Anal. Calcd for $C_{31}H_{25}SiBrO_2$: C, 69.27; H, 4.69. Found: C, 69.16; H, 4.80.

Rearrangement of the Deuterated Silylcarbinols. A. With Sodium–Potassium Alloy. A solution of 8.4 g (0.024 mol) of carbinols, $[\alpha]^{22}D$ −3.16° (*c* 7.41, benzene), diastereomer ratio 68:32, in 250 ml of dry ether, was treated with several drops of 1:5 sodium–potassium alloy under nitrogen. The reaction was followed by tlc on Eastman Chromogram silica gel sheets (Type K301R2) using benzene and was complete in 1.5 hr. The material was filtered to remove the alloy, and after washing with water and 5% HCl and drying, removal of the solvent under reduced pressure gave crude alkoxysilane, $[\alpha]^{22}D$ −14.1° (*c* 6.48, cyclohexane). The material was distilled in a Kugelröhr at about 150–210° (0.09 mm) to give an oil, $[\alpha]^{22}D$ −15.7° (*c* 8.18, cyclohexane). Attempts to establish the ratio of diastereomers by nmr spectroscopy (in CCl_4, $CDCl_3$, acetone, (+)-α-phenylethylamine, or (+)-2-octanol) failed, even at 100 MHz, since the Si–Me signals overlapped: ir (CCl_4) 4.67 (C–D), 6.88 (C–Ph), 7.01 and 8.97 (Si–Ph), 9.17 (Si–O–C, broad) μ; nmr (CCl_4) δ 6.9–8.15 (Ar), 4.70 (C–H, broad), 0.71 (Si–Me, no separate signals for diastereomers observed).

B. With Triethylamine. A solution of 4.6 g (0.013 mol) of carbinols, $[\alpha]^{20}D$ −2.98° (*c* 11.15, benzene), in 23 ml of chloroform and 4 ml of triethylamine was stirred for 6 days at 47–60°. After removal of the amine by extraction with dilute hydrochloric acid, the crude product, $[\alpha]^{20}D$ −14.80° (*c* 12.6, cyclohexane), was distilled as before to give 4.2 g (91%) of oil, $[\alpha]^{20}D$ −17.68° (*c* 11.1, cyclohexane).

Reduction of the Alkoxysilanes. To 1.22 g (0.032 mol) of lithium aluminum hydride in 30 ml of dibutyl ether was added 5.4 g (0.0152 mol) of the above mixture of alkoxysilanes, $[\alpha]D$ −15.7°, in 25 ml of ether. The diethyl ether was distilled off until the temperature was 81°, and the mixture was heated for 21 hr. Acetone was added to destroy the excess lithium aluminum hydride, water was added, and the ether layer was separated. This and the ether extracts of the aqueous layer were washed with 5% HCl and water, and then dried over anhydrous magnesium sulfate. The ether was distilled off through a Vigreux column and the benzyl-α-*d* alcohol was distilled through a 6-in. spinning band column, bp 72–82° (8 mm). The benzyl-α-*d* alcohol was purified by glc on a 10-ft Carbowax column on Chromosorb G at 162°. The rotation of an 8.19% solution in pure benzyl alcohol was $\alpha^{22}D$ 0.011 ± 0.0005°, corresponding to $[\alpha]^{22}D$ +0.129 ± 0.005° for pure deuterated alcohol. This corresponds to an enantiomeric ratio of 54:46. The pot residue was eluted from a silica gel column with carbon tetrachloride, and, after being crystallized once from hexane, gave 3.11 g (82%) of (−)-1-naphthylphenylmethylsilane, $[\alpha]^{20}D$ −34.5° (*c* 11.45, hexane).

Similar reduction of the 4.2 g of alkoxysilanes ($[\alpha]^{20}D$ −17.68°) followed by glc on an 8-ft SE 54 column on Chromosorb G at 115° gave 0.1386 g of deuteriocarbinol. A 12.41% w/w solution in benzyl alcohol had $\alpha^{20}D$ 0.096 ± 0.0005°, corresponding to $\alpha^{20}D$ 0.740 ± 0.005° for pure deuterated material, with a 73:27 ratio of enantiomers.

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Equilibria for the Reaction of Cysteine and Derivatives with Formaldehyde and Protons[1,2]

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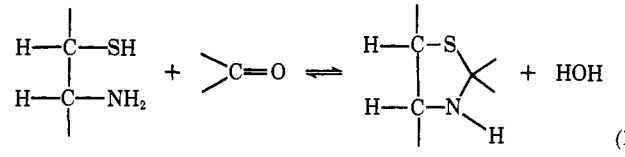
Abstract: Equilibrium constants for the major formaldehyde adducts of cysteine have been estimated from pH, spectrophotometric, and free thiol measurements of cysteine or a derivative at various formaldehyde concentrations. Equilibrium constants for the formation of *N*-hydroxymethyl-*S*-methylcysteine, *N*,*N*-dihydroxymethyl-*S*-methylcysteine, *S*-hydroxymethyl-*N*-acetylcysteine, and thiazolidine-4-carboxylate (TC) from formaldehyde and cysteine or a derivative are 15.9 M^{-1}, 16.0 M^{-2}, 700 M^{-1}, and 1.14×10^8 M^{-1} for adducts from compounds with neutral thiol and/or amino groups. Equilibrium constants for neutral and anionic *N*-hydroxymethyl-TC formation from neutral and anionic TC and formaldehyde are 7.0 and 0.7 M^{-1}, respectively. The microscopic proton dissociation constants for the eight species of cysteine which exist in the pH range 0–14 have been obtained at 25° and ionic strength 1.0 *M* from spectrophotometric measurements at different pH values, from studies on the proton dissociation of cysteine derivatives, and from mathematical relations between the constants. These data permit the calculation of the composition of cysteine solutions for precise interpretation of equilibrium and kinetic data for reactions involving cysteine.

Although reactions of carbonyl compounds with β-aminothiols, particularly cysteine, to form thiazolidines (eq 1) have been the subject of numerous previous studies,[3] measurements have not been reported



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(2) Abbreviations used are: CEE = L-cysteine ethyl ester; CME = L-cysteine methyl ester; CYS = L-cysteine; DTNB = 5,5′-dithiobis-(2-nitrobenzoic acid); EDTA = ethylenediaminetetraacetic acid; F = formaldehyde hydrate; HEPES = *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; HMTC = *N*-hydroxymethylthiazolidine-4-carboxylate; 2-MEA = 2-mercaptoethylamine; NAC = *N*-acetyl-L-cysteine; SEC = *S*-ethyl-L-cysteine; SMC = *S*-methyl-L-cysteine; TC = thiazolidine-4-carboxylate.

(3) (a) M. P. Schubert, *J. Biol. Chem.*, **111**, 671 (1935); (b) M. P. Schubert, *ibid.*, **114**, 341 (1936); (c) K. Shinohara, *ibid.*, **110**, 2((1935); (d) S. Ratner and H. T. Clarke, *J. Amer. Chem. Soc.*, **59**, 2((1937); (e) G. E. Woodward and E. F. Schroeder, *ibid.*, **59**, 16((1937); (f) D. Heyl, S. A. Harris, and K. Folkers, *ibid.*, **70**, 34((1948); (g) F. E. King, J. W. Clarke-Lewis, and R. Wade, *J. Chei Soc.*, 880 (1957); (h) V. duVigneaud, E. J. Kuchinskas, and A. Ho vath, *Arch. Biochem. Biophys.*, **69**, 130 (1957); (i) Y. Matsuo, *J. Ame Chem. Soc.*, **79**, 2011 (1957); (j) F. Bergel and K. R. Harrap, *J. Chei Soc.*, 4051 (1961); (k) M. V. Buell and R. E. Hansen, *J. Amer. Chei Soc.*, **82**, 6042 (1960); (l) D. Mackay and D. M. Shepard, *Biochi*

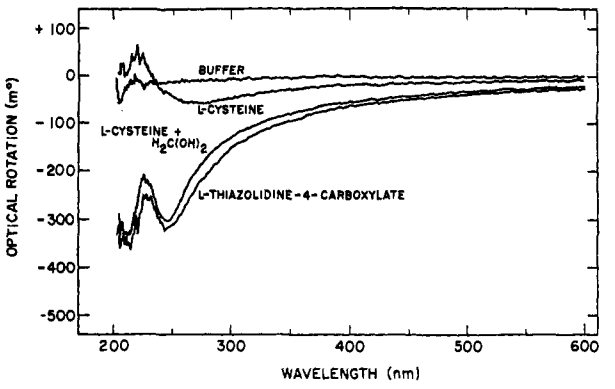


Figure 1. ORD curves for 0.01 *M* cysteine (pH 4.28), 0.01 *M* cysteine plus 0.10 *M* formaldehyde (pH 4.28), and 0.01 *M* thiazolidine-4-carboxylate (pH 4.45), ionic strength 1.0 *M*, 26–28°; 1.0-cm pathlength, scan speed 26 nm/min. Top line is base line of 0.1 *M* sodium acetate buffer, pH 4.46.

