Figure 7. Location of disulfide bonds in chymotrypsinogen.[45]

bonds per chain) and pepsinogen (three disulfide bonds) are not reduced substantially when the disulfide bonds remain intact. The intrinsic viscosity of serum albumin also remains quite high when one considers the fact that it contains 17 disulfide bonds per chain. A question arises perhaps in the case of chymotrypsinogen (five disulfide bonds), for which $[\eta] = 11.0$ cc/g when disulfide bonds are intact, compared to the value of 26.8 cc/g given in Table I. The locations of the disulfide bonds of this protein are shown in Figure 7.

Additional evidence on this question is provided by optical rotatory dispension data, to be presented in a subsequent paper. These data suggest that structured beads do not exist in any of the proteins we have investigated. The effect of the presence of the disulfide bonds on the rotation is quite small, and preliminary examination of the data suggests that the effect for each protein (including chymotrypsinogen) consists simply of a fixed change in molar rotation for each disulfide bond broken.

It should be noted, on the other hand, that synthetic polypeptides such as polyleucine and polyphenylalanine retain structured regions in 6 M GuHCl, at least at room temperature.[48,49] Structured regions may therefore be expected in proteins, too, if long segments of a polypeptide chain consist entirely or predominantly of highly hydrophobic amino acid residues.

(48) H. E. Auer and P. Doty, *Biochemistry*, **5**, 1716 (1966).
(49) H. J. Sage and G. D. Fasman, *ibid.*, **5**, 286 (1966).

Acid–Base Titrations in Concentrated Guanidine Hydrochloride. Dissociation Constants of the Guanidinium Ion and of Some Amino Acids[1]

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Abstract: This paper shows that concentrated guanidine hydrochloride is a suitable medium for acid–base titrations, not significantly different from concentrated solutions of alkali metal chlorides, except at high pH, where the dissociation of the guanidinium ion becomes appreciable. The pK for this dissociation, in 6 M solution, was found to be 13.74 at 25°. The pK's of a number of amino acids have been determined in 6 M guanidine hydrochloride and were found to be quite close to the corresponding pK values in dilute salt solutions. The results indicate in particular that intramolecular electrostatic interactions, between charged groups on amino acid molecules, are not greatly affected by the presence of high concentrations of guanidine hydrochloride.

It has recently been suggested in a paper from this laboratory[2] that proteins dissolved in concentrated guanidine hydrochloride (GuHCl)[3] solutions lose their characteristic native structure and assume a random conformation similar to that which simple organic polymers usually possess in solution. A detailed study of several proteins under these conditions is in progress, and some of the initial results are reported in papers which accompany this one.[4]

It is of special interest to investigate the acid–base properties of proteins in this solvent medium, as the complex interactions which introduce anomalies into the hydrogen ion equilibria of native proteins[5,6] should

disappear. However, as only fragmentary information exists on the suitability of concentrated GuHCl solutions as a medium for acid–base titrations,[7] a preliminary investigation with simpler acids and bases appeared desirable. This paper reports the results of such an investigation. The results prove interesting for their own sake, as an example of acid–base equilibria in concentrated salt solutions, quite apart from their subsequent use in the interpretation of protein titration curves.

Experimental Section

Guanidine Hydrochloride. The GuHCl used in this study was prepared from commercial guanidine carbonate (Eastman). The carbonate was first recrystallized from chilled 60% aqueous ethanol, and converted to the chloride by addition of 20% HCl to a lasting pH 4. The mixture was flash evaporated at 40° until crystals began to form. It was then cooled, and the crystals were filtered off. The crystals were dissolved in hot absolute alcohol and

(2) C. Tanford, K. Kawahara, and S. Lapanje, *J. Biol. Chem.*, **241**, 1921 (1966).
(3) The abbreviations GuH⁺ and Gu will be used to designate the guanidinium ion and the uncharged guanidine molecule, respectively.
(4) C. Tanford, K. Kawahara, and S. Lapanje, *J. Am. Chem. Soc.*, **89**, 729 (1967); Y. Nozaki and C. Tanford, *ibid.*, **89**, 742 (1967).
(5) C. Tanford, *Advan. Protein Chem.*, **17**, 69 (1962).
(6) J. Steinhardt and S. Beychok, *Proteins*, **2**, 140 (1964).

(7) J. W. Donovan, M. Laskowski, Jr., and H. A. Scheraga, *J. Mol. Biol.*, **1**, 293 (1959).

recrystallized by addition of benzene. A second recrystallization was performed, either from methanol or water.[8]

Standard Acid and Base. Standard HCl solutions (0.1, 0.5, and 1.0 M) were prepared from constant-boiling HCl according to the procedure of Foulk and Hollingsworth.[9] Standard KOH solutions (0.1 and 0.5 M) were prepared by a modification of the procedure of Powell and Miller.[10] A 1 M solution of KOH was prepared by flash evaporation of a 0.5 M solution under nitrogen gas.

Other Reagents. All other reagents were the best available commercial products. Tryptophan and histidine were recrystallized before use.

Sample solutions were prepared by weighing out reagents and making up to desired volume in a volumetric flask. An alternative procedure is to calculate the volume from the densities of GuHCl solutions reported elsewhere.[11] Refractive index measurement[12] provides a usable technique for determining the concentration of GuHCl solutions containing no other reagents.

