[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, CORNELL UNIVERSITY, ITHACA, N. Y.]

Thermodynamic Data from Difference Spectra.[1,2] II. Hydrogen Bonding in Salicylic Acid and its Implications for Proteins

BY J. HERMANS, JR.,[3a] S. J. LEACH[3b] AND H. A. SCHERAGA

RECEIVED NOVEMBER 20, 1962

The standard free energies and heats of ionization of salicylic acid, ethyl salicylate and the corresponding *para* compounds have been determined. From these, approximate values of the standard enthalpy and entropy of formation of the intramolecular hydrogen bonds in these compounds are obtained. The relation between the thermodynamic parameters for ionization of salicylic acid and the stability of side-chain hydrogen bonds in proteins is discussed, with particular reference to data for the protein ribonuclease.

Introduction

In order to account for the relative stabilities of helical and randomly coiled conformations of polypeptide chains, and for the effect of side-chain hydrogen bonding thereon, it is necessary to know the numerical values of the standard enthalpy and entropy of formation of a hydrogen bond in polypeptides and proteins. This paper is concerned with the standard enthalpy of formation, ΔH^0, of a hydrogen bond in aqueous solution. There are several types of hydrogen bonds, *e.g.*, OH. . . O, NH. . .O, etc., and ΔH^0 varies with the nature of the donor and acceptor groups, the medium and the presence of neighboring groups. Since a range of values[4] is quoted for ΔH^0, it appeared desirable to obtain an estimate of ΔH^0 from studies of ionization in model compounds in which internal hydrogen bonds are known to exist in aqueous solution. The compounds selected were the hydroxybenzoic acids and the corresponding ethyl esters. It is worth noting that the steric restrictions present in salicylic acid may also arise in proteins (where the donor and acceptor side-chain groups may be somewhat rigidly positioned by interactions with neighboring non-polar groups), thereby affecting the ionization behavior of the polar groups.[5-7]

Since the model compounds investigated here are aromatic, the pK's of the ionizable groups are determinable at various temperatures from ultraviolet difference spectra.[8] Potentiometric titration experiments were also carried out. No attempt was made to determine pK's in reversible cells since the corresponding experiments on proteins (for which low molecular weight compounds serve as models) are carried out as titrations. Of course, the pK's determined from potentiometric titrations and from ultraviolet difference spectra depend on ionic strength; this causes no difficulty since the pK's of ionizable groups in proteins and in model compounds are always compared at the same ionic strength.

In the potentiometric titrations, limits on the precision of measuring the (apparent) activity coefficients of OH^- and H^+ ion make it difficult to determine accurate concentrations of the ionized and un-ionized forms of the substances being titrated at very high and very low pH; hence, there is a limitation on the accuracy of the computed pK values. In the spectrophotometric method, the concentrations of the conjugate acid and base forms are determined directly and this difficulty does not arise. Thus, we consider the spectrophotometric values of the pK's of salicylic acid more reliable than those determined by potentiometric titration.

Experimental

Materials.—*o*-Hydroxybenzoic (salicyclic) acid (m.p. 158.2–159°) and *p*-hydroxybenzoic acid (m.p. 212.5–213.5°) were the twice recrystallized A.R. grade chemicals (Eastman Kodak Co.). Benzoic acid was a N.B.S. standard sample, and the A.R. grade phenol (m.p. 40.5°, b.p. 169–170° at 744 mm.) was redistilled twice from zinc powder. All four substances were checked for purity by potentiometric titration and found to be 98.5–100% pure. Ethyl salicylate (m.p. −2 to 0°) and *p*-hydroxy ethylbenzoate (m.p. 116–118°), both Eastman Kodak Co. products (purified), were used without further purification.

All other reagents used were A.R. grade. For the potentiometric titrations, use was made of CO_2-free glass-distilled (then deionized) water. KOH solutions were freed of carbonate and stored in polyethylene bottles.

Potentiometric Titrations.—The solutes were dissolved in CO_2-free water containing 0.1 M KCl, to give approximately 0.002 M solutions. Titrations were carried out in a cell thermostated at 0–1° or 25 ± 0.01°, maintaining an atmosphere of nitrogen over the solution and using a magnetic stirrer for mixing. Titrant (1.0 N KOH or HCl) was added from a 0.1-ml. Gilmont ultramicroburet and provision was made for a glass electrode. A KCl bridge connected the solution to a saturated calomel half-cell which was thermostated at 25° throughout all measurements.

The pH measurements were made using a Beckman GS meter (A scale) standardized as required at pH values of 4, 7 and 10. Meter readings were corrected for the difference in temperature between the glass and calomel electrodes by the factor $298/(273 + t)$ where t is the temperature inside the titration cell. "Apparent" activity coefficients for KOH and HCl were obtained by titrating CO_2-free water containing only 0.1 M KCl and finding the mean values of γ over each range. At 25° these were 0.83 and 0.78 at high and low pH, respectively, and these values were used to calculate the number of protons bound at each pH and temperature and hence the acid dissociation constants. The change in ionic strength during titration was considered negligible. For each compound, titration constants were calculated for each point on duplicate titration curves and the values quoted are arithmetic means.

Spectrophotometric Measurements.—A Beckman model GS pH meter (A scale) and a Beckman model DU spectrophotometer, equipped with photomultiplier and thermospacers, were used in all experiments. Values of optical density difference, ΔD, were obtained as a function of pH at each temperature, as described previously.[8] It was previously found[8] that the e.m.f. was linear in pH, using several standard buffers and 0.1 N HCl at the three temperatures, 0°, 25° and 45°. Therefore, this linearity was assumed to hold over the entire pH range used at these temperatures.