for the relevant equilibrium constants nor has the detailed mechanism of the reaction been elucidated. Thiazolidine formation is responsible for perturbations in the formol titration of cysteine,[3a-e,4,5] for the inhibition by carbonyl compounds of a variety of tests for the thiol group when applied to cysteine,[3b-d] and for the inhibition by cysteine of metabolic processes requiring pyridoxal derivatives,[3h] and is useful for protection of aminothiols by carbonyl compounds in synthetic work.[3g] Proteins containing sulfhydryl and amino groups in close proximity may bind biologically important carbonyl compounds in thiazolidine-like (substituted aldimine) linkages.[6-8]

During a reexamination of the mechanism of formation of TC from cysteine and formaldehyde,[9] which exhibits a complex pH–rate profile, knowledge of the composition of solutions of cysteine and formaldehyde at different acidities became necessary. The equilibrium constants for the binding of protons[10] and formaldehyde to the various microscopic species of cysteine and related compounds were determined at ionic strength 1.0 *M* and 25°.

Experimental Section

Materials. L-Cysteine hydrochloride monohydrate, SMC, NAC, and *N*-acetyl-*S*-benzyl-L-cysteine were used without further purification. Thiocholine iodide was converted to the chloride by passage through a Dowex-1 chloride column. The purity of compounds was confirmed by titration with base or DTNB[11] except for *N*-acetyl-*S*-benzylcysteine. Reagent grade formaldehyde in 36.6–37.2% solutions containing 10–12% methanol was used without further purification. The concentration of stock formaldehyde solutions of 1.0 *M* was confirmed by sulfite titration.[12] Carboxylic acids and their salts, inorganic salts, HEPES, and DTNB were of reagent grade and used without further purification.

Deionized water, 5×10^5 to 18×10^6 ohm cm specific resistance, was used throughout with potassium chloride to maintain ionic strength at 1.0 *M*.

TC was synthesized by the method of Ratner and Clarke,[3d] mp 189–191° dec (uncorr) (lit. 184–185°, [3d]195°[3b]). *Anal.* Calcd for $C_4H_7O_2NS$: C, 36.06; H, 5.29; N, 10.52. Found: C, 36.08; H, 5.41; N, 10.43. The ORD spectrum (Durrum-Jasco automatic spectropolarimeter) of TC is presented in Figure 1, and agrees with TC generated by mixing 0.1 *M* formaldehyde with 0.01 *M* cysteine at pH values 1.0, 4.3, 7.8, and 11.8 (1.0-cm path length). The nmr spectrum (Jeolco C60H) of TC in 1.0 *M* deuterium chloride in deuterium oxide reveals a triplet (95 Hz), singlet (115 Hz), and doublet (176 Hz), upfield with respect to chloroform as an external standard within a capillary tube, areas 1:2:2, J = 6.75 Hz, and is consistent with the structure of TC.

Methods. All titration data are reported as the negative logarithm of the apparent (macroscopic) or microscopic proton dissociation constants, pG' or pK', respectively.[10p]

Titrimetric. The titrimetric methods have been described.[13] Measurements of pH were carried out with a Radiometer 25 SE or 26 SE pH meter using GK 2021 C combined electrodes (Radiometer) calibrated with pH 4, 7, and 10 standard buffer solutions and 0.1 *N* hydrochloric acid.[14]

Proton dissociation constants for nonoverlapping pG' values were calculated (a) by methods previously described,[13] (b) from the negative slope of a plot of [acid] against [acid]/[H^+] in which the concentration of acid was determined from the amount of titrant after correction for a potassium chloride blank,[15] or (c) from data uncorrected for amounts of titrant consumed by potassium chloride blanks from the equation, $pG' = pH - \log \{([base] + [H^+])/([acid] - [H^+])\}$.[16]

The method of Noyes[16] was applied to cysteine and CME, compounds with overlapping pG' values due to the simultaneous proton dissociation from the -SH and $-NH_3^+$ groups.

Spectrophotometric titrations were conducted on Zeiss PMQ II or Gilford Model 2000 multiple sample absorbance recorder spectrophotometers equipped with cell compartments which were thermostated at 25 ± 0.1° as previously described.[13] Complete ultraviolet spectra were obtained with a Cary Model 14 or a Hitachi Coleman Model 124 recording spectrophotometer.

Spectrophotometric titrations of cysteine and CME were conducted at a single wavelength in the range 230–240 nm based on the absorbance of the thiolate anions. Values of the fraction of the total species as the anion, α_{RS^-}, were obtained in the pH range 3.8–12.0 for a constant total concentration of compound, from $\alpha_{RS^-} = (A_x - A_a)/(A_b - A_a)$ in which A_a, A_b, and A_x are the absorbances of undissociated thiol forms, thiolate anion forms, and mixtures of the various forms at a given pH value, respectively.

Biophys. Acta, **59**, 553 (1962); (m) D. Mackay, *Arch. Biochem. Biophys.*, **99**, 93 (1962); (n) J. C. Peskin and B. B. Love, *Biochim. Biophys. Acta*, **78**, 751 (1963); (o) K. Mizuno, K. Ozawa, and Y. Kuno, *Exp. Eye Res.*, **5**, 276 (1966); (p) K. Mizuno, Y. Kuno, and K. Ozawa, *Jap. J. Ophthalmol.*, **10**, 85 (1966); (q) H. A. Rothschild and E. S. G. Barron, *J. Biol. Chem.*, **209**, 511 (1954); (r) T. W. Birch and L. J. Harris, *Biochem. J.*, **24**, 1080 (1930).

(4) D. French and J. T. Edsall, *Advan. Protein Chem.*, **2**, 277 (1945).

(5) R. G. Kallen and W. P. Jencks, *J. Biol. Chem.*, **241**, 5864 (1966).

(6) (a) S. Shaltiel, J. L. Hedrick, and E. H. Fischer, *Biochemistry*, **5**, 2108 (1966); **8**, 2429 (1969); (b) G. F. Johnson and D. J. Graves, *ibid.*, **5**, 2906 (1966).

(7) (a) P. Fasella, *Annu. Rev. Biochem.*, **36**, 185 (1967), and references therein; (b) W. B. Dempsey and H. N. Christensen, *J. Biol. Chem.*, **237**, 1113 (1962).

(8) J. Heller, *Biochemistry*, **7**, 2914 (1968).

(9) R. G. Kallen, *J. Amer. Chem Soc.*, **93**, 6236 (1971).

(10) (a) R. K. Cannan and B. C. J. G. Knight, *Biochem. J.*, **21**, 1384 (1927); (b) H. Borsook, E. L. Ellis, and H. M. Huffman, *J. Biol. Chem.*, **117**, 281 (1937); (c) L. R. Ryklan and C. L. A. Schmidt, *Arch. Biochem.*, **5**, 89 (1944); (d) R. E. Benesch and R. Benesch, *J. Amer. Chem. Soc.*, **77**, 5877 (1955); (e) M. A. Grafius and J. B. Neilands, *ibid.*, **77**, 3389 (1955); (f) G. Gorin and C. W. Clary, *Arch. Biochem. Biophys.*, **90**, 40 (1960); (g) G. Gorin, *J. Amer. Chem. Soc.*, **78**, 767 (1956); (h) J. C. Evans and G. L. Ellman, *Biochim. Biophys. Acta*, **33**, 574 (1959); (i) D. Garfinkel and J. T. Edsall, *J. Amer. Chem. Soc.*, **80**, 3823 (1958); (j) H. Lindley, *Biochem. J.*, **74**, 577 (1960); **82**, 418 (1962); (k) D. A. Doornbos, *Pharm. Weekbl.*, **102**, 269 (1967); (l) E. L. Elson and J. T. Edsall, *Biochemistry*, **1**, 1 (1962); (m) E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids, and Peptides," Reinhold, New York, N. Y., 1943, Chapters 4 and 5; (n) M. Calvin in "Gluthathione," S. Colowick, R. Lazarow, E. Racker, D. R. Schwartz, E. Stadtman, and H. Waelsch, Ed., Academic Press, New York, N. Y., 1954, p 3; (o) R. Benesch, R. E. Benesch, and W. I. Rogers in ref 10n, p 31; (p) J. T. Edsall and J. Wyman, "Biophysical Chemistry," Vol. 1, Academic Press, New York, N. Y., 1958, pp 452–453, 464–465, 481–487, 496–504; (q) R. Cecil in "The Proteins," H. Neurath, Ed., Vol. 1, 2nd ed, 1963, p 399; (r) D. P. Wrathall, R. M. Izatt, and J. J. Christensen, *J. Amer. Chem. Soc.*, **86**, 4779 (1964). Several of the pK_a' values from this reference appear to be in substantial disagreement with the present and other work (see also ref 10s); (s) E. W. Wilson, Jr., and R. B. Martin, *Arch. Biochem. Biophys.*, **142**, 445 (1971), and references therein.

