetra- and hemihexa(6.5)-hydrates. A solid believed to be octahydrate exists but we did not succeed in cooling it to low temperatures. The original objective was to make use of the third law of thermodynamics to find out if the acid or any of its hydrates retained disorder at limiting low temperatures, due to random hydrogen bonding or other effects leading to lack of internal crystalline perfection.

In starting this problem it was hoped that most of the data needed to calculate the entropies of formation of the several hydrates from each other at ordinary temperatures were already available. We were soon disillusioned. We do not mean to imply that previous work on aqueous sulfuric acid does not include many examples of very good work but rather that the significant application of the equation

$$\Delta S = (\Delta H - \Delta F)/T$$

to the present problem requires data of very great accuracy. For example a combined error of the order of 100 cal. mole^{-1} in ($\Delta H - \Delta F$) at 298°K. leads to an error of several tenths of a cal. deg.$^{-1}$ mole^{-1} in ΔS, and this small amount can have significance in theoretical explanations of disorder in crystals. We may say at once that no evidence of residual disorder at low temperatures

(1) This work was supported in part by the National Science Foundation and by the Office of Naval Research, United States Navy.