Reasonable accuracy in the pH measurements at pH values above 12 was obtained by using a separate glass electrode with a low sodium ion error for these measurements. This electrode was stored at pH 10 and never brought below pH 9. As proof of the proper functioning of this electrode, we cite the fact that 0.1 N KOH gave readings in agreement (±0.03 unit) with the values quoted by Bates[9] for 0.1 N NaOH at 0°, 25° and 45°.

(1) This investigation was supported by research grant H-1662 from the National Heart Institute, National Institutes of Health, U. S. Public Health Service, and by research grant GB-75 from the National Science Foundation.

(2) Presented, in part, before the Division of Biological Chemistry at the 134th National Meeting of the American Chemical Society, Chicago, Ill., September, 1958.

(3) (a) Present address: Research and Development Division, American Viscose Corp., Marcus Hook, Penna. (b) On leave from the Division of Protein Chemistry, C.S.I.R.O., Parkville N.2 (Melbourne), Victoria, Australia, 1956–1957.

(4) Reviewed by H. A. Scheraga, *Ann. Rev. Phys. Chem.*, **10**, 191 (1959); see also "The Proteins," Chapt. 8, Ed. H. Neurath, Academic Press, Inc., New York, N. Y., in press.

(5) J. Hermans, Jr., and H. A. Scheraga, *J. Am. Chem. Soc.*, **83**, 3293 (1961).

(6) H. A. Scheraga, *J. Phys. Chem.*, **65**, 1071 (1961).

(7) G. Némethy, I. Z. Steinberg and H. A. Scheraga, *Biopolymers*, **1**, 43 (1963).

(8) J. Hermans, Jr., J. W. Donovan and H. A. Scheraga, *J. Biol. Chem.*, **235**, 91 (1960); this is paper I in this series.

(9) R. G. Bates, "Electrometric pH Determinations, John Wiley and Sons, Inc., New York, N. Y., 1954, p. 87.

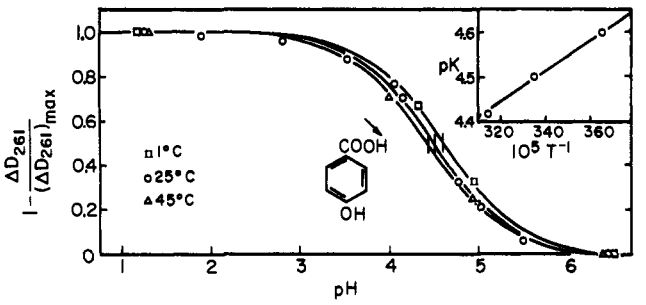

Fig. 1.—The pH-dependent difference spectrum for the ionization of the carboxyl group (arrow) of p-hydroxybenzoic acid. The curves are theoretical ones of the form of eq. 1. The inset is a plot of pK *vs.* $1/T$.

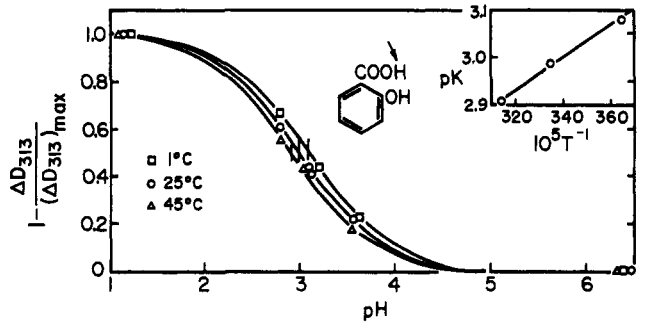

Fig. 2.—Same as Fig. 1, but for the carboxyl group of salicylic acid.

Stock solutions in water of the compounds to be investigated were first prepared. These were then diluted with appropriate buffer solutions. Concentrations of the final solutions varied from 1×10^{-4} to 6×10^{-4} M. Readings on the spectrophotometer were than made (using 1-cm. quartz cells) at the chosen temperature. Directly after this the pH of each solution was measured at the same temperature.

The following buffers were used: citrate, acetate, phosphate, tris-(hydroxymethyl)-aminomethane (TRIS), carbonate and borate. The concentration of the buffer was always 0.01 M in the final solutions.

The same pH-dependence of ΔD of salicylic acid solutions at high pH (at 25° and 1°) was observed at ionic strengths of 0.1 and 1. The measurements on the esters are in the absence of any added salt, with the exception of the buffer; the other measurements are on solutions containing 0.1 mole of KCl per liter.

Some difficulty was encountered with the esters, which hydrolyze in aqueous solution. The following method was, therefore, adopted: Stock solutions were prepared with absolute ethanol as the solvent. One ml. of these was then diluted to 100 ml. with buffered solution at the temperature at which the spectrophotometric measurement was made, thereby diluting out the alcohol. By keeping the esters in alcohol, and minimizing the time between the dilution with water and the spectrophotometric measurements, it was possible to reduce hydrolysis to less than 5% during the course of a measurement at 25° at extremely high pH. At lower pH values, where the rate of hydrolysis is smaller, this effect was negligible. At 0° no hydrolysis was detected at any pH; however, at 45° there was considerable error due to hydrolysis, and such data were not used for the determination of ΔH^0.

Results

The values of the apparent pK (*i.e.*, at finite ionic strength; see Introduction) were determined from the relation[8]

$$pK = p\mathrm{H} - \log \frac{x}{1 - x} \qquad (1)$$

at constant wave length and temperature where $x = \Delta D/\Delta D_{max}$, ΔD is the value of the optical density of one solution measured with respect to another at lower pH (where $x \sim 0$), and ΔD_{max} is the value of ΔD for a solution at a high pH (where $x \sim 1$). A plot of the experimental data for x *vs.* pH is fitted with a theoretical curve of the form of eq. 1, thereby determining the pK.