(11) G. L. Ellman, *ibid.*, **82**, 70 (1959).

(12) J. F. Walker, "Formaldehyde," 3rd ed, American Chemical Society Monograph, Reinhold, New York, N. Y., 1964.

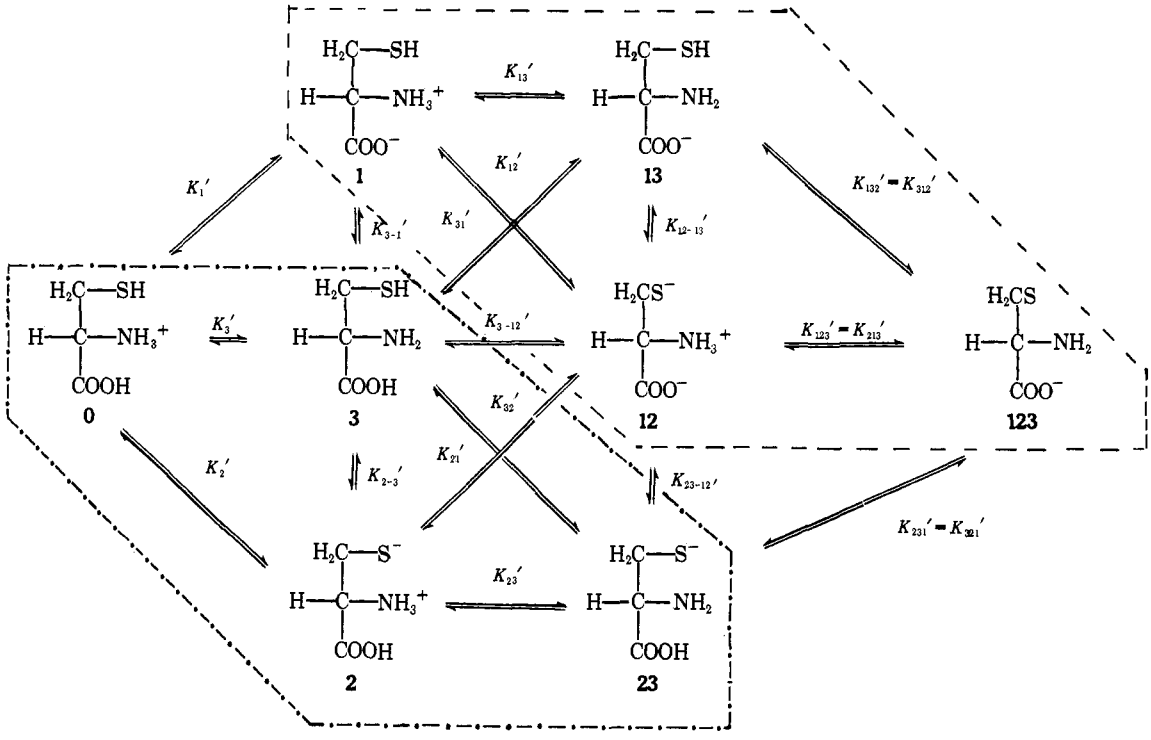
(13) R. G. Kallen and W. P. Jencks, *J. Biol. Chem.*, **241**, 5845 (1966).

(14) V. Gold, "pH Measurements," Wiley, New York, N. Y., 1956.

(15) B. H. J. Hofstee, *Science*, **131**, 39, 1068 (1960).

(16) A. Albert and E. P. Serjeant, "Ionization Constants of Acids and Bases," Wiley, New York, N. Y., 1962.

Scheme I. Proton Dissociation and Tautomerization Equilibria of Cysteine[a]



[a] For cysteine: $G_1' = K_1' + K_2' + K_3'$, $G_1'G_2'G_3' = K_1'K_{12}'K_{123}' = K_2'K_{23}'K_{231}'$, etc. $1/G_3 = 1/K_{132}' + 1/K_{123}' + 1/K_{231}'$.

The microscopic proton dissociation constants were calculated for each of the pH values from the α_{RS^-} values, the macroscopic proton dissociation constants (known from the independent titration), and eq 2 and 3 for cysteine[10p] and CME, respectively.

$$K_{12}' = \alpha_{RS^-}([H^+] + G_2') - (1 - \alpha_{RS^-})G_2'G_3'/[H^+] \quad (2)$$

$$K_2' = \alpha_{RS^-}([H^+] + G_1') - (1 - \alpha_{RS^-})G_1'G_2'/[H^+] \quad (3)$$

The remaining microscopic proton dissociation constants were calculated from $K_{123}' = G_2'G_3'/K_{12}'$, $K_{13}' = G_2' - K_{12}'$, and $K_{132}' = G_2'G_3'/K_{13}'$ for cysteine (Scheme I), and from $K_{23}' = G_1'G_2'/K_2'$, $K_3' = G_1' - K_2'$, and $K_{32}' = G_1'G_2'/K_3'$ for CME.[17]

Formol Titration of Amines. Formol titrations of SMC were carried out as described[5] except that 10^{-4} *M* EDTA was present and the solutions were maintained under nitrogen when necessary.

Equilibrium Constant for Thiazolidine Formation. A reaction mixture (50 ml), which contained 1.5–7.5 × 10^{-3} *M* TC, 0.001 *M* EDTA, 0.01 *M* potassium phosphate buffers at pH values 5.56, 6.56, and 7.45, and KCl, was maintained at 25° protected from exposure to light. Aliquots, 2.7 ml, were removed at various times and added to 0.3 ml of 1.0 *M* potassium phosphate buffer, 90% free base, and 0.02 ml of 0.01 *M* DTNB for assay of the free thiol content by the ΔA_{412}, utilizing a cysteine standard curve, with correction for a blank at pH 7.6 (ϵ 13,600 M^{-1} cm^{-1}).[11] A 5 × 10^{-5} *M* cysteine solution was stable under these conditions for the time required to approach equilibrium. TC fails to react significantly with DTNB and calculations based on the appropriate equilibrium constants indicate that no significant *N*-hydroxymethylcysteine or hemithioacetal accumulates under the assay conditions (see below).

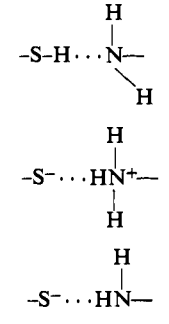
Results

Proton Dissociation Equilibria. The results for the proton dissociation equilibria for cysteine and derivatives will be presented in terms of Scheme I[18] for the various microscopic states of ionization and the relevant microscopic proton dissociation constants.[17] Data from previous work are summarized in Tables I and II.[22]

Cysteine Methyl Ester (CME) and Cysteine. Two equivalents of titrant are consumed in titrations of CME and cysteine in the pH range 4–11 (Figure 2). The titration data form a smooth curve, devoid of a clearly defined plateau after the addition of about 1 equiv of titrant, and analysis of these data was by the method of Noyes[16] for overlapping pG' values (Table I). Titration of L-cysteine from pH 4.01 to 1.0 reveals a single titratable group (Table I). Absence of hysteresis in forward and reverse titrations indicates no significant instability of cysteine or CME.

The spectrophotometric titration data of cysteine and CME in the pH range 3.8–12.0 (Figure 2) yield the mi-

(18) Forms involving intramolecular hydrogen bonds[10o,19]



which could be responsible for perturbed macroconstants[19,20] but are present in very low concentrations, if at all,[21] are not included.

(17) For derivatives of cysteine the macroscopic proton dissociation constants are numbered sequentially from the most acidic titratable group(s). For example, the proton dissociation scheme for cysteine methyl ester utilizes the lower left region (-·-·-) of Scheme I, by replacing the carboxylic acid proton with a methyl group, where $G_1' = K_2' + K_3'$ and $1/G_2' = 1/K_{32}' + 1/K_{23}'$ since microscopic proton dissociation constants subscripted 1 and the macroscopic constant G_3' are not applicable.

(19) R. E. Davis, S. P. Molnar, and R. Nehring, *J. Amer. Chem. Soc.*, 91, 97 (1969).

(20) (a) M. Laskowski, Jr., and H. A. Scheraga, *ibid.*, 76, 6305 (1954); (b) M. M. Tuckerman, J. R. Mayer, and F. C. Nachod, *ibid.*, 81, 92 (1959).