Electrometric Titration. A Radiometer PHM 4 pH meter was used, in conjunction with a Radiometer glass electrode (G202B) or a Beckman glass electrode (40498). The calomel reference electrode was a home-made one of the free junction type. Potassium acid phthalate was used as pH standard.

Acid and base were added by means of Agla syringe burets with a capacity of 0.5 ml. The buret tip, the electrodes, and a nitrogen inlet were all fitted into a jacketed vessel with a capacity of about 20 ml. The actual volume of solution used for titration was about 3 ml. Solutions were stirred, sometimes continuously and sometimes intermittently, by means of a magnetic stirrer.

All measurements were made at $25.00 \pm 0.05°$, with nitrogen passing over the solution. The nitrogen was first passed through a solution of approximately the same composition as the solution to be titrated, so as to give it about the same vapor pressure of water.

Spectrophotometric Titration. The spectrophotometric titration of tyrosine was carried out in a Cary 15 spectrophotometer. A difference spectral technique was used, employing four cells. The sample beam passes through two cells, one containing sample plus added base, the other containing solvent alone plus KCl solution (so as to dilute the GuHCl to the same extent as the added base does). In the reference beam, the KCl solution was added to the cell containing the sample and the base to the cell containing solvent alone. The peak of the difference spectrum was at 295 mμ and the absorbance at this wavelength was measured as a function of pH. The temperature of the measurements was $25.0 \pm 1.0°$.

Results and Discussion

Purity and Stability of GuHCl. Commercial preparations of GuHCl generally contain impurities. As many as seven detectable impurities were found in one sample by Fridovich.[13] One of them (a potent inhibitor of the enzyme xanthine oxidase) was identified as ammeline. Ammeline and two other substituted triazines have been reported as impurities by Engelbrecht, et al.[14] Since substances of this kind contain titratable groups, and since they have intense ultraviolet absorption bands,[15] it was important to show that GuHCl, as prepared by the procedure given above, is relatively free of such impurities.

The absence of detectable titratable impurities was demonstrated by direct titration of GuHCl. These experiments led to consistent values of activity coefficients for H^+ and OH^- ions, which were independent of pH, as will be shown below. In addition, we carried out careful titrations of a number of substances (at concentra-

(8) A detailed procedure may be obtained by writing to the authors.

(9) C. W. Foulk and M. Hollingsworth, *J. Am. Chem. Soc.*, **45**, 1270 (1923).

(10) J. Powell and M. Miller, *J. Chem. Educ.*, **34**, 330 (1957).

(11) K. Kawahara and C. Tanford, *J. Biol. Chem.*, **241**, 3228 (1966).

(12) W. Kielley and W. F. Harrington, *Biochim. Biophys. Acta*, **41**, 401 (1960).

(13) I. Fridovich, *Biochemistry*, **4**, 1098 (1965).

(14) R. M. Engelbrecht, H. E. Mosely, W. P. Donahoo, and W. R. Rolingson, *Anal. Chem.*, **29**, 579 (1957).

(15) R. C. Hirt and D. J. Salley, *J. Chem. Phys.*, **21**, 1181 (1953); R. C. Hirt and R. G. Schmitt, *Spectrochim. Acta*, **12**, 127 (1958).

tions of the order of 0.01–0.03 M) in 6 M GuHCl. Values of the apparent pK were calculated from all parts of the resulting titration curves, from α (degree of dissociation) = 0.020 to α = 0.980. The observed pK values were found to be identical to within ± 0.01 in all such experiments. The presence of less than 5×10^{-5} M titratable impurity, with a pK lying within ± 2 pK units of the pK of the substance being titrated, would have been readily detected by deviations in the observed pK with changing α.

Titrations of acetic acid, glutamic acid, histidine, glycine, lysine, and tyrosine were used in this way to demonstrate the absence (to a level close to 10^{-5} M) of impurities with pK values ranging from about 1.5 to 11.5.

The absence of detectable amounts of substituted triazines and other absorbing impurities was demonstrated spectrophotometrically; 6 M GuHCl, prepared as described above, was found to absorb strongly only below 225 mμ, below which wavelength the absorbance rises extremely steeply. The absorbance at 225 mμ is about 0.1, and it falls off slowly with increasing wavelength. Most of the absorbance above 225 mμ presumably represents the tail of the GuHCl absorption bands, and part of it is undoubtedly due to Cl^- ions, as concentrated solutions of HCl and NaCl also show some absorbance between 225 and 300 mμ. It should be noted that melamine and other contaminants which have been identified in commercial GuHCl have absorption maxima at 225 mμ or above, with molar absorptivities of 10,000 or greater.[15] Even if all the observed absorbance at 225 mμ and above were due to impurities, the maximum possible concentration of any of these substances in 6 M GuHCl would be about 10^{-5} M. Actual concentrations must be much lower.

It was found that concentrated GuHCl solutions could be kept without detectable change for at least several days at neutral pH. At high alkaline pH, however, where the concentration of the uncharged base, guanidine, becomes significant (see below), the slow formation of an impurity was observed. The absorption spectrum of this substance showed a sharp peak at 232 mμ, and it was formed at a constant rate over a period of several days, indicating that it was a product of GuHCl itself. The substance was found to have little or no absorbance at low pH: the disappearance of absorbance corresponded to a pK of about 3.3. Both the absorption maximum and the pK suggest that the substance being formed may be biguanide which has been reported[15] as having little absorbance as a doubly charged positive ion and an absorption peak at 230 mμ (molar absorbancy 12,400) as a singly charged positive ion. The reported pK for conversion from one form to the other is 3.2.