If ΔD_{max} is not determinable directly, *e.g.*, if a sufficiently high pH (to make $x \rightarrow 1$) or low pH (to make $x \rightarrow 0$) cannot be attained, then the data can be treated by an alternative method. Differentiation of eq. 1 gives

$$(dx/dp\mathrm{H})_{x=1/2} = (\ln 10)/4 = 0.576 \qquad (2)$$

If data are available at least up to the inflection point of the titration curve (*i.e.*, where $x = {}^1/_2$ and $d\Delta D/dp\mathrm{H}$ has its maximum value) then ΔD_{max} is determinable,[10] with the aid of eq. 2 in the form

$$\Delta D_{max} = (d\Delta D/dp\mathrm{H})_{max}/0.576 \qquad (3)$$

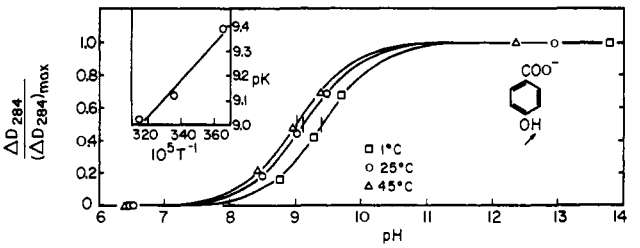

Fig. 3.—Same as Fig. 1, but for the hydroxyl group of p-hydroxybenzoic acid.

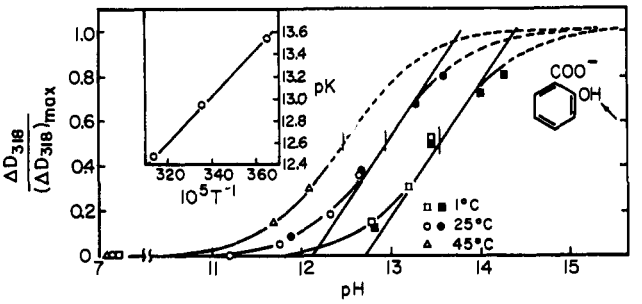

Fig. 4.—Same as Fig. 1, but for the hydroxyl group of salicylic acid. The solid and open symbols correspond to ionic strengths of 1.0 and 0.1, respectively. The straight lines are the experimentally determined maximum slopes.

TABLE I

SPECTROPHOTOMETRIC DATA AT 25°

Compound	Molarity × 10^4	Ionizing group	Reference pH[a]	Measured[b] pH	ΔD^c_{max}	λ_{max}, mμ
p-Hydroxybenzoic acid	5.8	COOH	6.40	1.19	0.414	261
	2.9	OH	6.40	12.94	.510	284
Salicylic acid	1.28	COOH	6.30	1.12	.180	313
	1.28	OH	7.10	...	.240[d]	318
Ethyl p-hydroxybenzoate	1.10	OH	6.10	11.96	.275	295
Ethyl salicylate	1.10	OH	7.10	11.99	589	335

[a] At this pH the degree of dissociation of a carboxyl group is essentially unity, and that of the phenolic hydroxyl group is essentially zero for each compound. [b] To obtain ΔD_{max}. [c] All values of ΔD_{max} are positive with the neutral solution as the reference. [d] Obtained by use of eq. 3. The same value was obtained from the data at 0°; hence ΔD_{max} at 45° was assumed also to be 0.240.

Once ΔD_{max} is determined in this manner, then a theoretical x *vs.* pH curve can be computed. This procedure was used in the case of the ionization of the hydroxyl group of salicylic acid. In all other cases, ΔD_{max} could be obtained directly from experimental data.

The values of ΔH^0 were computed by means of the van't Hoff equation

$$-\Delta H^0 = R\, d \ln K/d(1/T) \qquad (4)$$

Spectrophotometric data at 25° for the various ionizing groups are given in Table I. Corresponding data

(10) L. J. Reed and J. Berkson, *J. Phys. Chem.*, **33**, 760 (1929).

TABLE II

APPARENT pK'S OF IONIZING GROUPS[a]

Compound	COOH group: Diff. spec.	COOH group: Potentiometric titration	COOH group: Lit.	OH group: Diff. spec.	OH group: Potentiometric titration	OH group: Lit.
C_6H_5COOH		4.22 (0.5°, 25°)	4.20–4.23 (25°)[c,d]			
C_6H_5OH					10.24 (0.5°)	
					9.86 (25°)	9.81–10.01 (25°)[d]
p-OHC_6H_4COOH	4.60 (1°)	4.59 (0.5°)		9.39 (1°)	9.45 (0.5°)	
	4.50 (25°)	4.48 (25°)	4.54–4.62 (25°)[e,f]	9.11 (25°)	9.09 (25°)	9.13 (30°)–9.39 (25°)[f,g]
	4.42 (45°)			9.02 (45°)		
o-OHC_6H_4COOH	3.08 (1°)	3.07 (0.5°)		13.55 (1°)	13.12 (0.5°)[b]	
	2.99 (25°)	2.83 (25°)	2.82–2.98 (25°)[e,f,h]	12.95 (25°)	12.62 (25°)[b]	12.4 (30°)–13.4 (25°)[g,i]
	2.91 (45°)			12.48 (45°)		
p-$OHC_6H_4COOC_2H_5$				8.44 (1°)		
				8.34 (25°)		
o-$OHC_6H_4COOC_2H_5$				10.34 (1°)		
				9.92 (25°)		
				9.65 (45°)		

[a] See text for ionic strength. The temperature (in °C.) is indicated in parentheses. [b] Accuracy in doubt. [c] C. K. Rule and V. K. La Mer, *J. Am. Chem. Soc.*, **60**, 1981 (1938). [d] H. C. Brown, D. H. McDaniel and O. Häfliger, in "Determination of Organic Structures by Physical Methods," Ed. E. A. Braude and F. C. Nachod, Academic Press Inc., New York, N. Y., 1955, p. 567. [e] J. F. J. Dippy, *Chem. Rev.*, **25**, 151 (1939). [f] B. Jones and J. C. Speakman, *J. Chem. Soc.*, 19 (1944). [g] C. T. Abichandani and S. K. K. Jatkar, *J. Indian Inst. Sci.*, **21A**, 417 (1938). [h] B. Hok, *Svensk Kem. Tidskr.*, **65**, 182 (1953). [i] N. Konopik and O. Leberl, *Monatsh.*, **80**, 420 (1949).

