Nuclear Magnetic Resonance Studies of the Acid–Base Chemistry of Amino Acids and Peptides. I. Microscopic Ionization Constants of Glutathione and Methylmercury-Complexed Glutathione

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Abstract: The extent of ionization of the two carboxylic acid groups, the sulfhydryl group, and the ammonium group of fully protonated glutathione was determined as a function of pH from the chemical shifts of selected carbon-bonded protons. The results indicate that the two carboxylic acid groups ionize simultaneously in the pH range 0.5 to 6.0 while the sulfhydryl group and the ammonium group ionize simultaneously in the pH range 7 to 12. From the pH dependence of the extent of ionization of the four acidic groups, eight microscopic ionization constants which describe the acid–base chemistry of glutathione at the molecular level were obtained. The L-glutamyl carboxylic acid group of fully protonated glutathione is 10.8 times as acidic as the glycyl carboxylic acid group, while the sulfhydryl group of doubly protonated glutathione is 1.57 times as acidic as the ammonium group. The four microscopic ionization constants which describe the ionization of the two carboxylic acid groups of the sulfur-coordinated methylmercury complex of glutathione and the ionization constant of the ammonium group were also determined from chemical shift measurements. The L-glutamyl carboxylic acid group of the fully protonated methylmercury complex is 10.7 times as acidic as the glycyl carboxylic acid group, while the ionization constant of the ammonium group is the same as the microscopic constant of the corresponding reaction of doubly protonated glutathione. A method is presented for evaluating the microscopic ionization constants of diprotic acids when the fractional ionization of each of the acidic groups undergoing ionization simultaneously can be determined as a function of pH.

The acid–base chemistry of amino acids and peptides is usually characterized in terms of macroscopic ionization constants obtained from pH titration data. When the acidities of the substituent groups are markedly different, the constants can be assigned to the individual groups. If, however, ionization occurs simultaneously at two or more groups, the macroscopic constants are a composite of the microscopic constants for ionization from the individual groups.[1] In such cases, it is not possible to describe the acid–base chemistry at the molecular level in terms of macroscopic constants.

Microscopic ionization constants have not been reported for the majority of amino acids and peptides, which contain two or more acidic groups of comparable acidity, presumably because the methods used in studying their acid–base chemistry generally do not monitor proton ionization at the molecular level. A detailed knowledge of the microscopic ionization of such amino acids and peptides is, however, often essential for an understanding of their chemistry. For example, in the determination of formation constants of metal complexes of these ligands, the microscopic ionization constants may be required when not all the groups which ionize simultaneously are involved in metal complexation. Of the amino acids having two substituent groups of comparable acidity, cysteine has been studied the most extensively. By using the macroscopic constants of cysteine in conjunction with the macroscopic constants of derivatives in which the sulfhydryl proton and the amino protons were replaced by alkyl groups, the microscopic constants were evaluated.[2,3] The assumption that the alkyl substituent has the same effect on the acidity of other protons as the proton it replaced limits the accuracy of microscopic constants determined by this method.[4] The microscopic ionization of cysteine has also been characterized by using the macroscopic constants in conjunction with the extent of ionization of the sulfhydryl proton as determined by ultraviolet absorption spectroscopy,[5–7] Raman spectroscopy,[8] and calorimetry.[9] Assumptions are necessary in each of these methods, and the reliability of the calorimetric results has recently been questioned.[7]

The sensitivity of the chemical shifts and spin–spin coupling constants of nonlabile carbon-bonded protons to the ionization of nearby acidic functional groups makes nuclear magnetic resonance (nmr) spectroscopy a direct method for studying acid–base chemistry at the molecular level.[10–18] When exchange of

(1) For a discussion of microscopic ionization constants, see: J. T. Edsall and J. Wyman, "Biophysical Chemistry," Academic Press, New York, N. Y., 1958, p 487.
(2) R. L. Ryklan and C. L. A. Schmidt, *Arch. Biochem.*, **5**, 89 (1944).
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(15) J. L. Sudmeier and C. N. Reilley, *Anal. Chem.*, **36**, 1698 (1964).
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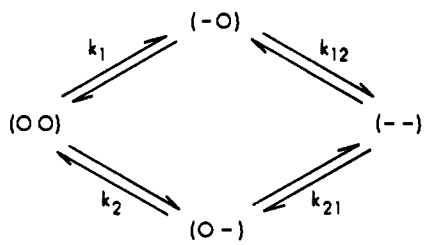
the acidic proton between protonated and ionized forms is rapid on the nmr time scale, the chemical shifts of the exchange-averaged resonances provide a measure of the fractional ionization.[10] Such data have previously been applied to the study of microscopic ionization schemes.[11,15,16,18] Shrager, *et al.*, in a study of the ionization of L-histidine and several L-histidine derivatives and dipeptides, described a curve-fitting procedure for determining ionization constants from nmr titration curves.[18]

In this paper, we describe a general method for evaluating microscopic ionization constants for diprotic acids from the fractional ionization of each of the acidic groups as a function of pH. The method is then applied to the tripeptide glutathione (γ-L-glutamyl-L-cysteinylglycine) and to the sulfur-coordinated methylmercury complex of glutathione. The microscopic ionization schemes of fully protonated glutathione and of methylmercury-complexed glutathione were elucidated by nmr. Ionization of each of the acidic substituent groups was monitored as a function of pH by using the averaged chemical shifts of selected carbon-bonded protons; the carbon-bonded protons were chosen so that, at a given pH, the chemical shift behavior of a given set of protons would reflect the ionization of a single acidic group. From these data, the microscopic ionization constants were evaluated.