In one solution of 6 M GuHCl, at pH 11.5, appearance of absorbance at 232 mμ was followed for 3 days. The rate dA_{232}/dt was found to be about 0.009/hr. If the substance being formed is indeed biguanide, this corresponds to a rate of formation of 7×10^{-7} mole/l. hr.

Apparent Activity Coefficient of H$^+$ Ions. Apparent activity coefficients of hydrogen ions were determined by measurement of the pH of solutions of HCl in concentrated GuHCl. The apparent activity coefficient (γ'_H) is defined conventionally[16] as the ratio of the

(16) C. Tanford, in "Electrochemistry in Biology and Medicine," T. Shedlovsky, Ed., John Wiley and Sons Inc., New York, N. Y., 1955, Chapter 13.
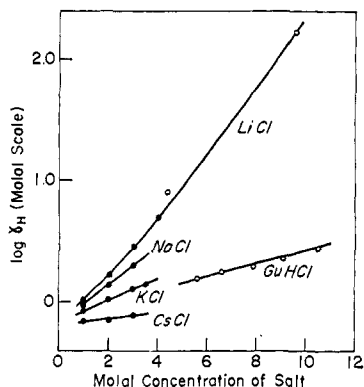
Figure 1. Apparent activity coefficients of hydrogen ions in solutions of GuHCl and other chlorides, at 25°. Points shown as filled circles are based on $\gamma_\pm$ values determined in cells without liquid junction, with assumptions given in the text. Open circles are based on pH measurements with the glass electrode, assuming pH = $-\log C_H\gamma_H$.

apparent activity (antilog of $-$pH) to the stoichiometric concentration of added HCl (C^0_{HCl}), i.e.

$$pH = -\log C^0_{HCl} - \log \gamma'_H \quad (1)$$

The activity and activity coefficient are designated as "apparent" because they differ from the hydrogen ion activity and activity coefficient based on measurements in cells without liquid junction. The data were determined on the basis of molar concentrations (i.e., C^0_{HCl} in eq 1 represents the molar concentration of HCl), because molar concentration units are generally used in the experimental study of protein titration curves. In order to compare the log γ'_H values obtained with literature data for other concentrated solutions, it is, however, desirable to have log γ'_H values on a molal scale (i.e., where C^0_{HCl} in eq 1 represents the molal concentration of HCl). To make the conversion, we have assumed that the small amount of HCl present has a negligible effect on the density of the solutions, and have calculated molal concentrations from the densities of appropriate solutions of GuHCl alone.[11] Apparent activity coefficients on both scales are summarized in Table I. Measurements were made on HCl

Table I. Apparent Activity Coefficients of H$^+$ Ions in Aqueous Guanidine Hydrochloride at 25°

| —GuHCl concn[a]— | | —Log γ'_H— | |
M	m	M scale	m scale
4.0	5.6	0.34	0.19
4.5	6.6	0.42	0.25
5.0	7.8	0.49	0.29
5.5	9.1	0.58	0.36
6.0	10.5	0.70	0.45

[a] Results are independent of pH, at least down to pH 0.7, which represents the lower limit of measurement.

solutions ranging from 0.001 to 0.06 M. No effect of the HCl concentration on γ'_H could be detected. This is the expected result since γ'_H depends on interactions with all other ions, and the concentration of ions resulting from the presence of HCl is a negligibly small fraction of the total.

It is of interest to compare the behavior of HCl in GuHCl solutions with its behavior in other salt solutions. To make this comparison we have assumed that the concentration of free H$^+$ ions is equal to C^0_{HCl}. (There would seem to be no possible way in which GuHCl could act as a base.) We have also assumed that the contribution of the liquid junction potential between the GuHCl solution and the KCl solution of the calomel electrode is negligibly small. This assumption should be valid within the precision of the comparison we are making, which is certainly no better than ± 0.02. The contribution of the liquid junction potential to log γ'_H in 0.15 M KCl is of the order of 0.02,[16] and the contribution should diminish as the ionic strength increases, since liquid junction potentials generally decrease with increasing ionic strength. The foregoing assumptions are equivalent to assuming that γ'_H is essentially the same as γ_H, the activity coefficient of hydrogen ions defined in the conventional way.

For purposes of comparison, we have used tabulated values of $\gamma_\pm$ for HCl in concentrated salt solutions, which are available in the literature.[17] To estimate γ_H from these data, we have assumed that the activity coefficient of Cl$^-$ (γ_{Cl}) depends only on the total molality of salt, and that it is equal to the activity coefficient of K$^+$ (γ_K) at the same molality. Using $\gamma_\pm$ values for KCl, and setting $\gamma_\pm = \gamma_{Cl}$, we thus obtain γ_{Cl} values applicable to HCl solutions. Setting 2 log $\gamma_\pm$ for HCl solutions equal to log γ_H + log γ_{Cl} then enables us to calculate γ_H. These assumptions clearly represent an approximation.