TABLE III

STANDARD FREE ENERGIES, ENTHALPIES AND ENTROPIES OF IONIZATION AT 25° FROM DATA[a,b] OF TABLE II

Compound	COOH group: ΔF^0	COOH group: ΔH^0	COOH group: ΔS^0	OH group: ΔF^0	OH group: ΔH^0	OH group: ΔS^0
	(kcal./mole)		e.u.	(kcal./mole)		e.u.
C_6H_5COOH	5.7	0.0 (0.1)[c,d,e]	−19.1			
C_6H_5OH				13.3	5.8 (5.4, 5.6)[f,g]	−25.1
p-OHC_6H_4COOH	6.2	1.6 (0.4)[c,d]	−15.4	12.6	3.4	−30.8
o-OHC_6H_4COOH	4.1	1.5	− 8.7	17.9	10.5	−24.8
p-$OHC_6H_4COOC_2H_5$				11.5	1.5	−33.5
o-$OHC_6H_4COOC_2H_5$				13.7	7.1	−22.1

[a] All data are from spectrophotometric titrations except those for benzoic acid and phenol which are from potentiometric titrations. [b] Literature values are in parentheses. [c] G. Briegleb and A. Bieber, *Z. Elektrochem.*, **55**, 250 (1951). [d] T. L. Cottrell, G. W. Drake, D. L. Levi, K. J. Tully and J. H. Wolfenden, *J. Chem. Soc.*, 1016 (1948). [e] E. J. Cohn and J. T. Edsall, in "Proteins, Amino Acids and Peptides as Ions and Dipolar Ions," Reinhold Publishing Corp., New York, N. Y., 1943. [f] H. M. Papee, W. J. Canady, T. W. Zawidski and K. J. Laidler, *Trans. Faraday Soc.*, **55**, 1734 (1959). [g] E. H. Binns, *ibid.*, **55**, 1900 (1959).

at 0° and 45° are only slightly different, and are omitted from the table. The spectrophotometric titration curves, and the corresponding theoretical curves are shown in Fig. 1–6; in Fig. 3–6 the data are plotted as x *vs.* pH, but in Fig. 1 and 2 they are plotted as $(1 - x)$ *vs.* pH. The values of the pK's obtained from the spectrophotometric and potentiometric titration curves are shown in Table II, together with data found in the literature.

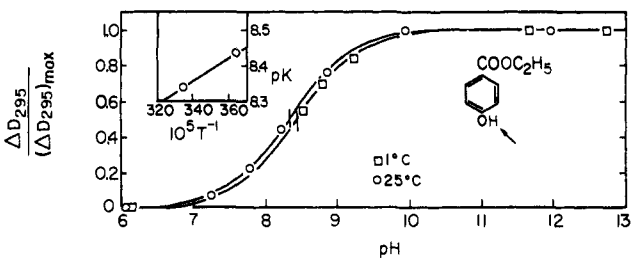

Fig. 5.—Same as Fig. 1, but for the hydroxyl group of ethyl p-hydroxybenzoate.

The insets in Fig. 1–6 show plots of pK *vs.* $1/T$. The slopes of these lines were used to calculate values of ΔH^0 according to eq. 4. Standard free energies and entropies of ionization were obtained from the equations

$$\Delta F^0 = -RT \ln K \quad (5)$$

$$\Delta F^0 = \Delta H^0 - T\Delta S^0 \quad (6)$$

The thermodynamic parameters are given in Table III; the data for benzoic acid and phenol were derived from potentiometric titrations, all others from spectrophotometric titrations. Data for ΔH^0 for the COOH group of o- and p-hydroxybenzoic acids, derived from potentiometric titrations (but not shown in Table III), are in agreement with those obtained from the spectrophotometric titrations.

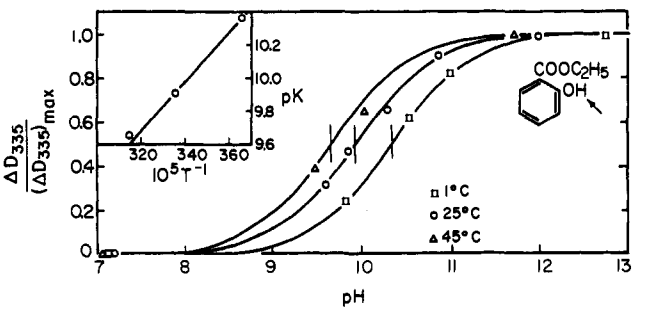

Fig. 6.—Same as Fig. 1, but for the hydroxyl group of ethyl salicylate.

Accuracy of the Data.—The reported pK values are estimated to be accurate[8] to within ±0.02 pK unit, and the reported ΔH values to within at worst ±1 kcal./mole. The high pK's of salicylic acid are probably accurate within the larger range ±0.04 because of the particular way ΔD_{max} had to be obtained from the experimental data and because of the increased unreliability of the pH measurements at high pH. Since much of the error in the high pK's of salicylic acid derived from these causes is systematic and of the same

magnitude at each temperature, the error in ΔH is not increased correspondingly and may be put at ± 1.5 kcal./mole.

Anticipating the treatment these data will receive in the discussion, the following may be said of the values obtained there. Since the first step performed is a subtraction of the ionization data for two compounds, the systematic error in the values obtained is eliminated to a large extent, so that the error in these *differences* in ΔF values is ± 0.1 kcal./mole, and in the *differences* in ΔH values is ± 1 kcal./mole. The steps of the analysis performed after this initial subtraction are based on assumptions which are justifiable; however, it is not possible to evaluate the magnitude of the error thereby introduced.