Theoretical Section

In this section, a method is presented for evaluating microscopic ionization constants of diprotic acids from fractional ionization data. In its application to glutathione, the extent of ionization was determined from chemical shift measurements; the method is general, however, and can be used to evaluate microscopic constants when the extent of ionization is obtained by other means.

The microscopic ionization scheme of a diprotic acid can be represented schematically as



where the symbol on the left within a given set of parentheses indicates the state of protonation of acidic group 1 and the symbol on the right that of group 2; O represents a protonated group and − an ionized group. The ionization reaction to which a given equilibrium constant refers is indicated by the subscript; the last number in the subscript denotes the group involved in the ionization step under consideration while the preceding number denotes the group from which the proton has already ionized. The microscopic ionization constants are thus defined as

$$k_1 = \frac{[H^+][(-O)]}{[(OO)]} \quad (1)$$

$$k_2 = \frac{[H^+][(O-)]}{[(OO)]} \quad (2)$$

$$k_{12} = \frac{[H^+][(--)]}{[(-O)]} \quad (3)$$

$$k_{21} = \frac{[H^+][(--)]}{[(O-)]} \quad (4)$$

Let F_1 represent the fraction of molecules from which the proton of acidic group 1 has ionized, and F_2 the fraction of molecules from which the proton of acidic group 2 has ionized. Then

$$F_1 = \frac{[(-O)] + [(--)]}{[(OO)] + [(-O)] + [(O-)] + [(--)]} \quad (5)$$

and

$$F_2 = \frac{[(O-)] + [(--)]}{[(OO)] + [(-O)] + [(O-)] + [(--)]} \quad (6)$$

Substitution of eq 1–4 into eq 5 and 6 leads to

$$F_1 = \frac{k_1/k_2 + k_{21}/[H^+]}{[H^+]/k_2 + k_1/k_2 + 1 + k_{21}/[H^+]} \quad (7)$$

and

$$F_2 = \frac{1 + k_{21}/[H^+]}{[H^+]/k_2 + k_1/k_2 + 1 + k_{21}/[H^+]} \quad (8)$$

Combination of eq 7 and 8 followed by rearrangement yields

$$F_1/F_2 = ((1 - F_1/F_2)/[H^+])k_{21} + k_1/k_2 \quad (9)$$

Ionization constant k_{21} and the ratio k_1/k_2 thus can be obtained from the slope and intercept of a plot of F_1/F_2 *vs.* $(1 - F_1/F_2)/[H^+]$. Ionization constant k_2 can then be calculated from the extent of ionization of acidic group 2 as a function of pH with eq 10 by using the values determined above for k_{21} and the ratio k_1/k_2. Equation 10 was obtained by rearrangement of eq 8. Ionization

$$k_2 = \frac{F_2[H^+]}{1 + k_{21}/[H^+] - F_2k_1/k_2 - F_2 - F_2k_{21}/[H^+]} \quad (10)$$

constant k_1 is then calculated from the ratio k_1/k_2 and the value determined above for k_2, and finally k_{12} is calculated using the relation $k_{12} = k_2k_{21}/k_1$, the derivation of which is given in ref 1.

Experimental Section

Chemicals. Glutathione (Nutritional Biochemicals Corp.) was used as received. Methylmercuric hydroxide (Alfa Inorganics) was purified and the stock solution standardized by an nmr titration procedure described previously.[19] A stock solution of tetramethylammonium (TMA) nitrate was prepared by titration of a 25% aqueous solution of TMA hydroxide (Eastman Organic Chemicals) with HNO_3 to a neutral pH.

pH Measurements. All pH measurements were made with an Orion Model 801 pH meter equipped with a standard glass electrode and a fiber-junction, saturated calomel reference electrode. Saturated potassium acid tartrate, 0.050 *M* phosphate, and 0.01 *M* sodium tetraborate solutions, pH values 3.56, 7.00, and 9.18, were used to standardize the pH meter. pH measurements were converted to hydrogen ion concentrations[20] using activity coefficients calculated from the Davies equation.[21] p_cH will be used to represent the negative logarithm of the hydrogen ion concentration.