Some idea of the validity of the foregoing assumptions can be obtained with the aid of pH measurements of HCl in concentrated LiCl, made by Rosenthal and Dwyer.[18] The procedure used by these workers is essentially the same as we have used for HCl in GuHCl, and log γ'_H values on the molar and molal scales can be computed from their data. At somewhat lower concentrations of LiCl, $\gamma_\pm$ values from precise emf measurements in cells without liquid junction are available,[17] and they can be used to calculate log γ_H values as outlined above. Both calculations are shown in Figure 1, and the agreement between them is seen to be very satisfactory.

Examination of all the data of Figure 1 shows that the behavior of HCl in GuHCl is comparable to the behavior of HCl in solutions of the chlorides of the alkali metals. The data in GuHCl appear to be roughly intermediate between comparable data for KCl and CsCl.

Apparent Activity Coefficient of OH$^-$ Ions. The apparent activity coefficient of hydroxyl ions is defined, conventionally,[16] in a manner similar to that used for hydrogen ions. We first define an apparent activity of hydroxyl ions from the relation $a'_{OH} = K^0_w/a'_H$, where K^0_w is the ion product $a_H a_{OH}$ for water at unit activity. Where pOH = $-\log a'_{OH}$ we define γ'_{OH} by the relation

$$pOH = pK^0_w - pH = -\log C^0_{KOH} - \log \gamma'_{OH} \quad (2)$$

and determine log γ'_{OH} from pH measurements on solutions of KOH (at concentration C^0_{KOH}) in concentrated

(17) H. S. Harned and B. B. Owen, "The Physical Chemistry of Electrolytic Solutions," Reinhold Publishing Corp., New York, N. Y., 1950.

(18) D. Rosenthal and J. S. Dwyer, J. Phys. Chem., 66, 2687 (1962).

GuHCl. The results of such measurements are shown in Table II. They are strikingly different from the data for log γ'_H given in Table I. Whereas log γ'_H is positive, log γ'_{OH} is negative. When we take into account the fact that the data represent logarithms of the activity coefficient, the difference is really quite large. Whereas γ'_H is considerably greater than unity, γ'_{OH} < 0.1. The major reason for this difference is the removal of OH⁻ ions from solution by combination with GuH⁺ ions to form the free base Gu, as will be shown below.

Table II. Apparent Activity Coefficients of OH⁻ Ions in Aqueous Guanidine Hydrochloride at 25° [a]

| —GuHCl concn— | | Log γ'_{OH} | |
M	m	M scale	m scale
5.0	7.8	−0.975	−1.165
5.5	9.1	−1.015	−1.230
6.0	10.5	−1.045	−1.290

[a] These data, like those of Table I, are independent of pH.

It should be observed that the definition of log γ'_{OH} given above is chosen for a practical purpose, to permit ready calculation of the free concentration of OH⁻ ions from pH measurements. With this definition, however, it cannot be assumed that γ'_{OH} is essentially equal to γ_{OH}, the activity coefficient of hydroxyl ions as normally defined, whereas it could be assumed that γ'_H as defined by eq 1 is essentially equal to γ_H.

To obtain γ_{OH} values from our data with the same degree of approximation as was used to obtain γ_H, i.e., neglecting effects of the liquid junction potential and setting pH = −log a_H, but making no other assumptions, we must use the correct expression[19] for the dissociation constant of water in eq 2, i.e.

$$K^0_w = a_H a_{OH}/a_{H_2O} \qquad (3)$$

where a_{H_2O} is the activity of water in the concentrated GuHCl solution. We must also take into consideration the fact that the GuH⁺ ion is an acid, which may be converted to the free base, guanidine, at high pH, with the accompanying removal of OH⁻ from the solution. As a result, the free concentration of OH⁻ ions cannot be considered to be the same as the added concentration of KOH.

To calculate the effect of the dissociation of GuH⁺ on the concentration of hydroxyl ions we define the dissociation constant as

$$K'_G = a_H C_{Gu}/C_{GuH^+} \qquad (4)$$

Since formation of each molecule of Gu removes one OH⁻ ion, the true concentration of OH⁻ ions, C_{OH}, will be $C_{OH} = C^0_{KOH} - C_{Gu}$. Combining these relations, and setting $a_{OH} = \gamma_{OH}C_{OH}$, we obtain

$$C^0_{KOH}/C_{OH} = 1 + K'_G C_{GuH^+} \gamma_{OH}/K^0_w a_{H_2O} \qquad (5)$$

Thus the apparent activity coefficient defined by eq 2 is given by

$$\gamma'_{OH} = \gamma_{OH}C_{OH}/a_{H_2O}C^0_{KOH} \qquad (6)$$

(19) R. A. Robinson and R. H. Stokes, "Electrolyte Solutions," Butterworth and Co., London, 1955.
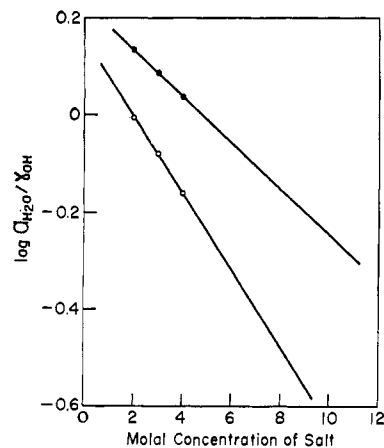
Figure 2. The value of log a_{H_2O}/γ_{OH} in concentrated GuHCl, estimated for use in eq 7 (Table III). The upper line gives an estimate based on an assumed equivalence between GuHCl and KCl, the lower line is based on equivalence between GuHCl and CsCl.

or, by combination with eq 5

$$\log \gamma'_{OH} = -\log\left(\frac{a_{H_2O}}{\gamma_{OH}} + \frac{K'_G C_{GuH^+}}{K^0_w}\right) \qquad (7)$$

Equation 7 is rigorously true, except for neglect of the liquid junction potential.