(21) J. T. Edsall, *Biochemistry*, 4, 28 (1965).

(22) The variations in pG_3' values result primarily from differences in ionic strength and are not unreasonable based on rough calculations from a simplified form of the Debye–Hückel equations.[10i]

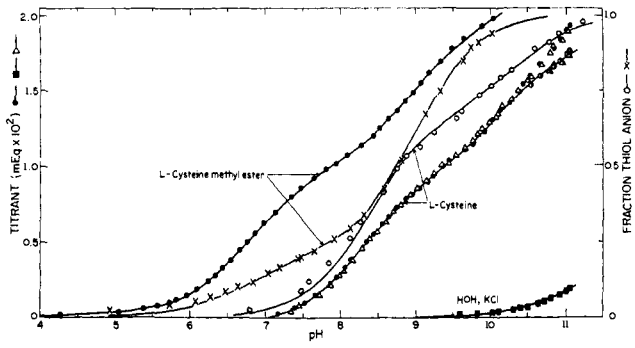


Figure 2. Titration curves of L-cysteine methyl ester and L-cysteine. CME: (●) titration of 10 ml of 0.01 *M* solution with 1.0 *N* NaOH; (×) spectrophotometric titration 1.0×10^{-4} *M* at 230 nm. L-Cysteine: titration of 10 ml of 0.01 *M* solution, (●) forward with 1.0 *N* sodium hydroxide, (Δ) reverse with 1.0 *N* hydrochloric acid, (◑) and (Δ) are forward and reverse titration data corrected for water, potassium chloride blank (■), respectively; (○) spectrophotometric titration 1.5×10^{-4} *M* at 240 nm. Ionic strength 1.0 *M*, 25°. Fraction thiol anion, α_{RS^-}, is $(A_x - A_a)/(A_a - A_b)$ (see Experimental Section). Solid lines are calculated based on values in Table II and eq 2 and 3.

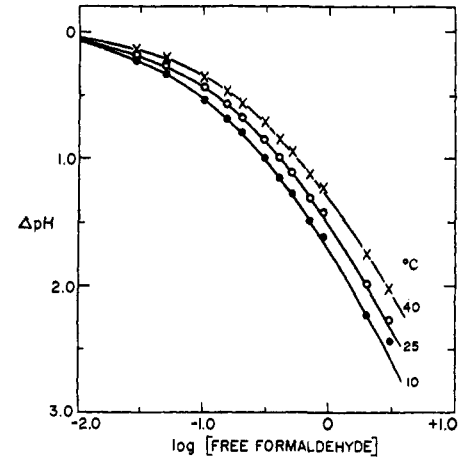


Figure 3. The change in the pH of 0.01 *M* *S*-methyl-L-cysteine buffers, 10% free base, as a function of the logarithm of the free formaldehyde hydrate (F_F) concentration; ionic strength 1.0 *M*: (●) 10°, (○) 25°, (×) 40°. The solid, theoretical lines are calculated from eq 4 and the values in Table III.

croscopic proton dissociation constants from eq 2 and 3, respectively (Table II). The solid lines drawn through the data in Figure 2 are calculated on the basis of the

Table I. Macroscopic Proton Dissociation Constants for Cysteine and Derivatives at 25°

Compd	pG_1'	pG_2'	pG_3'	Ref
S-Methyl-L-cysteine	2.22 (19)	8.88 (28)		*a*
	c	8.75		*e*
S-Ethyl-L-cysteine	2.03	8.60		*f*
N-Acetyl-L-cysteine	3.08 (29)	9.51 (52)		*a*
L-Cysteine methyl ester	6.77[b] (51)	8.99[b] (51)		*a*
L-Cysteine ethyl ester	6.69[d]	9.17[d]		*g*
L-Cysteine-betaine	*c*	8.65		*h*
N-Acetyl-*S*-benzyl-L-cysteine	3.07 (17)			*a*
L-Cysteine	2.00 (13)	8.38[b] (71)	10.37[b] (71)	*a*
	c	8.30	10.40	*h*
	1.71	8.33	10.78	*i*
	c	8.27	10.42	*j*
	c	8.07	9.95	*k*
Thiazolidine-4-carboxylate	1.63 (25)	6.24 (54)		*a*
	1.51	6.21		*f*

[a] This work; number in parentheses following pG′ value is the number of determinations, ionic strength 1.0 *M*. [b] By the method of Noyes;[16] ranges for CME, pG_1', 6.72–6.82; pG_2', 8.96–9.02 (650 pairwise calculations); ranges for cysteine, pG_2', 8.34–8.44; pG_3', 10.30–10.45 (298 pairwise calculations). [c] Not determined. [d] Calculated from microconstants, 23°. [e] Reference 10e. [f] Reference 3d; ionic strength 0.1–0.2 *M*. [g] Reference 10d; ionic strength *ca.* 0.05 *M*. [h] Reference 10e; ionic strength 0.15 *M*. [i] Reference 10b; ionic strength 0. [j] Reference 10g; ionic strength 0.1 *M*. [k] Reference 10r; ionic strength 1.0 *M*.

macroscopic and microscopic proton dissociation constants contained in Tables I and II for the titration and spectrophotometric data, the latter utilizing eq 2 and 3.

The microscopic ionization constants for L-cysteine enclosed in the dashed lines in Scheme I are calculated from the relations, $K_{12}' = G_2' - K_{13}'$, $K_{132}' = G'_2G_3'/K_{12}'$ (Table II) with (i) pK_{13}' assigned the pG_2' value of 8.88 (SMC), *i.e.*, Wegscheider's principle[10p] (see Discussion), or (ii) K_{12}', obtained from spectrophotometric titrations of cysteine. The microscopic proton dissociation constants for species of cysteine enclosed within the dot–dashed lines are based on pK_a' values for CME.

S-Methyl-L-cysteine (SMC) and N-Acetyl-L-cysteine (NAC). Titration of SMC and NAC in the pH range 1.5–12.0 reveals two titratable groups for each compound (Table I).

Equilibria for Formaldehyde Adduct Formation. Carbinolamine Formation from Formaldehyde and SMC. The equilibrium constants, L_1' and L_2', for the addition of 1 and 2 mol of formaldehyde to the amino group of cysteine to form the mono- and dihydroxymethyl adducts (Scheme II) could not be obtained due to the further rapid reaction to form TC and were, therefore, determined for SMC by the formol titration technique.[5]

The change in pH of SMC buffers at various formaldehyde concentrations (Figure 3) is described by eq 4,

$$pH_0 - pH_{obsd} = \Delta pH = \log (1 + L_1'[F_F](1 + L_3'[F_F])) \quad (4)$$

where pH_{obsd} and pH_0 are the pH values in the presence and the absence of formaldehyde, respectively, $L_1' = [RNHCH_2OH]/[RNH_2][F_F]$, $L_3' = [RN(CH_2OH)_2]/[RNHCH_2OH][F_F]$, and F_F is free, hydrated formaldehyde. Calculated curves based on the equilibrium constants in Table III and eq 4 are shown as solid lines (Figure 3).

Plots of log L_1' or log L_3' against the reciprocal of the absolute temperature are linear (not shown) and the enthalpy changes, obtained from the slope of such a plot,[23] are −3.3 and −4.8 kcal/mol for ΔH_1 and ΔH_3, respectively, in agreement with those for similar compounds.[24]

Equilibrium Constants for Thiazolidine Formation from Formaldehyde and Cysteine. The apparent equilibrium constants for TC formation from cysteine

(23) W. J. Moore, "Physical Chemistry," 3rd ed, Prentice-Hall, Englewood Cliffs, N. J., 1962.
(24) P. Le Hénaff, *C. R. Acad. Sci. Paris*, 258, 3690 (1964).