Nmr Measurements. Nmr spectra were obtained on a Varian

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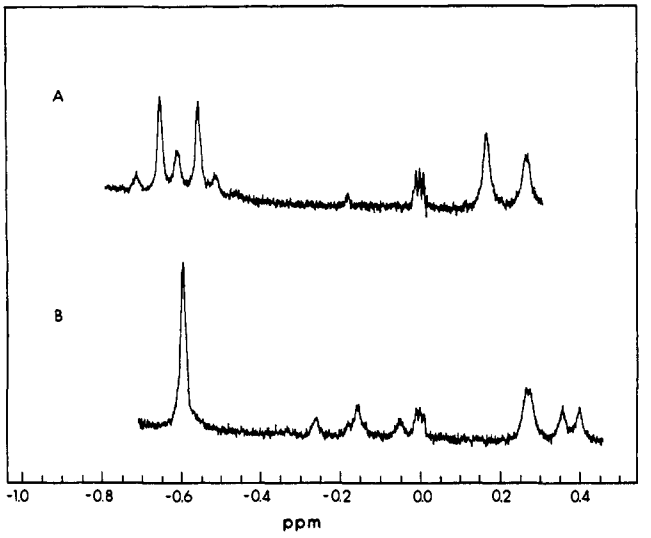


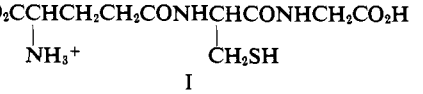
Figure 1. Proton nmr spectra (60-MHz) of the glycyl methylene, the L-cysteinyl methylene, and the L-glutamyl methine protons at (A) p_cH 5.60 and (B) p_cH 10.32. The triplet at 0.00 ppm is due to the protons of the reference TMA (0.15 *M* glutathione, $T = 25°$).

A-60-D high-resolution spectrometer at a probe temperature of $25 \pm 1°$.[22] Spectra were recorded at sweep rates of 0.2 Hz/sec.

Chemical shifts are reported in ppm relative to the central resonance of the TMA triplet; positive shifts indicate resonances upfield from TMA. The central resonance of TMA is 3.17 ppm downfield from the methyl resonance of sodium 3-(trimethylsilyl)-1-propanesulfonic acid (TMS*). Solutions used in the nmr measurements were prepared in distilled water from the requisite amounts of crystalline glutathione and methylmercuric hydroxide stock solution under a nitrogen atmosphere to minimize oxidation of the sulfhydryl group of glutathione. TMA nitrate was added to each solution at a concentration of approximately 0.01 *M* as a reference compound. The solutions at pH less than 6 were prepared from a 50-ml solution whose pH was adjusted to an initial value of ~0.5 with HNO_3. KOH was then added and samples were withdrawn at the appropriate pH values. The ionic strength of the solutions used in the evaluation of the microscopic ionization constants of the carboxylic acid groups varied from 0.31 to 0.42 *M* owing to changes in the concentrations of partially ionized forms of glutathione. Solutions at pH greater than 6 were prepared from a 50-ml solution whose initial pH was 4.7. KOH was added to increase the pH, and samples were withdrawn at the appropriate pH values. The ionic strength of the solutions used in the evaluation of the microscopic ionization constants of the sulfhydryl and ammonium groups varied from 0.20 to 0.55 *M*. In the calculation of hydrogen ion concentration from pH, the ionic strengths used were those calculated for the specific pH values; the concentrations of the partially ionized forms of glutathione used in the calculation of each ionic strength were calculated from the macroscopic ionization constants.[23]

Results

Ionization Scheme of Glutathione. The formula of fully protonated glutathione is



I

Ionization of the acidic protons was followed by observing the resonance patterns for the nonlabile methylene protons of the glycine and L-cysteine residues and the nonlabile methine proton of the L-glutamic acid residue, referred to hereafter as the A, B, and C protons, as a function of pH. The resonance pattern for the A protons consists of a doublet up to pH ~7.5 due

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(23) N. W. Pirie and K. G. Pinhey, *J. Biol. Chem.*, **84**, 321 (1929).

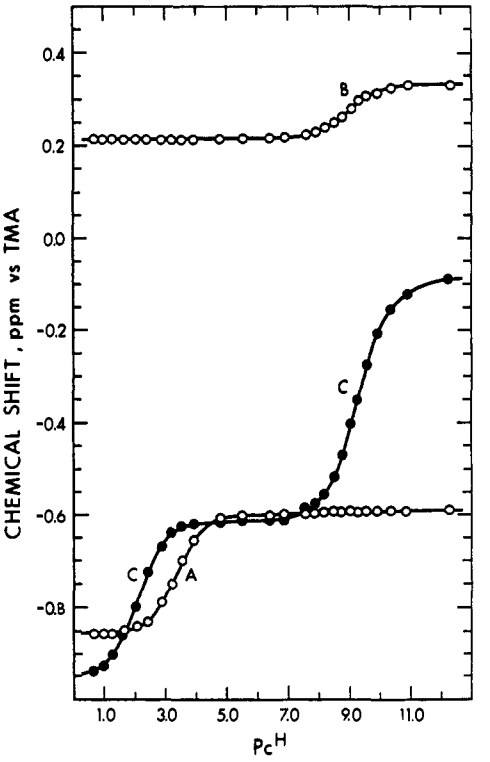

Figure 2. p_cH dependence of the chemical shifts of the (A) glycyl methylene protons, (B) L-cysteinyl methylene protons, and (C) L-glutamyl methine proton of glutathione. The curves connecting the points are theoretical curves predicted using the microscopic ionization constants in Table I (0.15 *M* glutathione, $T = 25°$).