Equation 7 shows that the large negative values of log γ'_{OH} could be due to very small values of γ_{OH}. However, activity coefficients of ions in concentrated salt solutions are generally greater rather than less than unity, as are the γ_H values shown in Figure 1. With a_{H_2O} < 1, the first term in brackets should therefore be <1. On the other hand, K'_G is known[20] to be of order 10⁻¹⁴, i.e., it has about the same magnitude as K^0_w, so that the second term is likely to be the predominant one when C_{GuH^+} is large, as is true here. Thus log γ'_{OH} is likely to be a reflection of the weakly acidic properties of GuH⁺ and can be used to determine a value for K'_G.

The Acid Dissociation Constant of GuH⁺. In order to estimate the dissociation constant of GuH⁺ by means of eq 7, it is necessary to estimate values for the term a_{H_2O}/γ_{OH}. We have done this by assuming that this factor, in GuHCl, will have the same order of magnitude as the values of this same factor in KCl and in CsCl, basing this assumption on the behavior of log γ_H shown in Figure 1. The values of a_{H_2O} were calculated from the osmotic coefficients of these salts,[19] and the values of γ_{OH} from values of $\gamma_\pm$ for KOH in KCl and for CsOH in CsCl, as given by Harned and Owen.[17] We made assumptions similar to those used in estimating γ_H, namely that $\gamma_K = \gamma_{Cl}$ in KCl solutions, which leads at once to a value for γ_K, and hence for γ_{OH} in KOH–KCl solutions. We also assumed that γ_{Cl} in CsCl solutions has the same value as in KCl solutions of the same molality. This permits evaluation of γ_{Cs} from $\gamma_\pm$ for CsCl, and hence, of γ_{OH} in CsOH–CsCl solutions.

The results, given in Figure 2, show that there is considerable difference between the ratio a_{H_2O}/γ_{OH} as determined from the two sets of data, but this is not important, for the important conclusion is that this

(20) N. F. Hall and M. R. Sprinkle, *J. Am. Chem. Soc.*, **54**, 3470 (1932).
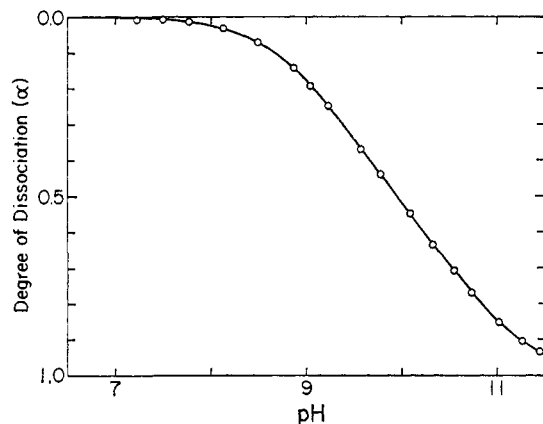
Figure 3. Electrometric titration of DL-lysine in 6 M GuHCl at 25°. The value of α is based on the titration of *two* groups in the pH range covered. The curve is a theoretical curve, according to eq 8, with $pK_2 = 9.29$ and $pK_3 = 10.63$.

ratio is small (as predicted), so that its contribution to the right-hand side of eq 7 is almost negligible. As a matter of fact, we could have used a_{H_2O}/γ_{OH} values based on $\gamma_{\pm}$ values for NaOH in NaCl and still have obtained pK'_G values which differ by only 0.02 from those actually calculated in Table III.

Table III. Calculation of pK'_G at 25° [a]

GuHCl concn, M	a_{H_2O}/γ_{OH}, M units		K'_G/K^0_w	
	KCl[b]	CsCl[b]	KCl[b]	CsCl[b]
5.0	0.46	0.24	1.80	1.84
5.5	0.37	0.18	1.81	1.85
6.0	0.30	0.14	1.80	1.82
			Av	1.82
			pK^0_w	13.996[c]
			pK'_G	13.74

[a] From eq 7. [b] In the columns headed "KCl" a_{H_2O}/γ_{OH} is based on $\gamma_{\pm}$ for KOH in KCl, in the columns headed "CsCl" it is based on $\gamma_{\pm}$ for CsOH in CsCl. [c] Reference 19.

The calculation of pK'_G from these data is shown in Table III. The ratio K'_G/K^0_w is seen to be virtually independent of the choice of sources for a_{H_2O}/γ_{OH}. It is also seen that the same value is obtained at all three concentrations of GuHCl. The small variation in log γ'_{OH} seen in Table II is compensated for by the variation in a_{H_2O}/γ_{OH}. The resulting value of pK'_G, 13.74 at 25°, is very close to the value of 13.65 determined in dilute solutions of GuHCl by Hall and Sprinkle[20] more than 30 years ago.