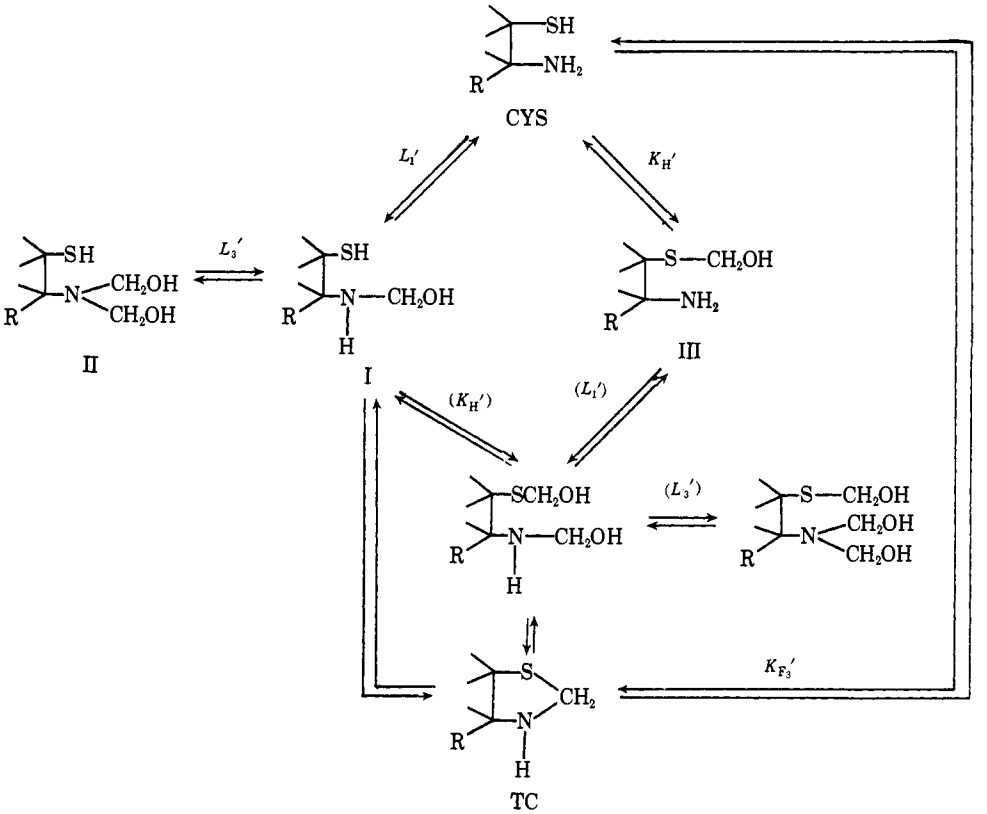
Table II. Microscopic Proton Dissociation Constants for Cysteine and Derivatives at 25°, Ionic Strength 1.0 *M*

Constant[a]	Cysteine									NAC[b]	CME[c]	
	This work									This work	This work	CEE
	d	*e*	*f*	*g*	*h*	*i*	*j*	*k*	*l*			*j*
pK_1'	2.00	2.00	1.70							3.08		
pK_2'	7.44	7.44	7.45							8.48	7.44	7.45
pK_3'	6.88	6.88	6.77								6.88[m]	6.77
pK_{12}'	8.55	8.54	8.53	8.50	8.65	8.49	8.53	8.66	8.45	9.51		
pK_{13}'	8.88	8.90	8.86	8.85	8.66	8.75	8.86	8.60	8.31			
pK_{21}'	3.10	3.10	2.79							4.11		
pK_{23}'	8.32	8.32	8.41								8.32	8.41
pK_{31}'	4.00	4.02	3.80									
pK_{32}'	8.88	8.88	9.09								8.88	9.09
pK_{123}'	10.20	10.22	10.36	10.35	10.05	10.21	10.36	10.45	9.58			
pK_{132}'	9.87	9.85	10.03	10.00	10.04	9.95	10.03	10.51	9.73			
pK_{231}'	4.99	4.99	4.74									
pK_{3-12}'	3.67	3.66	3.47									
pK_{3-1}'	−4.88	−4.88	−5.06									
pK_{2-3}'	−0.56	−0.56	−0.68									
pK_{12-13}'	0.33	0.36	0.33	0.35	0.01	0.26	0.33	−0.06	−0.15			
pK_{23-12}'	−5.22	−5.23	−5.62									

[a] See Scheme I, (s) = spectrophotometric titration; temperatures and ionic strengths for referenced data as in Table I except ref 10d, 25°, *ca.* 1.0–3.0 *M*. [b] No NH_3^+ group; pK_{12}' (s); pK_2' estimated as 8.48 = 9.51 − 1.03, from the effect of a β-carboxylic acid on the thiol proton dissociation of cysteine; $pK_{12}' - pK_2' = 1.10$ and $pK_{132}' - pK_{32}' = 0.97$, av 1.03 (*cf.* ref 10p, p 466); $pG_1' + pG_2' - pK_2' = pK_1' + pK_{12}' - pK_2'$. For NAC ethyl ester pK_{12}' was estimated kinetically to be 8.53 at 25°, ionic strength 0.1 *M* (R. Cecil and J. R. McPhee, *Biochem. J.*, **60**, 496 (1955). For *N*-acetyl-*S*-benzyl-L-cysteine pG_1' is 3.08 (*cf.* $pK_1' = 3.08$ for NAC). [c] No COOH group; pK_2' (s). [d] Calculated from macroscopic constants in Table I based on SMC. [e] (s). [f] Reference 10p. [g] Raman ir spectrometric titration.[101] [h] Based on cysteine betaine; ref 10e. [i] Based on SMC; ref 10e. [j] (s) (ref 10d). [k] Based on SEC (ref 10c). [l] Reference 10r. [m] pK_3' 6.50 at 20° (J. P. Danehy and C. J. Noel, *J. Amer. Chem. Soc.*, **82**, 2511 (1960)).

and formaldehyde were determined from the concentration of thiol, detected by titration with DTNB, when the hydrolysis of TC reaches equilibrium following total TC present initially and the free thiol present at equilibrium; the concentration of free formaldehyde hydrate is equal to the amount of free cysteine. Calcu-

Scheme II. Formaldehyde Equilibria of Cysteine or Derivatives[a]



[a] For SMC: $L_1' = ([I][HOH])/([RNH_2][F])$; $L_2' = ([II][HOH]^2)/([RNH_2][F]^2)$; $L_3' = L_2'/L_1' = ([II][HOH])/([RNHCH_2OH][F])$; R = . For NAC: $K_H' = ([III][HOH])/([RSH][F])$. For cysteine: $K_{F_3}' = ([TC^-][HOH])/([CYS^-][F])$; F = formaldehyde hydrate.

dilution (Figure 4). The amount of TC present at equilibrium was calculated from the difference between lated apparent equilibrium constants are contained in Table IV.

Table III. Equilibrium Constants for Adduct Formation from Formaldehyde and SMC, NAC, and Cysteine at Ionic Strength 1.0 M[a]

Equilibrium	T, °C	SMC[b]	NAC	CYS
$L_1' = ([RNHCH_2OH][HOH])/([RNH_2][F])$	10	21.0		
	25	15.9		26.8[c]
	40	11.8		
$L_3' = ([RN(CH_2OH)_2][HOH])/([RNHCH_2OH][F])$	10	1.54		
	25	1.02		
	40	0.69		
$K_H' = ([RSCH_2OH][HOH])/([RSH][F])$	25		700[d]	
$K_{F_1}' = [TC^+]/([CYS^+][F])$	25			2.24×10^5 [e,f]
$K_{F_2}' = [TC^\pm]/([CYS^\pm][F])$	25			$5.25 \pm 0.5 \times 10^5$ [g]
$K_{F_3}' = [TC^-]/([CYS^-][F])$	25			$1.14 \pm 0.01 \times 10^8$ [g]
$K_{TF_1}' = [HMTC^-]/([TC^-][F])$	25			7.0
$K_{TF_2}' = [HMTC^\pm]/([TC^\pm][F])$	25			0.7

[a] Water activity 1.0, see Scheme III; $HMTC^-$ and $HMTC^\pm$ are anionic and net neutral N-hydroxymethyl-TC; for abbreviations see ref 2. [b] From Figure 3, $pG_2' = 9.23$, 8.88, 8.19 at 10, 25, and 40°, respectively. [c] Determined by kinetic methods, pH 10–11 (ref 9). [d] Determined by spectrophotometric equilibrium measurements (R. G. Kallen and M. M. Frederick, manuscript submitted to *J. Org. Chem.*). [e] From $K_{F_1}' = K_{F_2}'G_1'/K_{TC_1}'$ where $G_1' = [CYS^\pm][H^+]/[CYS^+]$ and $K_{TC_1}' = [TC^\pm][H^+]/[TC^+]$ (see Scheme III). [f] Phosphate buffer 0.01 M, 0.01 M EDTA. [g] From eq 5.