to spin–spin coupling to the nitrogen-bonded proton of the glycyl peptide linkage; at higher pH's the doublet collapses to a single line due to the increased rate of peptide proton exchange.[24-26] The doublet centered at −0.603 ppm in spectrum A in Figure 1 and the singlet at −0.595 ppm in spectrum B are due to the glycyl methylene protons. The resonance pattern for the B protons consists of the A part of an approximately A_2X pattern up to pH ~7.5, as illustrated by the doublet centered at 0.219 ppm in spectrum A, while at higher pH values they give rise to the AB part of an ABX pattern as illustrated by the multiplet at 0.328 ppm in spectrum B. Not all lines in the multiplet were resolvable, and consequently a complete analysis of the spectrum was not attempted; rather the chemical shift at the center of the pattern was used as a measure of ionization of the sulfhydryl proton. The resonance pattern for the C proton consists of an approximately first-order triplet, as illustrated by the patterns at −0.608 ppm in spectrum A and −0.156 ppm in spectrum B, over the entire pH range studied in the present work; the chemical shift of the central resonance was used as a measure of ionization of the L-glutamyl carboxylic acid group and the ammonium group.

The p_cH dependence of the chemical shifts of the A, B, and C protons is shown by the experimental points through which the solid curves are drawn in Figure 2. At p_cH 0.5 glutathione is fully protonated. As the p_cH is increased from 0.5, the averaged resonance pattern for the C proton shifts upfield (indicated by curve C in

(24) A. Berger, A. Loewenstein, and S. Meiboom, *J. Amer. Chem. Soc.*, **81**, 62 (1959).
(25) M. Sheinblatt, *ibid.*, **88**, 2123 (1966).
(26) D. L. Rabenstein and S. Libich, *Inorg. Chem.*, 11, 2960 (1972).

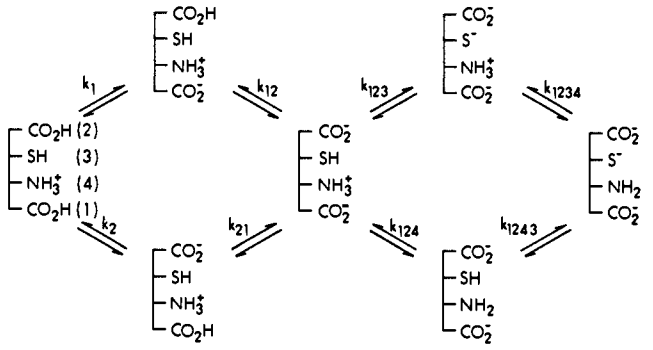

Figure 3. Microscopic ionization scheme for glutathione, considering only the predominant species present. See text for labeling of the acidic groups and definition of the subscripts.

Figure 2) while the chemical shifts of the other resonance patterns remain relatively constant, indicating the most acidic group of fully protonated glutathione to be the L-glutamyl carboxylic acid group. As the p_cH is increased further, the resonance patterns for the A and C protons both shift upfield in the p_cH range 2–4, indicating simultaneous ionization from the L-glutamyl and glycyl carboxylic acid groups. It is assumed that ionization of the protonated L-glutamyl ammonium group is negligible in this p_cH range, based on the acidity of the protonated amino group of L-glutamic acid,[1] while the lack of any shift in the resonance pattern for the B protons indicates no measurable ionization of the sulfhydryl group. As the p_cH is increased further, the resonance patterns for the B and C protons shift upfield starting at $p_cH \sim 7$, indicating simultaneous ionization of the L-cysteinyl sulfhydryl group and the L-glutamyl ammonium group. At $p_cH > 12$, ionization is complete as indicated by the constant chemical shifts of the B and C resonance patterns. At no p_cH in the range 0.5–12 is there any indication of ionization of the nitrogen-bonded proton of the peptide linkage, in agreement with previous studies on peptides.[14,25,26]

The chemical shift data thus indicate that ionization of the acidic protons of fully protonated glutathione occurs predominantly *via* the microscopic scheme shown in Figure 3. The ionization reaction to which a given equilibrium constant refers is indicated by the subscript; the subscripts are used in the same manner as in the previous section. The L-glutamyl carboxylic acid group is denoted by the number 1, the glycyl carboxylic acid group by 2, the sulfhydryl group by 3, and the ammonium group by 4, as indicated by the fully protonated form of glutathione in Figure 3. The complete ionization scheme[1] also includes species resulting from ionization of the sulfhydryl and ammonium groups of fully protonated glutathione. However, the extent of ionization of the sulfhydryl and ammonium groups in the pH range 0.5–6 is negligible relative to ionization of the carboxylic acid groups; thus the concentration of species other than those in Figure 3 is negligible.