Dissociation Constants of Amino Acids. The dissociation constants of several amino acids were determined, and the results are summarized in Table IV. The standard nomenclature is used: pK_1, pK_2, and pK_3 refer to the successive thermodynamic constants of the amino acids, in order of decreasing acidity. The constants pk_{12}, etc., used for tyrosine, are microscopic constants, as defined by Edsall, *et al.*[21] The constants determined in 6 M GuHCl are designated by primed symbols because they represent apparent equilibrium constants, in which the hydrogen ion activity is taken as the negative antilog of the pH, and the ratio of basic

(21) J. T. Edsall, R. B. Martin, and B. R. Hollingsworth, *Proc. Natl. Acad. Sci.*, **44**, 505 (1958).

Table IV. Dissociation Constants of Amino Acids at 25°

		pK' in 6 M GuHCl	In absence of GuHCl pK' $I =$ 0.10– 0.16[a]	pK^0 $I = 0$[b]
Glycine	pK_1	2.30	2.43[c]	2.35[c]
	pK_2	9.55	9.69[c]	9.78[c]
L-Tryptophan	pK_1	2.20	2.30[d]	
	pK_2	9.20	9.35[d]	
L-Glutamic acid	pK_1	2.13	2.30[e]	2.16[f]
	pK_2	4.12	4.28[e]	4.32[f]
L-Histidine	pK_2	6.22	6.05[g]	6.04[h]
DL-Lysine	pK_2	9.29	(9.03)[i]	
	pK_3	10.63	(10.57)[i]	
L-Tyrosine	pK_2	8.97	9.11[j]	9.19[j]
	pK_3	10.12	10.13[j]	10.47[j]
	pk_{12}	9.60	9.63[j]	9.71[j]
	pk_{13}	9.09	9.28[j]	9.35[j]
	pk_{123}	9.49	9.70[j]	9.95[j]
	pk_{132}	10.00	10.05[j]	10.31[j]
Reference Compounds				
Acetic acid		4.55[k,l]	4.65[m]	4.76[m]
Imidazole		7.18[k]	7.11[n]	
n-Butylamine		10.68[k]	10.70[o]	
Phenol		10.22[k]	9.85	9.98

[a] pK' refers to the apparent pK, as normally measured in a cell with liquid junction, as defined by eq 4 for example, in dilute aqueous salt solution, ionic strength 0.10 to 0.16, 25°, except that the data for lysine are at ionic strength 0.01. Where literature data are at a different temperature or ionic strength, appropriate corrections were made where data on which to base such corrections (*e.g.*, heats of ionization) were available. [b] pK^0 refers to the true thermodynamic pK, obtained by extrapolation of data to pure water. [c] Numerous literature data for glycine agree very closely with the values given here, which represent the data of E. J. King, *J. Am. Chem. Soc.*, **73**, 155 (1951). It should be noted that King's pK' values are based on the molality of H⁺ ions and have been corrected to the same basis used for the other constants of this table, in which the H⁺ ion activity is used. [d] A. Albert, *Biochem. J.*, **47**, 531 (1950); D. D. Perrin, *J. Chem. Soc.*, 3125 (1958); *ibid.*, 290 (1959). [e] R. F. Lumb and A. E. Martell, *J. Phys. Chem.*, **57**, 960 (1953). [f] A. Neuberger, *Biochem. J.*, **30**, 2085 (1936). [g] N. C. Li and R. A. Manning, *J. Am. Chem. Soc.*, **77**, 5225 (1955). [h] C. L. A. Schmidt, W. K. Appelman, and P. L. Kirk, *J. Biol. Chem.*, **88**, 285 (1930). [i] A. Albert, *Biochem. J.*, **50**, 690 (1952). Ionic strength 0.01. [j] References 21 and 22. [k] Reference 7. [l] We have determined this constant also, obtaining a value of 4.54. [m] Reference 23. [n] Several determinations are in close agreement. The value given is that of C. Tanford and M. L. Wagner, *J. Am. Chem. Soc.*, **75**, 434 (1953). [o] J. Bjerrum, *Chem. Rev.*, **46**, 381 (1950).

to acidic forms is expressed in concentration units rather than activity units, as in eq 4.

The thermodynamic dissociation constants listed in the table were determined by direct titration in 6 M GuHCl, at an amino acid concentration of 0.01–0.03 M, except in the case of tyrosine, for which limited solubility required use of a lower concentration (0.005 M was used). Typical data are those for lysine, shown in Figure 3. These data are replotted in Figure 4 in the form of a plot of pH $-$ log $[\alpha/(1 - \alpha)]$ *vs.* α, where α is the degree of dissociation calculated on the basis of two dissociable protons per molecule in the pH range being investigated. This function has the form (for lysine or tyrosine)

$$\text{pH} - \log \frac{\alpha}{1 - \alpha} = pK'_3 + \log \frac{1 + 2a_H/K'_2}{2 + a_H/K'_3} \quad (8)$$

where a_H is the negative antilog of the measured pH. At low pH (*i.e.*, $\alpha \rightarrow 0$) the right-hand side extrapolates
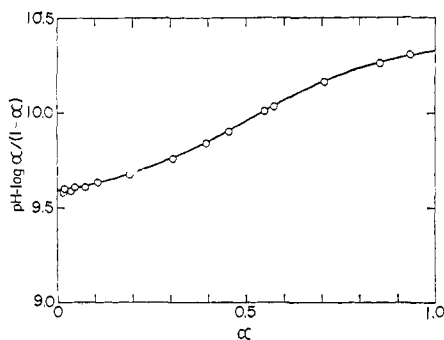
Figure 4. The data of Figure 3, plotted to show the apparent pK as a function of α. The curve is theoretical, based on eq 8, with pK_2 = 9.29 and pK_3 = 10.63.

to pK'_2 + log 2, at high pH (*i.e.*, $\alpha \rightarrow$ 1) it becomes equal to pK'_3 − log 2. Preliminary values of pK'_2 and pK'_3 were determined from visual extrapolation of the data of Figure 4 to α = 0 and α = 1, and they were refined by applying eq 8 to all the data in the figure.