The observed rate constants for the attainment of equilibrium from the hydrolysis of TC[25] (Figure 4) are in the approximate range expected from the apparent equilibrium constant for TC formation and the

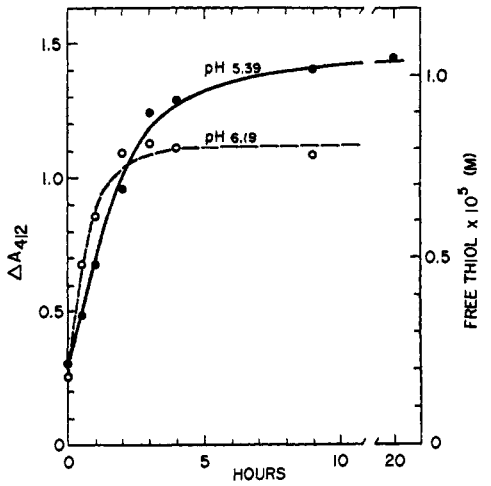


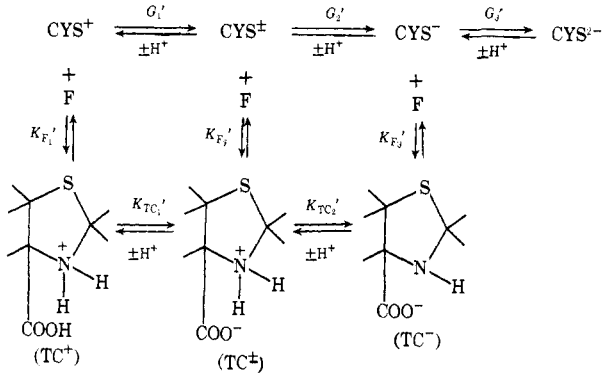
Figure 4. Approach to equilibrium of thiazolidine-4-carboxylate (TC) hydrolysis to cysteine and formaldehyde at pH 5.39 (●) and 6.19 (○) at 25°. Initial TC concentration 7.5×10^{-3} M, potassium phosphate buffers 0.01 M, ionic strength 1.0 M with KCl, 0.01 M EDTA. Free thiol was determined by the DTNB method.

observed rate of TC formation from cysteine and formaldehyde.[9] At pH 5.73 the equilibrium position was approached from the direction of TC synthesis from cysteine and formaldehyde and $K_{F_{app}}' = 7 \times 10^5$ M^{-1} (average) (*cf.* Table IV).

The 1:1 stoichiometry of the formaldehyde–cysteine adduct is indicated by the agreement between the ORD (Figure 1) and nmr spectra for the comparison of synthetic TC with TC generated from cysteine and formaldehyde and by the constancy of the equilibrium constants over a fivefold range of initial TC concentration. The apparent equilibrium constants for TC formation, $K_{F_{app}}'$, above pH 4 are related to pH-independent equilibrium constants expressed in terms of macroscopic ionization states for cysteine and TC by eq 5 (see Discussion and Scheme III), where α_{TC^-} is the fraction of TC as the anion.

$$1/K_{F_{app}}' = (1 - \alpha_{TC^-})/K_{F_2}' + \alpha_{TC^-}(1 + G_3'/[H^+])/K_{F_3}' \quad (5)$$

Scheme III[a]



[a] At pH > 4: $K_{F_{app}}' = ([TC^\pm] + [TC^-])/\{([CYS^\pm] + [CYS^-] + [CYS^{2-}])[F]\}$; $K_{F_1}' = [TC^+]/([CYS^+][F])$; $K_{F_2}' = [TC^\pm]/([CYS^\pm]\cdot[F])$; $K_{F_3}' = [TC^-]/[CYS^-][F])$; F = formaldehyde hydrate.

At much higher formaldehyde concentrations a second formaldehyde molecule adds to cysteine to form N-hydroxymethyl-TC (see below).

N-Hydroxymethyl-thiazolidine-4-carboxylate Formation. From the formol titration of TC at high enough concentration of TC that its hydrolysis was insignificant, the equilibrium constants for neutral and anionic N-hydroxymethyl-TC formation from neutral and anionic TC and formaldehyde were calculated (Table III).

Discussion

Proton Dissociation Scheme for Cysteine. Methods for determination of the seven microconstants for cysteine required for the complete description of the pH dependence of the concentrations of the eight species in solution (Scheme I) have been presented and discussed elsewhere.[10p] In this study we have utilized two independent methods: (1) Wegscheider's principle of equivalence and (2) spectroscopic titration (see below).[26]

(25) A. A. Frost and R. G. Pearson, "Kinetics and Mechanism," 2nd ed, Wiley, New York, N. Y., 1961.

(26) Additional approaches to the determination of the microconstants of cysteine have utilized the pH dependence of the rate of reaction of the thiol group of cysteine with chloroacetamide[10j] or enthalpic

Table IV. Equilibrium Constant Determination for Thiazolidine-4-carboxylate Formation at 25°, Ionic Strength 1.0 M[a]

Initial TC × 10^3 M	Concentration — Equilibrium — Cysteine[b] × 10^5 M			TC × 10^3 M			$K_{F_{app}}'$[c] × 10^{-5} M^{-1}		
	pH 5.57	pH 6.56	pH 7.45	pH 5.57	pH 6.56	pH 7.45	pH 5.57	pH 6.56	pH 7.45
7.5	10.8	5.96	2.62	7.39	7.44	7.47	6.33	20.9	146
6.0	9.96	5.18	2.27	5.90	5.95	5.98	5.94	22.2	116
4.0	7.61	4.28	1.83	3.92	3.96	3.98	6.76	21.6	119
2.0	5.31	2.91	1.28	1.95	1.97	1.99	6.91	23.3	121
1.0	3.77	1.93	0.83	0.96	0.98	0.99	6.75	26.3	144
0.5	2.69	0.98	0.60	0.47	0.49	0.49	6.49	24.6	136
						$K_{F_{app}}'$(av) × 10^{-5} M^{-1}	6.53	23.2	130
						Stand dev	0.32	1.84	12.0

[a] pH maintained with potassium phosphate buffers 0.01 M; 0.01 M EDTA present. [b] $[H_2C(OH)_2]_{eq} = ([CYS^{\pm}] + [CYS^-] + [CYS^{2-}])_{eq}$. [c] $K_{F_{app}}'$ defined in Scheme III (see Tables I and III and eq 5).

(1) In the former method, the proton dissociation constant of a derivative in which a proton is replaced by a methyl group[30] and cannot dissociate is used to estimate the corresponding microscopic dissociation constant.[10p,31] In practice the Wegscheider principle appears to have been valid for the use of an ester group as a model for the undissociated carboxylic acid group in the cases of dicarboxylic acids,[10p] glutamic acid,[10p,31c] tyrosine,[31a,b,d] and, in our work, cysteine. Furthermore, in the case of mercaptoacetic acid[31d] and now cysteine, the *S*-methyl analogs appear to be valid models for the −SH group. This is indicated by the agreement of both the present data and that of Grafius and Neilands,[10e] based on SMC, with the data from spectroscopic techniques in the present and earlier[10d] studies.

(2) Spectroscopic titration, which may directly detect the net ionization state of a specific group, for example the thiolate anion which is uv active[10d] or −SH which is Raman infrared active,[10l] can provide data for the calculation of microconstants based on the assumption in the former case that the molar absorptivity is not significantly altered as the ionization state of neighboring groups changes. In the cases of cysteine[10d,g,32,33] and 2-mercaptoethylamine[10d,19,33] there are small shifts of the wavelength of the maximum ultraviolet absorbance (231–237 nm) as a function of pH in the alkaline region. These shifts have been attributed to the changing state of ionization of the adjacent amino group as indicated by the fact that the position of the maximum is independent of pH in the case of ionization of aliphatic thiols not possessing an adjacent amino group, for example, mercaptoacetic acid[10d] (*cf.* ref 33). The occurrence of such shifts of the wavelength of the maximum absorbance has led to doubts about the validity of the use of the spectrophotometric method.[10f,g,r,33,34]

Data on the spectral and acid–base properties of thiols (Table V) show that the logarithms of the molar absorptivities for aliphatic thiols fall within the range 3.65–3.81, despite differences in the maximum wavelength, pK_a' value, and the nature of the substituents.[35] From the relative constancy of the molar absorptivities of thiol anions and the spectrophotometric inertness of ammonium proton dissociation, for example, alanine[10d] and our data for SMC, we conclude that the molar absorptivities of the various thiolate anions of 2-MEA and cysteine are similar and may be nearly identical. Furthermore, pK_a' calculations are not very sensitive to the difference in the molar absorptivity between the thiolate zwitterion ($RCHNH_3^+$-CH_2S^-) and anion ($RCHNH_2CH_2S^-$), $\epsilon_{\pm}$ and ϵ_-, respectively, and involve only an additional term, log $[(\epsilon_{\pm} - \epsilon_0)/(\epsilon_- - \epsilon_0)]$, in the logarithmic form of eq 2 and 3, where ϵ_0 is the molar absorptivity of species with un-ionized thiol groups. Thus, the maximal variation in molar absorptivities revealed in Table V leads to maximal differences in pK_a' of only 0.16 when ϵ_0 is relatively small as in the present cases.