Determination of the Microscopic Ionization Constants of Glutathione. The fractional ionization of each of the acidic groups of glutathione was determined as a function of p_cH from the chemical shift data in Figure 2. It was assumed that a change in the chemical shift of a particular set of carbon-bonded protons is due only to ionization of the adjacent acidic groups. Thus, the change in the chemical shift of the C protons in the p_cH range 0.5–6 is assumed to be due solely to ionization of the L-glutamyl carboxylic acid group while the change in the chemical shift of the A protons is assumed to be due solely to ionization of the glycyl carboxylic acid group. The validity of this assumption can be seen from the pH independence of the chemical shift of the B protons during ionization of the two carboxylic acid groups and the negligible change in the chemical shift of the A protons during ionization of the amino and sulfhydryl groups. Also, the results of studies on the pH dependence of the chemical shifts of carbon-bonded protons of polyglycine peptides support this assumption.[14,26]

Microscopic ionization constants k_1, k_2, k_{12}, and k_{21} were evaluated from fractional ionization data for the p_cH range 0.5–6 using the methodology described in the Theoretical Section. Over this p_cH range, fully protonated glutathione behaves as a diprotic acid with the simultaneous ionization of the L-glutamyl carboxylic acid group and the glycyl carboxylic acid group. The fractional ionization of the L-glutamyl carboxylic acid group, F_1, was determined at a given pH from the chemical shift of the C proton, δ_c, while the fractional ionization of the glycyl carboxylic acid group, F_2, was determined from the chemical shift of the A protons. To illustrate, the determination of F_2 from δ_A will be described. At a given p_cH, δ_A is equal to the average of the chemical shifts of the forms in which the glycyl carboxylic acid group is ionized and protonated, $\delta_{A,I}$ and $\delta_{A,P}$, weighted according to the fractional concentrations of the ionized and protonated forms.

$$\delta_A = F_2\delta_{A,I} + (1 - F_2)\delta_{A,P} \qquad (11)$$

Rearrangement of eq 11 leads to

$$F_2 = \frac{\delta_A - \delta_{A,P}}{\delta_{A,I} - \delta_{A,P}} \qquad (12)$$

At a given p_cH, F_2 was calculated directly from the chemical shift of the A protons using eq 12. Microscopic constant k_{21} was determined to be 4.71×10^{-3} with a standard deviation of 0.98×10^{-4} from the least-squares slope of the plot of F_1/F_2 *vs.* $(1 - F_1/F_2)/[H^+]$ in Figure 4 while k_1/k_2 was determined from the least-squares intercept to be 10.8 with a standard deviation of 0.20. Microscopic constants k_1, k_2, and k_{12} were then calculated from these values for k_{21} and the ratio k_1/k_2; the results are given in Table I. The uncertainties are the standard deviations.

Table I. Microscopic Ionization Constants of Glutathione and the Sulfur-Coordinated Methylmercury Complex of Glutathione[a,b]

	Glutathione	Methylmercuric glutathione
pk_1	2.09 ± 0.05	2.19 ± 0.05
pk_2	3.12 ± 0.05	3.23 ± 0.05
pk_{12}	3.36 ± 0.10	3.37 ± 0.12
pk_{21}	2.33 ± 0.01	2.34 ± 0.02
pk_{123}	8.93 ± 0.04	
pk_{124}	9.13 ± 0.04	9.11 ± 0.02
pk_{1234}	9.28 ± 0.10	
pk_{1243}	9.08 ± 0.02	

[a] At 25°. [b] Uncertainties are the standard deviations.

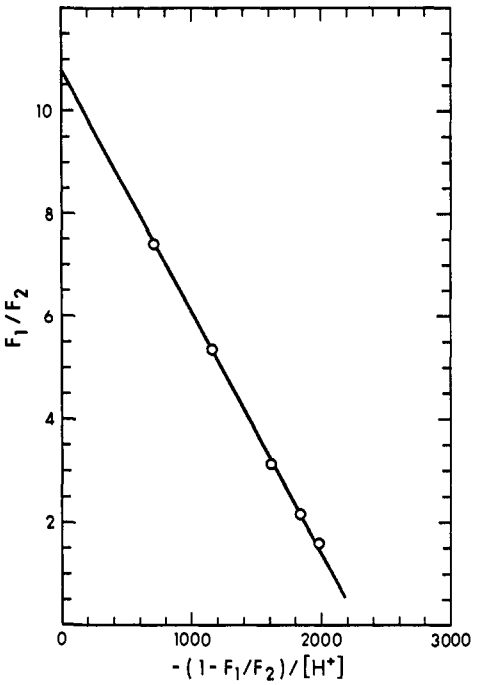

Figure 4. Evaluation of k_{21} and k_1/k_2 for glutathione at 25°.