Discussion

The discussion is directed toward two questions: (1) what information can be obtained from the data of Table III about hydrogen bonds in salicylic acid and ethyl salicylate, and (2) how applicable are these results to side-chain hydrogen bonds in proteins?

Reactivity of Substituents.—Before discussing the effect of hydrogen bonding on ionization, we must recognize that the compounds investigated here are aromatic and contain groups whose ionization is affected by substituents on the benzene ring.[11-13] The resulting pK can be expressed by the equation

$$pK = pK^0 - \rho\sigma \qquad (7)$$

where K is the observed ionization constant, K^0 is that for the unsubstituted compound, ρ is a constant characteristic of the ionizing group, and σ is a constant characteristic of the substituent group. Recently it has been possible to split the $\rho\sigma$ term into contributions from various polar and resonance effects.[13] However, the effect of hydrogen bonding has not heretofore been included quantitatively as a specific part of the $\rho\sigma$ term. We shall thus attempt to evaluate the contributions of several polar and resonance effects[13] to $\rho\sigma$, and regard the remainder as being due to hydrogen bonding. Even this simplified approach cannot be rigorously followed, since enough data[13] are not available. Therefore, we shall indicate the additional assumptions which have to be made in order to obtain the heat of formation of the hydrogen bond in salicylic acid.

The effects responsible for altering the reactivity are[13]: resonance effect, steric resonance effect, steric effect, resonance polar effect, and polar effect. In order to evaluate these effects we shall compare the *ortho* and *para* compounds. In general, most of the above effects are similar in *o*- and *p*-substituted compounds and will, therefore, be eliminated by subtracting the thermodynamic parameters for the *p*-compound from those of the *o*-compound.[13,14] In particular, this is true of the first effect mentioned; the second and third effects are absent since there is no steric hindrance between the hydroxyl and carboxyl (or ethylcarboxy) groups.[13] The resonance polar effect will be partly eliminated in the subtraction of data for *p*- and *o*-compounds; we shall *assume* that it does not play a role here. We are, therefore, left with the polar effect, which is due to charge and dipole interactions, and the hydrogen bonding effect whose magnitude we wish to calculate.

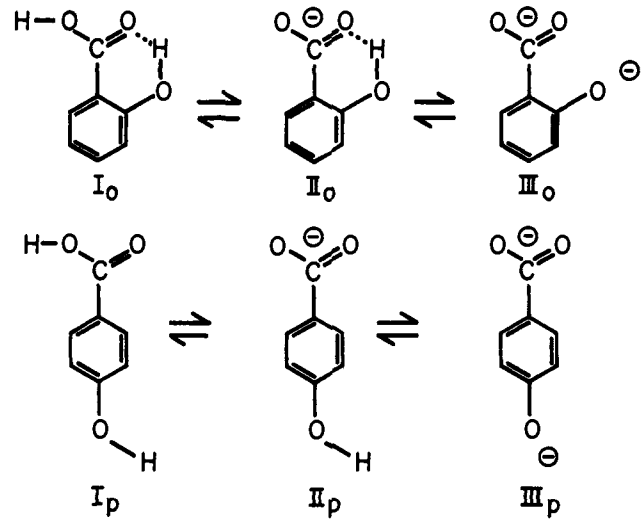

Fig. 7.—Ionic forms of *o*- and *p*-OHC_6H_4COOH molecules in solution.

The polar effect alone is too small to account for the very large difference in pK between salicylic and *p*-hydroxybenzoic acid. This can be seen by considering the pK's of *o*- and *p*-phthalic[15] and *o*- and *p*-aminobenzoic[16] acids. Although the substituent effects are large, as indicated by the considerable differences in pK's between the substituted and unsubstituted compounds, the observed *differences* between the *o*- and *p*-compounds are small compared to those observed for salicylic acid.

The following considerations will allow us to express the above argument in mathematical form. The ionization equilibria in the *o*- and *p*-hydroxybenzoic acids are represented in Fig. 7. The hydrogen bond in form II_0 is usually included to account for the pK's of salicylic acid. The inclusion of a hydrogen bond in form I_0 will be justified below.[17] In any event, it is reasonable to expect that the hydrogen bond in form II_0 will be more stable than that in form I_0 because of electrostatic interactions in form II_0. Obviously, there is no intramolecular hydrogen bond in any of the other forms in Fig. 7. In order to represent the thermodynamic parameters for formation of the hydrogen bonds, we shall use the subscripts 1, 2, 3, 4 to represent the OH, COO^-, COOH and $COOC_2H_5$ groups, respectively; thus ΔH^0_{12} and ΔS^0_{12} would be the standard enthalpy and entropy, respectively, of *formation* of a hydrogen bond between OH and COO^- groups.

The polar effect arises from electrostatic interactions between charged groups or between a charged group and a dipole. It is included in the ionizations we are discussing, as can be seen from a consideration of the cyclic process: $II_p \rightarrow II_0 \rightarrow III_0 \rightarrow III_p \rightarrow II_p$ for which the over-all enthalpy change is zero. The enthalpy change for the process $II_p \rightarrow II_0$ may be taken as the enthalpy of formation of a hydrogen bond, and will be equated with ΔH^0_{12}.[19] Therefore

(11) L. P. Hammett, "Physical Organic Chemistry," McGraw-Hill Book Co., Inc., New York, N. Y., 1940, p. 184.

(12) H. H. Jaffé, *Chem. Rev.*, **53**, 191 (1953).

(13) R. W. Taft, in M. S. Newman, "Steric Effects in Organic Chemistry," John Wiley and Sons, Inc., New York, N. Y., 1956, p. 556.