In the case of cysteine the assumption that the molar absorptivities of the various thiolate anion species are quite similar appears to be correct on the following grounds. (a) The Raman infrared spectrum of cysteine shows no significant shift of the wave number of the −SH vibration as the state of ionization of the adjacent amino group is altered[10l] and analysis of the pH dependence of the peak height has provided independent estimates of the microconstants which are in good agreement with those obtained by the ultraviolet spectrophotometric method.[10d] (b) There is excellent agreement between the microscopic pK_a' values obtained by the spectrophotometric method and by the application of Wegscheider's principle of equivalence based on SMC (Table II)[37] (*cf.* ref 10p).

measurements.[10r] A discussion of the complicated interplay of factors which enter into the determination of nucleophilic reactivity and the use of reactivity to estimate basicity,[27] and a somewhat misapplied[28] attempt to utilize the microconstants for aminothiols in a kinetic study of their reactions with acrylonitrile,[29] have appeared.

(27) W. P. Jencks, "Catalysis in Chemistry and Enzymology," McGraw-Hill, New York, N. Y., 1969.

(28) R. G. Kallen, manuscript in preparation.

(29) M. Friedmann, J. F. Cavins, and J. S. Wall, *J. Amer. Chem. Soc.*, 87, 3672 (1965).

(30) The use of amide groups as models for undissociated carboxylic acid groups is less satisfactory both in theory and practice[31a] (*cf.* ref 31b).

(31) (a) R. B. Martin, J. T. Edsall, D. B. Wetlaufer, and B. R. Hollingworth, *J. Biol. Chem.*, 233, 1429 (1958), and references therein; (b) E. Brand, W. H. Goldwater, and L. Zucker, Abstracts, 106th National Meeting of the American Chemical Society, New York, N. Y., Sept 1944, p 19B; (c) A. Neuberger, *Biochem. J.*, 30, 2085 (1936); (d) J. T. Edsall, R. B. Martin, and B. R. Hollingsworth, *Proc. Nat. Acad. Sci. U. S.*, 44, 505 (1958).

(32) (a) G. A. Anslow and M. L. Foster, *J. Biol. Chem.*, 97, 37 (1932); (b) J. R. Loofbourow, cited in G. H. Beaven and E. R. Holiday, *Advan. Protein Chem.*, 7, 319 (1952); (c) C. Fromageot and G. Schnek, *Biochim. Biophys. Acta*, 6, 113 (1950).

(33) R. H. DeDeken, J. Broekhuysen, J. Béchet, and A. Mortier, *ibid.*, 19, 45 (1956).

(34) One effort to resolve the uncertainty about molar absorptivities by solvent and ionic strength effects on the spectrophotometric titrations of cysteine has met with only partial success.[10f]

(35) In the cases of NAC and gluthathione a small contribution of the amide absorbance is present.[36]

(36) W. B. Gratzer in "Poly-α-amino Acids," G. D. Fasman, Ed., Vol. 1, Marcel Dekker, New York, N. Y., 1967, p 177.

(37) The tautomerization constant expressing the ratio of species

Table V. Molar Absorptivities of Thiol Anions in Aqueous Solution

Compound	λ_{max}, nm	log ϵ[a]	Ref	pK_a'	Ref
2-Propanethiol	240	3.73	*j*	10.6	*j*
				10.65	*o*
n-Butanethiol	238	3.7	*k*	10.65	*o*
2-Methyl-2-propanethiol	243	3.71	*l*	11.05	*s*
2-Mercaptoethanol	233	3.75	*m*	9.51	*p*
				9.5	*q*
	235	3.71	*j*	9.48	*r*
				9.6	*j*
				9.43	*s*
2-Mercaptoacetate	240[c]	3.87	*j*	10.24	*t*
	237	3.65	*b*	10.32	*n*
				10.40	*j*, *r*
2-Mercaptopropionate	237	3.70	*j*	10.20	*j*, *r*
Mercaptosuccinate	238	3.67	*b*		
	238	*d*	*j*		
N-Acetylcysteine	232	3.75	*b*	9.51	*b*
Glutathione	234 (s)	3.78	*j*	9.12	*q*
				9.20	*n*
2-Mercaptoethylamine	232–236	*e*	*n*	8.35	*n*, *r*
				8.11	*t*
	230–235	*d*	*j*	8.6	*q*
L-Cysteine	231–237	3.62	*n*	8.54,[f] 9.85[g]	*b*
	231–236	3.68	*j*		
	236	3.68	*b*	7.44,[h] 8.32[i]	*b*
L-Cysteine methyl ester	234	3.73	*b*		
Thiocholine	229	3.81	*b*	8.17	*b*

[a] ϵ (cm^{-1} M^{-1}), (s) = shoulder. [b] This work, 25°, ionic strength 1.0 *M*, pH 11.8 ± 0.1. [c] At pH 12.4; shifts of the wavelength of maximum absorbance were observed in the course of thiol ionization and further shifts to longer wavelength were observed beyond the pH region of the ionization. [d] Not reported. [e] In range 3.60–3.78. [f] pK_{12}'. [g] pK_{132}'. [h] pK_2'. [i] pK_{23}'. [j] Reference 33. [k] L. H. Noda, S. A. Kuby, and H. A. Lardy, *J. Amer. Chem. Soc.*, **75**, 913 (1953). [l] M. J. Murray, *Anal. Chem.*, **21**, 941 (1949). [m] K. L. Brown and R. G. Kallen, unpublished observations. [n] Reference 10d. [o] D. L. Yabroff, *Ind. Eng. Chem.*, **32**, 257 (1940). [p] G. E. Lienhard and W. P. Jencks, *J. Amer. Chem. Soc.*, **88**, 3982 (1966). [q] Reference 10n. [r] See Danehy and Noel, Table II, footnote *m*. [s] M. M. Kreevoy, E. T. Harper, R. E. Duvall, H. S. Wilgus, III, and L. T. Ditsch, *J. Amer. Chem. Soc.*, **82**, 4899 (1960). [t] Reference 10j.

The evaluation of the microconstants for cysteine at constant temperature and ionic strength enables calculation of the fraction of total cysteine as any species at pH values from 0 to 14 (see Appendix in ref 9 and Figure 3 in ref 10d) and, therefore, detailed analysis of kinetic data[9] or correction of pH-dependent equilibrium constants to pH independent values (see below). The success of such analyses is an additional confirmation of the correctness of these constants.

Thiazolidine-4-carboxylate Formation. The formation of TC from formaldehyde and cysteine, with consideration given to the two macroscopic proton dissociation constants of TC and the three macroscopic proton dissociation constants of cysteine, is depicted in Scheme III. Equation 5 is derived from Scheme III and accounts for the pH dependence of the apparent equilibrium constants at pH values greater than 4. Due to the instability of cysteine in long term experiments at alkaline pH and our main interest in the physiological pH range, the equilibrium constant determinations were restricted to the pH region 5–7.5.

The values of the equilibrium constants, K_{F_2}' and K_{F_3}' (Table III), for the formation of TC$^{\pm}$ from CYS$^{\pm}$ and TC^{-} from CYS^{-}, respectively, were obtained from the pH dependence of the apparent equilibrium constants for TC formation and eq 5 by a nonlinear least-squares fit[38] of the data contained in Table IV. Decreased adduct formation has been noted for the cysteine–acetone adduct with increasing acidity.[3e]

Since the carboxylic acid ionization constants differ little for cysteine (pG_1' = 2.00) and TC (pK_{TC_1}' = 1.63) (Table I), the equilibrium constant for TC formation, K_{F_1}' and K_{F_2}', for TC^{+} from CYS^{+} and TC$^{\pm}$ from CYS$^{\pm}$, must differ by approximately threefold, since $G_1'/K_{TC_1}' = K_{F_1}'/K_{F_2}'$ (Scheme III). The values of K_{F_2}' and K_{F_3}' indicate that the formation of TC$^{\pm}$ from CYS$^{\pm}$ is more than two orders of magnitude less favorable than the formation of TC^{-} from CYS^{-}. This may be attributed largely to the destabilizing influence of protonation on the nitrogen of the thiazolidine ring relative to the destabilization of cysteine by protonation on nitrogen. This observation in conjunction with consideration of the pK_a' values for the ammonium proton dissociation for proline[4,10p] (pK_a' = 10.60) and TC (pK_a' = 6.24) suggests that the larger sulfur atom in a five-membered ring, although of approximately the same electronegativity as carbon,[39] imposes greater sp^2 character in the nitrogen hybridization. The lower pK_a' value for TC of 6.24 may reflect the difficulty this thiazolidine nitrogen atom has in accommodating the sp^3 hybridization required for nitrogen protonation and, possibly, the loss of double bond–no bond reso-

12 to 13 (Scheme I) is about 2, in close agreement with several other groups[10d-f,l,p,s] (*cf.* ref 10c and 10e). The disagreement in tautomerization constants may be attributable to an incorrect pK_a' value (8.60)[10c] for SEC (Tables I and II) and, since inconsistency arises within the same investigation,[10e] to the probability that $-N^+(CH_3)_3$ is not a valid model for $-NH_3^+$. A similar discrepancy with a smaller effect of $-N^+(CH_3)_3$ than of the $-NH_3^+$ group on the ionization of neighboring groups has been observed in the analysis of the ionization scheme for tyrosine[3l,a,d] and is not unexpected in view of the rather complicated effects that methylation and hydroxymethylation[5] have on ammonium and substituted ammonium proton dissociation constants.