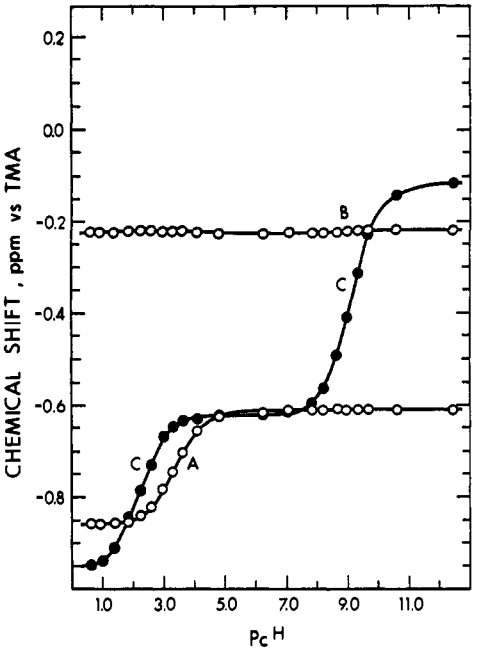

Figure 5. p_cH dependence of the chemical shifts of the (A) glycyl methylene protons, (B) L-cysteinyl methylene protons, and (C) L-glutamyl methine proton of methylmercury-complexed glutathione (0.15 M CH_3HgOH, 0.15 M glutathione, T = 25°).

Microscopic ionization constants k_{123}, k_{124}, k_{1234}, and k_{1243} were evaluated from fractional ionization data for the p_cH range 7–12. Over this p_cH range, doubly protonated glutathione behaves as a diprotic acid with the sulfhydryl and ammonium groups undergoing simultaneous ionization. The fractional ionization of the sulfhydryl group, F_3, was determined at a given pH from the chemical shift of the B protons while the fractional ionization of the ammonium group, F_4, was determined from the chemical shift of the C proton. Microscopic constant k_{1243} was determined to be 8.36 $\times$ 10^{-10} with a standard deviation of 0.40 $\times$ 10^{-10} from the least-squares slope of a plot of F_3/F_4 *vs.* $(1 - F_3/F_4)/[H^+]$ while k_{123}/k_{124} was determined from the least-squares intercept to be 1.57 with a standard deviation of 0.02. Microscopic constants k_{123}, k_{124}, and k_{1234} were then calculated from these values for k_{1243} and the ratio k_{123}/k_{124}; the results are given in Table I.[27]

The solid curves in Figure 2 represent the theoretical chemical shift *vs.* pH behavior of the A, B, and C protons of glutathione as predicted by the microscopic ionization constants in Table I.

Methylmercury-Complexed Glutathione. The chemical shifts of the A, B, and C protons of glutathione in a solution containing equimolar concentrations of methylmercuric hydroxide and glutathione are shown as a function of p_cH by the experimental points through which the solid curves are drawn in Figure 5. The p_cH dependence of the chemical shifts of the A and C proton is qualitatively the same as that shown in Figure 2, while the resonances for the B protons are shifted downfield over the entire pH range and show virtually no pH dependence. These results indicate that the methylmercuric ion is coordinated to the ionized sulfhydryl group of glutathione over the p_cH range 0.5–13. The pH dependence of the chemical shift of the methyl group of methylmercury and the spin–spin coupling constant for coupling between the methyl protons and mercury-199 also indicates methylmercury coordination to the ionized sulfhydryl group. The chemical shift and spin–spin coupling behavior of the methyl group of methylmercury bonded to the ionized sulfhydryl group of glutathione will be described in a later communication on detailed studies of the binding of methylmercury to sulfhydryl-containing amino acids and peptides.[28]

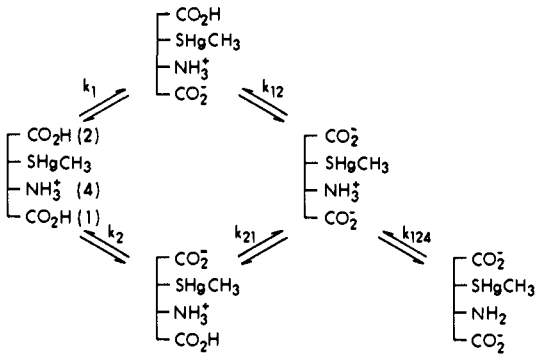

Figure 6. Microscopic ionization scheme for methylmercury-complexed glutathione, considering only the predominant species present. See text for labeling of the acidic groups and definition of the subscripts.