(14) It is of interest to note that the application of eq. 7 to the *p*-compounds (using ρ and σ values from ref. 12) yields the following values of pK: 4.58 for the COOH group of *p*-hydroxybenzoic acid (exptl. 4.50), 8.43 for the OH group of ethyl *p*-hydroxybenzoate (exptl. 8.34). This agreement is within the limits of applicability of eq. 7. A value of σ for a *p*-COO^- group was not available to us. From the experimental pK data we compute a value of $\sigma = 0.3$, which is a reasonable one.[12] Therefore, these *p*-compounds do not exhibit any abnormal behavior and should be good references with which to compare the corresponding *o* compounds.

(15) R. Kuhn and A. Wassermann, *Helv. Chim. Acta*, **11**, 44 (1928).

(16) A. M. Liquori and A. Ripamonti, *Gazz. chim. ital.*, **85**, 578 (1955).

(17) A form containing an OCOH...OH instead of an OH...OCOH hydrogen bond was not considered, since comparison of the pK's of *o*- and *p*-methoxybenzoic acid (4.09 and 4.49[18]) shows that the analogous OCOH ...OCH_3 bond has little stability.

(18) W. Ostwald, *Z. physik. Chem.*, **3**, 266 (1889); J. F. J. Dippy and R. H. Lewis, *J. Chem. Soc.*, 1426 (1932); J. F. J. Dippy and F. R. Williams, *ibid.*, 1888 (1934); E. E. Seeger and V. E. Bower, *J. Research Natl. Bur. Std.*, **64A**, 351 (1960).

(19) This procedure is valid only if essentially every molecule in form II_0 contains the hydrogen bond, *i.e.*, if K_{12} is very large.[20] In this particular

$$\Delta H^0_{12} = \Delta H^0_{(II_p \rightarrow III_p)} + \Delta H^0_{(III_p \rightarrow III_0)} - \Delta H^0_{(II_0 \rightarrow III_0)} \quad (8)$$

Similarly, for ΔS^0_{12} and ΔF^0_{12}. The first and third terms on the right-hand side of eq. 8 are standard enthalpies of ionization which are known. The second term represents a polar effect, that of the repulsion between the charges on the O^- and COO^- groups in form III_0 minus that of the smaller, similar repulsion in form III_p. The charge–dipole interaction in form II_0 is part of the hydrogen bond energy. An equation analogous to 8 can be written for ΔH^0_{14}, using the ionization data for the salicylic ester and the corresponding *p*-compound. For simplicity, we shall represent the enthalpy term due to the polar effect, $\Delta H^0_{(III_p \rightarrow III_0)}$, by $(\Delta H^0)'$. A similar term for the salicylic ester, arising from the electrostatic interaction between the O^- group and the $COOC_2H_5$ dipole, will be designated $(\Delta H^0)''$.

Equation 8 (and a corresponding one for the salicylic ester) and similar equations for the entropy changes reduce to [21]

$$\Delta H^0_{12} = -7.1 + (\Delta H^0)'; \quad \Delta S^0_{12} = -6.0 + (\Delta S^0)' \quad (9)$$

$$\Delta H^0_{14} = -5.6 + (\Delta H^0)''; \quad \Delta S^0_{14} = -11.4 + (\Delta S^0)'' \quad (10)$$

Up to this point we have refrained from considering the transformation $I_p \rightarrow I_0$, since it was uncertain whether or not form I_0 contains a hydrogen bond, even though the bond does exist in crystalline salicylic acid.[22] In the following analysis of our data we shall demonstrate that it does exist in aqueous solution, and obtain expressions for ΔH^0_{13} and ΔS^0_{13} analogous to eq. 9 and 10. Let us first *assume* that form I_0 *has no* hydrogen bond. By equating the thermodynamic parameters for the cyclic process $I_0 \rightarrow I_p \rightarrow II_p \rightarrow II_0 \rightarrow I_0$ to zero and expressing $\Delta H^0_{(II_p \rightarrow II_0)}$, etc., as ΔH^0_{12}, etc., we have

$$\Delta H^0_{12} = -0.1 + \Delta H^0_{(I_p \rightarrow I_0)}; \quad \Delta S^0_{12} = +6.7 + \Delta S^0_{(I_p \rightarrow I_0)} \quad (11)$$

Since the terms arising frm the polar effect, *i.e.*, $(\Delta H^0)'$, etc., and $\Delta H^0_{(I_p \rightarrow I_0)}$, etc. are small compared with those resulting from hydrogen bonding (see above), eq. 9 and 11 are incompatible. Therefore, we reject the assumption that the hydrogen bond does not exist in form I_0 and make the alternative assumption that it does, and that we may replace $\Delta H^0_{(I_p \rightarrow I_0)}$ by ΔH^0_{13}, the enthalpy of formation of a hydrogen bond; similarly for the entropy. With this assumption we obtain instead of eq. 11

$$\Delta H^0_{13} = \Delta H^0_{12} + 0.1; \quad \Delta S^0_{13} = \Delta S^0_{12} - 6.7 \quad (12)$$

and, substituting the values of ΔH^0_{12} and ΔS^0_{12} of eq. 9

$$\Delta H^0_{13} = -7.0 + (\Delta H^0)'; \quad \Delta S^0_{13} = -12.7 + (\Delta S^0)' \quad (13)$$

These expressions are in much better agreement with those of eq. 9 and 10, in spite of the fact that eq. 9, 10 and 13 refer to three different hydrogen bonds.