The microscopic constants shown for tyrosine were obtained by combining a direct electrometric titration with a spectrophotometric titration following exactly the procedure of Edsall and co-workers.[21,22] The direct spectrophotometric data are shown in Figure 5, and Figure 6 shows a plot of pH − log [$\alpha/(1 − \alpha)$] *vs.* α, where α is the degree of dissociation of the phenolic group. This function has the form

$$\text{pH} - \log \frac{\alpha}{1 - \alpha} = - \log \frac{k_{12}a_\text{H} + k_{13}k_{132}}{a_\text{H} + k_{13}} \quad (9)$$

At low pH ($\alpha \rightarrow$ 0) it becomes equal to pk_{12}, k_{12} being the dissociation constant of the phenolic group when the α-amino group is in its acidic (charged) form. At high pH ($\alpha \rightarrow$ 1) it becomes equal to pk_{132}, k_{132} being the dissociation constant of the phenolic group when the α-amino group is in its basic (uncharged) form.

Table IV shows, in addition to the data obtained in the present study, the pK values of four model compounds in 6 M GuHCl, as determined by Donovan, *et al.*,[7] and literature values for all of the dissociation constants in aqueous solution in the absence of GuHCl. The pK' values in this case refer to salt solutions at an ionic strength in the range 0.10 to 0.16, except the data for lysine, which refer to an ionic strength of about 0.01. The pK^0 values represent true thermodynamic constants obtained by extrapolation of experimental data to infinite dilution in pure water.

The results of Table IV are of considerable interest. They show that experimental pK values determined in 6 M GuHCl are suprisingly close to similar pK values determined in dilute aqueous salt solutions. In many instances the differences between pK^0 and pK' in the absence of GuHCl (*i.e.*, the difference between ionic strength zero and ionic strength near 0.1) is greater than the difference between pK' values in 6 M GuHCl and in the salt solutions of moderate ionic strength.

It is especially noteworthy that high concentrations of GuHCl are quite ineffective in suppressing intramolecular electrostatic interactions between charges on the amino acid molecules. In the data for L-tyrosine, for instance, the difference between pk_{12} and pk_{132}

(22) R. B. Martin, J. T. Edsall, D. B. Wetlaufer, and B. R. Hollingsworth, *J. Biol. Chem.*, 233, 1429 (1958).
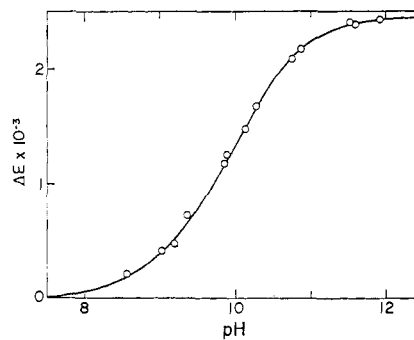
Figure 5. The dissociation of the phenolic group of tyrosine, in 6 M GuHCl at 25°, as measured by the change in absorbance ($\Delta\epsilon$) at 295 mμ. The degree of dissociation at any pH was calculated from these data as $\Delta\epsilon/2450$.
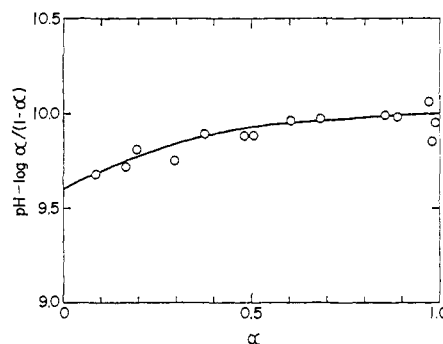

Figure 6. The data of Figure 5, plotted to show the apparent pK as a function of α. The curve is theoretical, based on eq 9, with pk_{12} = 9.60, pk_{13} = 9.09, pk_{132} = 10.00.

is a direct measure of the electrostatic interaction between the negatively charged phenolate group and the positively charged α-amino group. The difference is 0.60 at zero ionic strength, 0.48 at ionic strength 0.04, and 0.42 at ionic strength 0.16.[22] In 6 M GuHCl, the difference is 0.40, *i.e.*, virtually unaltered from the difference in 0.16 M salt.

Another example is provided by the interaction between the charges on the α-amino and α-carboxyl groups of glycine. One measure of this interaction is the difference between the pK_2 of glycine and the pK of the amino group of an ester of glycine. The pK of the latter is 7.70 in aqueous solution,[23] and ionic strength or the presence of GuHCl probably have little effect on this figure, to judge from the data for imidazole and butylamine. Assuming this pK to be invariant, the pK difference representative of the electrostatic interaction becomes 2.08 at ionic strength zero, 1.99 at ionic strength 0.1, and 1.85 in 6 M GuHCl.