The microscopic ionization scheme for methylmercury-complexed glutathione is shown in Figure 6. Using the procedure described above, the microscopic

(27) Microscopic ionization constants were also calculated in terms of hydrogen ion activities using the pH meter readings directly. The values so obtained are: for glutathione pk_1 = 2.19 ± 0.04, pk_2 = 3.22 ± 0.04, pk_{12} = 3.45 ± 0.10, pk_{21} = 2.42 ± 0.01, pk_{123} = 8.97 ± 0.04, pk_{124} = 9.17 ± 0.04, pk_{1234} = 9.35 ± 0.10, pk_{1243} = 9.08 ± 0.02; for methylmercuric glutathione pk_1 = 2.27 ± 0.05, pk_2 = 3.30 ± 0.05, pk_{12} = 3.48 ± 0.12, pk_{21} = 2.44 ± 0.02, pk_{124} = 9.20 ± 0.02.

(28) D. L. Rabenstein and M. T. Fairhurst, unpublished results.

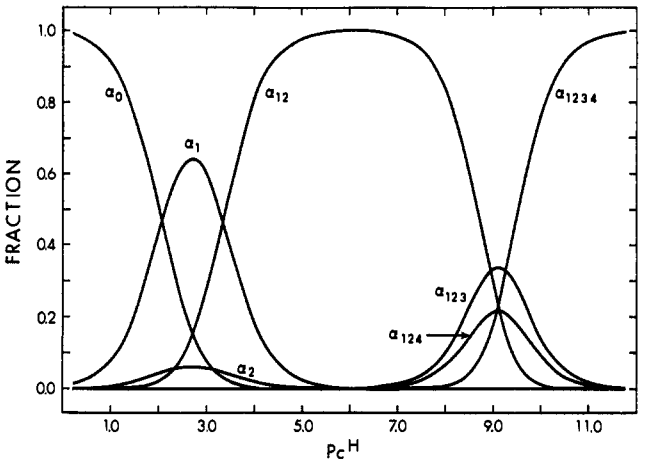

Figure 7. p_cH dependence of the distribution of the fractionally ionized forms of glutathione. α refers to the fractional concentration of the species indicated by the subscripts; see text for definition of the subscripts.

ionization constants listed in Table I for the carboxylic acid groups of methylmercury-complexed glutathione were obtained from the chemical shift data in Figure 5. Also, ionization constant k_{124} was determined from the mole fractions of complex in which the ammonium group is ionized and protonated (k_{124} = [H+][fraction ionized]/[fraction protonated]); the mole fractions were obtained from the chemical shift of the C proton in the p_cH range 7–11.5 using an equation analogous to eq 12. The value so obtained for k_{124} is given in Table I.

Discussion

Microscopic ionization constants for the sulfhydryl and ammonium groups of diprotonated glutathione have been determined by Martin and Edsall[29] by assuming the ionization constant of the ammonium group of *S*-methylglutathione to equal the microscopic constant of the ammonium group (k_{124}) of the diprotonated form of glutathione. The values obtained in the present work compare favorably with the reported results (pk_{123} = 8.92, pk_{124} = 9.20, pk_{1234} = 9.44, pk_{1243} = 9.16, μ = 0.16 *M*).

Jung, *et al.*,[30] observed the pH dependence of the carbon-13 chemical shifts of each of the carbon atoms of glutathione; however no attempts to quantitatively characterize the microscopic ionization scheme were reported. Benesch and Benesch[5] followed the ionization of the sulfhydryl group of glutathione by ultraviolet absorption spectroscopy and concluded from comparisons with pH titration data that the extent of ionization of the sulfhydryl and ammonium groups was approximately equal, but no microscopic constants were evaluated. From results obtained in this work, the sulfhydryl group of doubly protonated glutathione is 1.57 times as acidic as the ammonium group, while the L-glutamyl carboxylic acid group of fully protonated glutathione is 10.8 times as acidic as the glycyl carboxylic acid group.

The validity of the microscopic constants determined in this work can be established by comparison of the macroscopic constants obtained experimentally with those predicted from the microscopic constants. The macroscopic constants are related to the microscopic ionization scheme shown in Figure 3 by eq 13–16.

$$K_1 = k_1 + k_2 \quad (13)$$

$$K_2 = k_{12}k_{21}/(k_{12} + k_{21}) \quad (14)$$

$$K_3 = k_{123} + k_{124} \quad (15)$$

$$K_4 = k_{1234}k_{1243}/(k_{1234} + k_{1243}) \quad (16)$$

Predicted and experimental macroscopic constants are given in Table II.[31]

Table II. Predicted and Experimental Macroscopic Ionization Constants for Glutathione

	Predicted	Experimental		
pK_1	2.05	2.12[a]		
pK_2	3.40	3.53		3.59[c]
pK_3	8.72	8.66	8.74[b]	8.75
pK_4	9.49	9.62	9.62	9.65

[a] N. W. Pirie and K. G. Pinhey, *J. Biol. Chem.*, **84**, 321 (1929). [b] R. B. Martin and J. T. Edsall, *Bull. Soc. Chim. Biol.*, **40**, 1763 (1958). [c] N. C. Li, O. Gawron, and G. Bascuas, *J. Amer. Chem. Soc.*, **76**, 225 (1954).