Finally, consideration of the standard free energies of formation at 25° shows that the hydrogen bond in form I_0 is weaker than that in form II_0.

$$\Delta F^0_{12} = -5.3 \text{ kcal./mole} + (\Delta F^0)' \quad (14)$$

$$\Delta F^0_{13} = -3.2 \text{ kcal./mole} + (\Delta F^0)' \quad (15)$$

Further

$$\Delta F^0_{14} = -2.2 \text{ kcal./mole} + (\Delta F^0)'' \quad (16)$$

Polar Effect.—To obtain the numerical values of the thermodynamic parameters for the formation of the three hydrogen bonds under consideration, we must obtain estimates of the unknown primed terms in eq. 9, 10, and 13–16. Some information follows from the fact that it is quite reasonable to expect that the OH...COOH and OH...$COOC_2H_5$ hydrogen bonds should have similar properties, or that

$$\Delta H^0_{13} = \Delta H^0_{14} \text{ and } \Delta S^0_{13} = \Delta S^0_{14} \quad (17)$$

Therefore, it follows from eq. 10 and 13 and from eq. 15 and 16, that

$$(\Delta H^0)' = (\Delta H^0)'' + 1.4 \quad (18)$$

$$(\Delta S^0)' = (\Delta S^0)'' + 1.3 \quad (19)$$

$$(\Delta F^0)' = (\Delta F^0)'' + 1.0 \quad (20)$$

Equation 20 implies that it is more difficult to bring two charged groups together (in form III_0) than it is to bring a charged group and a dipole together (in the analog of form III_0 for the ester). While this is a reasonable conclusion (following from the assumption in eq. 17), the assumption in eq. 17 still does not permit us to evaluate $(\Delta H^0)'$ and $(\Delta H^0)''$. Furthermore, there is no way in which additional independent information relating the unknowns can be obtained from our ionization data.

In principle, $(\Delta F^0)'$ and $(\Delta F^0)''$ can be obtained by applying the theory of electrostatics if the size, shape and charge distribution of the molecule are sufficiently well known. If the two charges in each of forms III_0 and III_p are considered as point charges it can be seen from a consideration of the structure of these molecules[22] that these must be localized at distances r_1 and r_2 of about 3 Å. and 7.7 Å., respectively, from each other.

If these charges were separated by water alone, one would have

$$(\Delta F^0)' = \frac{e^2N}{D_1 r_1} - \frac{e^2N}{D_2 r_2} \quad (21)$$

where e is the charge of the electron, N is the Avogadro number, $D_1 = D_2 = 80$, the dielectric constant of water. Thus, one obtains

$$(\Delta F^0)' = 0.9 \text{ kcal./mole} \quad (22)$$

As a matter of fact, this is a lower limit, since the two charges are in each case placed in a "cavity" of low dielectric constant ($D \approx 2$), surrounded by water.[23] This means that the effective dielectric constants which should be introduced in eq. 21 may be considerably smaller than 80, and we must, therefore, write

$$(\Delta F^0)' \geq 0.9 \text{ kcal./mole} \quad (23)$$

The charge–dipole interaction represented by $(\Delta F^0)''$ can be subjected to a similar treatment. Assuming values of r, the distance between charge and dipole, of again 3 and 7.7 Å., a value of zero for the angle between the vector r and the vector of the dipole in both molecules and a value of 3×10^{-18} e.s.u. cm. for the OH dipole moment μ, and using the equation

$$(\Delta F^0)'' \geq \frac{\mu e N}{D_1 r_1^2} - \frac{\mu e N}{D_2 r_2^2} \quad (24)$$

one obtains

$$(\Delta F^0)'' \geq 0.3 \text{ kcal./mole} \quad (25)$$

The theory of Kirkwood and Westheimer[23] allows one to compute the effective dielectric constant which is to replace that of water in eq. 21 and the corresponding one for the charge–dipole interaction. Unfortunately, these calculations are restricted by the following: (a) Unless the molecule has considerable symmetry no closed expression for ΔF^0 is available. While the molecule of *p*-OH benzoic acid probably meets one of these symmetry conditions, that of salicylic acid does not. (b) The calculated free energies are extremely sensitive to small changes in the geometry of the model, and thus cannot be predicted accurately.

case $\Delta F^0_{12} = 12.6 - 17.9 = -5.3$ kcal./mole; therefore $K_{12} \gg 1$. This same type of inequality holds for the other hydrogen bonds considered here.

(20) M. Laskowski, Jr., and H. A. Scheraga, *J. Am. Chem. Soc.*, **76**, 6305 (1954).

(21) Numerical values of $\Delta H''$ and $\Delta F''$ are in kcal./mole, of $\Delta S''$ in e.u. = cal./deg.. mole.

(22) W. Cochran, *Acta Cryst.*, **6**, 260 (1953).

(23) J. G. Kirkwood and F. H. Westheimer, *J. Chem. Phys.*, **6**, 506, 513 (1938).

In order to obtain estimates of $(\Delta F^0)'$ and $(\Delta F^0)''$ and hence of ΔF^0_{12}, etc., in spite of these restrictions, we shall make some additional assumptions which seem reasonable. We shall let

$$(\Delta F^0)' = 3(\Delta F^0)'' \quad (26)$$

a relation holding for their minimum values (see eq. 23 and 25). With this relation[24] and eq. 20 we obtain

$$(\Delta F^0)' = 1.5 \text{ kcal./mole}; \ (\Delta F^0)'' = 0.5 \text{ kcal./mole} \quad (27)$$

values not much higher than the minimum ones. Also, it would not appear unreasonable that the ratio $(\Delta H^0)'/(\Delta H^0)''$ is also about 3.