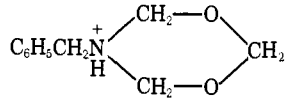
(38) For the data in the region pH 5.5–7.5, alternatively, a linear plot of $K_{F_{app}}'/\alpha_{CYS^-}$ against $\alpha_{CYS^0}/\alpha_{CYS^-}$ can be used in which α_{CYS^-} and α_{CYS^0} are the fraction of total cysteine with a single net negative charge and zero net charge, respectively. The slope of such a plot is K_{F_2}' and the ordinate intercept is K_{F_3}'.

(39) L. Pauling, "The Nature of the Chemical Bond," 3rd ed, Cornell University Press, Ithaca, N. Y., 1960.

nance contributions from $-S^- CH_2{=}{}^+N<$ canonical forms.[40]

N-**Hydroxymethyl Adduct Formation.** The equilibrium constants for the addition of the first and second formaldehyde molecules to the amino group of SMC indicate that the first addition occurs more than tenfold more readily than the second addition.[4,5] Since all tautomeric species are included in the equilibrium constant for monohydroxymethylamine formation, a small contribution for the formation of the zwitterionic species, $R^+NH_2CH_2O^-$,[41] may be present.

The equilibrium constants, $L_{1p}' = [R^+NH_2CH_2OH]/[R^+NH_3][F]$, for the formation of protonated monohydroxymethylamines from formaldehyde and N-protonated amino acids, are in the range 0.04–0.40 M^{-1} at 20° and water activity 1.0.[24,42] The pK_a' value for *N*-dihydroxymethylbenzylammonium ion has been calculated from the Taft equation to be 4.13[43] in rough agreement with the measured range 5.3–6.0 for triformalbenzylamine,[43] a difference in acidity from the



parent compound, benzylammonium ion (pK_a' = 9.34), of about 10^4. If extrapolation of these data to cysteine is approximately correct, from the relationships between the equilibrium constants, $K_{a_1}' = [RNH_2]\cdot a_{H^+}/[RNH_3^+]$, $L_2' = [RN(CH_2OH)_2]/[RNH_2][F]^2$, $K_{a_p}' = [RN(CH_2OH)_2]a_{H^+}/[R^+NH(CH_2OH)_2]$, the equilibrium constant L_{2p}', *i.e.*, $L_2'K_{a_1}'/K_{a_p}' = [R^+NH(CH_2OH)_2]/[R^+NH_3][F]^2$, is about 1.3×10^{-3} M^{-2} based on the value for SMC of L_2' of 16.2 M^{-1} (Table III). Thus, estimates of the equilibrium constants L_{1p}' and L_{2p}' for the formation of the protonated mono- and dihydroxymethyl adducts of protonated primary amines indicate that such species were minor components in all solutions studied and could be validly neglected in our preceding calculations.

There is kinetic evidence for the formation of imines and cationic imines between ordinary aliphatic amines and aliphatic aldehydes.[9,27,44] From studies of amines and formaldehyde in concentrated sulfuric acid, the ratio of the concentrations of unhydrated cationic Schiff base to hydrated, *i.e.*, $K_{SBH}' = [>C{=}N^+<]/[HOCNH^+]$, has been estimated to be about 10^{-2} and this low value probably accounts in part for the general failure to directly observe cationic imines in aqueous solutions[43] at equilibrium.

From recent studies of imines[45–48] derived from aliphatic amines and aliphatic aldehydes, the ratio of the concentration of neutral imine to carbinolamine for isobutyraldehyde is about 14.[47,48] This result is probably not directly applicable to systems involving formaldehyde, since it is expected that the [imine]/[carbinolamine] ratio may vary among various aliphatic aldehydes just as the [>C=O]/[aldehyde hydrate] ratio varies.[49,50] The ratio of neutral imine to carbinolamine is also probably different with aromatic amines and/or aromatic carbonyl compounds in which additional resonance stabilization of the imines occurs.

Biochemical Considerations. For simple carbonyl compounds, which may be bound to proteins as substituted aldimines in cases in which there exists the juxtaposition of an amino and a sulfhydryl group, thiazolidine-like linkages may result, since the stability order for formaldehyde adducts of cysteine is the following: thiazolidine > N-protonated thiazolidine > hemithioacetal > *N*-hydroxymethylcysteine > *N,N*-dihydroxymethylcysteine > *N*-hydroxymethylthiazolidine > N-protonated *N*-hydroxymethylthiazolidine. No direct measure of the stability of the Schiff base was obtained in this study but competition and other studies have indicated the much greater stability of the pyridoxal phosphate cysteine thiazolidine than that of ordinary pyridoxal phosphate Schiff bases.[3i,k]

The ability of borohydride to trap imine intermediates[51,52] has provided evidence for the existence of Schiff bases or rapidly equilibrating derivatives thereof (*e.g.*, carbinolamines or substituted aldimines) in covalently bound enzyme–substrate intermediates. Borohydride-reduced imines have been observed in cases in which the amine and carbonyl compound are both aliphatic, perhaps due to binding energy contribution of other parts of the substrate with enzyme which enhances the stability of imine forms.

The aminothiol–formaldehyde equilibria appear to be relevant to the success in the utilization of aldehydes in protein systems as preservatives, virucidal, bacteriocidal, tanning, and tissue fixation agents,[12] active-site probes,[53] the inhibition by penicillamine of structural protein cross-linking,[54] pyridoxal–phosphate binding, and model studies involving the condensation of retinaldehyde.[3n–p] 2-Iminothiazolidine-4-carboxylate, which results from the heterolytic cleavage of disulfides involving cysteine by cyanide, has been shown to underlie the molecular basis for the cyanide activation of papain.[55] Hydroxythiazolidine intermediates also occur in the *S–N*-acyl transfer reaction of *S*-acetyl-2-mercaptoethylamine and in the related hydrolysis of thiazolines.[56]

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(40) J. Hine and F. E. Rogers, *J. Amer. Chem. Soc.*, 90, 6701 (1968).
(41) J. Hine and F. C. Kokesh, *ibid.*, 92, 4383 (1970).
(42) (a) P. Le Hénaff, *Bull. Soc. Chim. Fr.*, No. 11, 3113 (1965); (b) P. F. Feraud and P. Le Hénaff, *C. R. Acad. Sci. Paris*, 265, 72 (1967).
(43) J. DeLuis, "The Chemistry of Formaldehyde Amine Condensation Products," Ph.D. Thesis, Pennsylvania State University, 1964; *Chem. Abstr.*, 63, 8184d (1965).
(44) R. G. Kallen and W. P. Jencks, *J. Biol. Chem.*, 241, 5851 (1966), and references therein.
(45) J. Hine and C. Y. Yeh, *J. Amer. Chem. Soc.*, 89, 2669 (1967).
(46) A. Williams and M. L. Bender, *ibid.*, 88, 2508 (1966).
(47) J. Hine, F. A. Via, J. K. Gotkis, and J. C. Craig, Jr., *ibid.*, 92, 5186 (1970).
(48) J. Hine, J. C. Craig, Jr., J. G. Underwood, II, and F. A. Via, *ibid.*, 92, 5194 (1970).
(49) R. P. Bell, *Advan. Phys. Org. Chem.*, 4, 1 (1966).
(50) R. P. Bell and P. G. Evans, *Proc. Roy. Soc., Ser. A*, 291, 297 (1966).
(51) (a) W. D. Riley and E. E. Snell, *Biochemistry*, 7, 3520 (1968); (b) D. S. Hodgins and R. H. Abeles, *Arch. Biochem. Biophys.*, 130, 274 (1969); (c) D. J. George and A. T. Phillips, *J. Biol. Chem.*, 245, 528 (1970).
(52) I. A. Rose, *Annu. Rev. Biochem.*, 35, 23 (1966).
(53) K. Uyeda, *Biochemistry*, 8, 2366 (1969).
(54) K. A. Piez, *Annu. Rev. Biochem.*, 37, 547 (1968).
(55) I. B. Klein and J. F. Kirsch, *Biochem. Biophys. Res. Commun.*, 34, 575 (1969).
(56) R. E. Barnett and W. P. Jencks, *J. Amer. Chem. Soc.*, 91, 2358 (1969).