The results in Table I clearly indicate that the state of ionization of neighboring substituent groups affects the acidity of a particular group in glutathione; each carboxylic acid group becomes less acidic by 0.24 p*k* unit when ionization occurs from the other carboxylic acid group, while the sulfhydryl and ammonium groups each become less acidic by 0.15 p*k* unit when ionization occurs from the other group. The direction of the change in acidity is as expected from the change in the charge on the substituent groups. The magnitudes of the changes in acidity are somewhat less than those reported for α-amino acids, in which the same substituent groups are undergoing simultaneous ionization, due to the increased proximity of the substituent groups in the α-amino acids. For glutamic acid,[32] the p*k* of the α-COOH changes from 2.15 to 2.62 when the γ-COOH ionizes while the p*k* of the γ-COOH changes from 3.85 to 4.32 when the α-COOH ionizes; for cysteine,[7] the p*k* of the –SH changes from 8.54 to 10.21 when the $-NH_3^+$ ionizes while the p*k* of the $-NH_3^+$ changes from 8.86 to 10.53 when the –SH ionizes.

The results in Table I also indicate that replacement of the sulfhydryl proton by the methylmercury cation has a negligible effect within experimental error on the acidities of the other substituent groups. This is likely due to the methylmercury cation being singly charged and somewhat removed from the other substituent groups.

The distribution of fractionally ionized forms of glutathione as a function of pH is of interest in view of the dependence of its chemical reactivity, in many cases, on the degree of ionization of specific functional groups. Using methods similar to those described by Laitinen,[33] the pH dependence shown in Figure 7 for the fractional concentrations was calculated from the microscopic

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(31) The experimental macroscopic constants are apparently mixed constants involving hydrogen ion activity and glutathione concentrations. Macroscopic constants predicted from mixed microscopic constants determined in this work[27] are: pK_1 = 2.15, pK_2 = 3.49, pK_3 = 8.76, pK_4 = 9.56.

(32) Reference 1, p 494.

(33) H. A. Laitinen, "Chemical Analysis," McGraw-Hill, New York, N. Y., 1960, p 35.

constants in Table I. The quantity α refers to the mole fraction of glutathione present at the stage of ionization indicated by the subscripts, where the subscripts are used in the same manner as with the microscopic constants. These calculations reveal that at the physiological pH of 7.4, the sulfhydryl proton is ionized in 2.8% of the glutathione.

This study represents the first part of a program directed toward characterizing the acid–base chemistry of amino acids and peptides at the molecular level. The microscopic ionization constants of glutathione and methylmercury-complexed glutathione were determined directly from chemical shift data without the use of macroscopic constants. The methods developed for evaluating the data are applicable to polyprotic systems in which the fractional ionization of each of the acidic groups undergoing simultaneous ionization can be determined as a function of pH. We are presently investigating the application of proton and carbon-13 nmr to the quantitative characterization of the acid–base chemistry of other amino acids and peptides.

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Electronic Spectra of Nucleic Acid Bases. I. Interpretation of the In-Plane Spectra with the Aid of All Valence Electron MO–CI Calculations

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Abstract: The in-plane part of the electronic spectra of nucleic acid bases as well as of their tautomers and ions is assigned by means of all valence electron SCF–MO–CI calculations. The various transitions of different bases are correlated using the nodal properties of the excited states. The spectra of pyrimidine and purine bases can both be divided into two groups, namely the cytosine and uracil type and the adenine and hypoxanthine type. Structurally, bases which show uracil or hypoxanthine type spectra differ from those which show cytosine or adenine type spectra by an additional proton at one of the nitrogen atoms of the six-membered ring. In pyrimidine bases, the presence of this proton leads to an increase in intensity of the transition lowest in energy and a decrease in intensity and shift toward higher energy of the second to lowest. The nodal patterns of both transitions are still similar in cytosine to those of pyrimidine, whereas in uracil they resemble more those found in an α,β-unsaturated ketone. In purine bases the excited states are more similar to those of purine itself but distinctly different from those of the underlying indole system. Adenine-type spectra are characterized by the fact that the lowest transition is localized mainly at N7=C8 in the five-membered ring, whereas the second spreads out over the fragment C2=N3—C4=C5—N7=C8 and corresponds to the lowest transition in a triene. In hypoxanthine-type spectra these two transitions are interchanged. Protonation at N7 in the five ring reverses this change and leads back to adenine-type spectra. We can exclude on the basis of our calculated spectra the possibility that protonation of adenine occurs at N7. The usual assumptions about the most stable tautomers are confirmed by a comparison of calculated and measured spectra. The results of the present calculation show that not only $n \rightarrow \pi^*$ transitions, but also $\pi \rightarrow \pi^*$ transitions, are profoundly affected by protonation and tautomerization. Hydrogen bonding in crystals might have a similar though smaller effect.

There have been numerous attempts to understand and correlate the electronic spectra of nucleic acid bases both experimentally[1–19] and theoretically.[20–30] The spectra of these compounds are of interest in themselves; furthermore, their understanding is necessary

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