Hydrogen Bond Strength.—Substituting the values for $(\Delta F^0)'$ and $(\Delta F^0)''$, and those for $(\Delta H^0)'$ and $(\Delta H^0)''$, and $(\Delta S^0)'$ and $(\Delta S^0)''$, obtained analogously, into eq. 9, 10 and 13–16, one obtains for the free energies of formation of the hydrogen bonds

$$\Delta F^0_{12} = -3.8 \text{ kcal./mole} \quad (28)$$

$$\Delta F^0_{13} = \Delta F^0_{14} = -1.7 \text{ kcal./mole} \quad (29)$$

and for the corresponding enthalpies and entropies

$$\Delta H^0_{12} = -5.0 \text{ kcal./mole}; \ \Delta S^0_{12} = -4.0 \text{ e.u.} \quad (30)$$

$$\Delta H^0_{13} = \Delta H^0_{14} = -4.9 \text{ kcal./mole}; \ \Delta S^0_{13} = \Delta S^0_{14} = -10.7 \text{ e.u.} \quad (31)$$

The values of ΔH^0 from eq. 30 and 31 are in the range previously quoted.[4]

Applicability as a Model for Proteins.—The side-chain hydrogen bonds of protein molecules will differ, of course, from those in salicylic acid. The analogous bond in proteins, that between tyrosyl and carboxyl side chains, does not involve the rigidity which exists in salicylic acid. In fact, the entropy of the hydrogen bonded groups is much lower than that of the non-hydrogen bonded ones (in the protein) because of the loss of rotational freedom about the single bonds in the side chains.[20] Therefore, the tyrosyl–carboxyl hydrogen bond in proteins will be much weaker than that in salicylic acid.

However, if rigidity (in the protein) can be achieved by other means, *e.g.*, from coöperative hydrophobic bonding,[6,7,25] then the only difference between the hydrogen bond in salicylic acid and in the protein would be the presence of the two substituents (OH and COOH) on the same benzene ring in salicylic acid but on different groups in the protein. This difference should be a minor one, and the hydrogen bonding and polar effects should be similar in both the protein and in salicylic acid. In such a case we should expect the thermodynamic parameters for the hydrogen bond in the protein to be similar to those found for salicylic acid. Also, the OH group should be hydrogen bonded to the carboxyl group in both the COOH and COO^- forms. Such a carboxyl group would have a pK lower than its normal value of 4.6, due to a polar effect (repulsion of H–ion by neighboring OH–group), but a standard enthalpy of ionization close to zero. Such a situation has been observed for ribonuclease.[5] In this protein, ionization of the abnormally strong carboxyl group ($pK \sim 2.5$, heat of ionization ~ 0 kcal./mole) is accompanied by a shift in the tyrosyl ultraviolet absorption spectrum, indicating the close proximity of this carboxyl to a tyrosyl group.

On the other hand the difference between ΔS^0_{12} and ΔS^0_{13} (eq. 30 and 31) would appear to make the OH· · · COOH bond in a protein sufficiently less stable than an OH· · ·COO^- one so that its occurrence in the absence of the rigidity provided by a non-polar environment is unlikely.

Conclusion.—While the analysis of the data presented here involves some assumptions, it is reasonable to conclude that the results obtained indeed represent the characteristics of the hydrogen bonds in these model compounds. The approach is a general one and should be valuable in treating similar model compounds.

While it is difficult to evaluate the errors in the computed thermodynamic parameters, as pointed out in the Results section, it is of interest that the enthalpy of hydrogen bond formation in salicylic acid (ΔH^0_{12}) is the same as that in the salicylic ester (ΔH^0_{14}). It may be noted that rather high values of ΔH^0_{12}, ΔH^0_{13} and ΔH^0_{14} are obtained. While the value of salicylic acid might *possibly* be in error because of the high pK involved, ΔH^0_{14} (for the ester) cannot contain such an error, since the pK ($\sim$10) is in the range where accurate data can be obtained.

The high values of ΔH^0 obtained for salicylic acid and its ester reflect some aspects of the specific local environment[7] of the particular donor and acceptor groups and the aromatic ring. From this point of view, the donor and acceptor groups of essentially every model compound will have their own specific environments, and corresponding ΔH^0 values. Therefore, it may be difficult to intercompare ΔH^0 values from different compounds. Nevertheless, the possibility of side-chain hydrogen bonding in proteins in aqueous solution is rendered plausible because of the ΔH^0 values obtained here.

(24) The results would not be much different if we assumed $(\Delta F^0)'/(\Delta F^0)''$ equal to 2 or to 4.

(25) H. A. Scheraga, "Protein Structure," Academic Press, Inc., New York, N. Y., 1961, p. 42.

[CONTRIBUTION FROM THE LAWRENCE RADIATION LABORATORY, UNIVERSITY OF CALIFORNIA, BERKELEY, CALIF.]

Isotopic Studies on the Radiation Decomposition of Crystalline Choline Chloride[1,2]

BY RICHARD M. LEMMON AND MARGARET A. SMITH

RECEIVED NOVEMBER 26, 1962

Carbon-14- and deuterium-labeled choline chlorides were used to study the mechanism of the radiolysis of crystalline choline chloride. These studies demonstrate that, during the highly-sensitive radiation decomposition: (1) the carbinol group of the ethanol moiety becomes the aldehyde group of the resultant acetaldehyde, (2) no hydrogens are transferred to or from the trimethylamino group, (3) the hydrogens of the ethanol moiety are highly mobile, and (4) intermolecular hydrogen transfers take place.

This paper is part of a continuing study of the extraordinary radiation sensitivity of crystalline choline chloride, $[(CH_3)_3NCH_2CH_2OH]^+Cl^-$, a compound that decomposes with G-values as high as 55,000.[3–5]

(1) The work described in this paper was sponsored by the U. S. Atomic Energy Commission.

(2) Presented before the 141st National Meeting of the American Chemical Society, Washington, D. C., March, 1962.

Earlier work had indicated the possibility of a symmetrical intermediate between choline chloride and one

(3) R. O. Lindblom, R. M. Lemmon and M. Calvin, *J. Am. Chem. Soc.*, **83**, 2484 (1961).

(4) R. M. Lemmon, P. K. Gordon, M. A. Parsons and F. Mazzetti, *ibid.*, **80**, 2730 (1958).

(5) R. M. Lemmon, M. A. Parsons and D. M. Chin, *ibid.*, **77**, 4139 (1955).