The high concentration of GuHCl may be somewhat more effective in suppressing the interaction between the two amino groups of lysine. Since there is no significant difference between the pK_2 values for glycine and α-amino-*n*-butyric acid,[24] the difference in pK_2 between lysine and glycine may be taken as a measure of this interaction. The difference in pK'_2 (Table IV), based on measurements for lysine at an ionic strength of 0.01, is 0.66. To judge from tyrosine, where data

(23) J. T. Edsall and J. Wyman, "Biophysical Chemistry," Vol. 1, Academic Press Inc., New York, N. Y., 1958.
(24) E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides," Reinhold Publishing Corp., New York, N. Y., 1943, p 84.

are available at ionic strengths of 0.004 and 0.16, a substantial reduction in $\Delta pK'_2$ may be anticipated if it is based on a lysine pK'_2 at higher ionic strength. (The directly comparable pK for tyrosine is pk_{123}, which drops from 9.95 to 9.70, as the ionic strength is raised from zero to 0.16. An *increase* of similar magnitude in pK'_2 for lysine may be anticipated.) As experimental data for lysine are not available at higher ionic strength, we can only guess what the value of $\Delta pK'_2$ would be at the higher ionic strength. A reasonable guess is 0.40. The $\Delta pK'_2$ in 6 M GuHCl, which is only 0.26, thus may represent an appreciable further decrease. It should be noted, however, that the interaction between the amino groups is by no means abolished entirely. The $\Delta pK'_2$ of 0.26 in 6 M GuHCl is still close to half the $\Delta pK'_2$ value at ionic strength 0.01.

Both the ineffectiveness of GuHCl in suppressing the interactions between charges of oppositive sign in glycine and tyrosine, and its somewhat greater effectiveness in suppressing the interaction between charges of like sign in lysine, may be simple consequences of the fact that the salt ions which provide the source of the ionic strength are large in size, having dimensions comparable to the distance between charges on the amino acid molecules. If the ionic strength of concentrated GuHCl is to provide an effective shield between charges of opposite sign, it is necessary that both GuH^+ and Cl^- ions have a high probability of being simultaneously in close proximity to the interacting charges. Steric considerations may well make this probability vanishingly small. For GuHCl to decrease the interaction between the positive charges of lysine, on the other hand, it is required only that a single Cl^- ion have a high prob-

ability of being in close proximity to the charges. Steric repulsions will clearly be less effective in this case.

Conclusions. It can be concluded from the results of this paper that concentrated GuHCl is a suitable medium for acid–base titrations, which does not differ significantly from concentrated solutions of alkali metal chlorides, such as KCl or CsCl. It is however usable only up to a pH of about 11 or 11.5. Prohibitive amounts of base are required to attain higher pH's because of the dissociation of the GuH^+ ion to uncharged Gu, and further difficulties arise from the apparent instability of Gu under these conditions. The fragmentary data given earlier suggest that the product formed from Gu may be biguanide. If so, its formation would result from the condensation of Gu with GuH^+, and loss of NH_3. The NH_3 would presumably be removed in any titrimetric experiment by the nitrogen gas normally employed to keep alkaline solutions free from CO_2. The formation of biguanide would thus be accompanied by some irreversibility in the uptake of base, and evidence for such irreversibility has in fact been found in titration studies carried above pH 11.

Concentrated solutions of GuHCl have remarkably little effect on the pK's of amino acids. In particular, intramolecular electrostatic interactions, which one might expect to be substantially reduced in a solvent medium of ionic strength 6.0, are in fact almost as strong as in dilute salt solutions, especially when the interacting charges are of opposite sign. This finding suggests that GuHCl may not be able to suppress electrostatic interactions in proteins entirely, even when the protein molecules are completely unfolded to a randomly coiled state.

Proteins as Random Coils. II. Hydrogen Ion Titration Curve of Ribonuclease in 6 M Guanidine Hydrochloride[1]

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Abstract: The hydrogen ion titration curve of ribonuclease in 6 M guanidine hydrochloride has been determined. The results differ significantly from titration data in a similar medium reported by Cha and Scheraga. All 32 known titratable groups of the protein (except for perhaps three phenolic groups) appear to be titrated with the pK values to be expected for fully exposed groups, subject to no interactions of any kind. Even electrostatic interactions appear to be reduced to less than experimentally significant magnitude. The three phenolic groups excepted from this generalization are subject to very weak interactions, leading to pK values about 0.4 higher than expected. These results are compatible with the conclusion, reached on the basis of other data, that ribonuclease and other proteins, in 6 M guanidine hydrochloride, behave as randomly coiled polypeptide chains even when their disulfide bonds are intact. An anomalous uptake of about one hydroxyl ion per protein molecule was observed at high pH and shown to be due to the occurrence of a β-elimination reaction at cystine residues.

E vidence presented in two earlier papers[2,3] suggests that proteins dissolved in concentrated guanidine hydrochloride (GuHCl) lose essentially all elements of

their native structure, and exist as randomly coiled chains in which no important noncovalent interactions remain. Although this conclusion was based primarily on data obtained under conditions where disulfide bonds of the proteins were broken, viscosity and optical

(2) C. Tanford, K. Kawahara, and S. Lapanje, *J. Biol. Chem.,* 241, 1921 (1966).

(3) C. Tanford, K. Kawahara, and S. Lapanje, *J. Am. Chem. Soc.,* 89, 729 (1967).