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Raman Spectra and Sulfhydryl Ionization Constants of Thioglycolic Acid and Cysteine*

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Raman spectra of thioglycolic acid and cysteine in different states of ionization are reported, with quantitative determinations of the intensities of the Raman bands in a photoelectric Raman spectrophotometer. Depolarization measurements are also reported. A method of calibrating polarization measurements with known reference substances is described. Ionization of the sulfhydryl group in thioglycolic acid not only abolishes the S-H stretching frequency at 2580 cm^{-1} but also is accompanied by marked increases in the intensities of a number of frequencies below 1000 cm^{-1}. In several of these frequencies, although not all, there is a parallel increase in the depolarization of the Raman bands. Apparent ionization constants (pK′ values) for the S-H group at high ionic strengths are calculated from the intensity of the 2580 cm^{-1} frequency as a function of pH, the reference standard of intensity being either (1) an attenuated beam from the primary light entering the Raman tube or (2) a reference line, assumed independent of pH, in the Raman spectrum of the substance itself. The two standards give results in reasonably close agreement; obviously the first is preferable in principle, but there are practical difficulties in applying it. The four microscopic pk values describing the ionization of the sulfhydryl and amino groups in cysteine were evaluated from the data, in the ionic strength range 0.5–1.0. The values found are in surprisingly close agreement with those obtained at much lower ionic strength, by Benesch and Benesch (1955), from ultraviolet absorption spectra.

We here report studies on the Raman spectra of two sulfhydryl compounds, thioglycolic acid and cysteine, involving quantitative determinations of the intensities and polarizations of the Raman bands. The effect of the ionization of the sulfhydryl group in both compounds, and of the carboxyl group in thioglycolic acid, are examined. The changes with pH in the intensity of the S-H stretching frequency near 2580 cm^{-1} permit the calculation of the apparent pK values of the sulfhydryl group. Earlier studies of the Raman spectrum of cysteine have been reported (Edsall *et al.*, 1950; Garfinkel and Edsall, 1958). However, these spectra were recorded photographically and the intensities of the Raman bands were estimated only qualitatively. While our own studies were in progress, Evans and Ellman (1959) reported some quantitative data which are in general accord with our own more extensive findings. Our data for cysteine permit the calculation of the four microscopic ionization constants—two for the amino and two for the sulfhydryl group—which were previously evaluated by Benesch and Benesch (1955) from ultraviolet absorption measurements. The relative faintness of the Raman spectra makes it necessary to work at relatively high concentrations, so that only apparent (pK′) values are obtained; and the quantitative precision of the measurements of Raman intensities is still inferior to that of ultraviolet spectra. Nevertheless, the agreement between the two sets of data is surprisingly good. In principle the use of the S-H stretching frequency in the Raman spectra has certain advantages, since it corresponds clearly to a specific vibrational mode of the ionizable group, whereas the interpretation of the ultraviolet absorption of the $-S^-$ group near 230 $m\mu$ is far more complex.

The Raman spectra of thioglycolic acid and the ions derived from it have not, to our knowledge, been previously determined. The study of this relatively simple molecule has revealed important changes in the frequencies, intensities, and polarizations of a large number of the Raman bands associ-

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ated with the ionization of the carboxyl and sulfhydryl groups.

Experimental

Cysteine hydrochloride was obtained from the Mann Research Laboratories, Brooklyn, N. Y., and was found to be of high purity, except for small traces of fluorescent impurities, which sometimes disturbed the measurements of the Raman spectrum. This fluorescence could be largely removed by treatment with Norite. Titration to more alkaline pH was carried out with KOH solution containing a small amount of KCN (approximately 1 mole per 150 moles KOH) to bind traces of heavy metal ions and inhibit auto-oxidation of the cysteine (Garfinkel and Edsall, 1958). The risk of oxidation was further greatly reduced by putting small measured volumes of a stock solution of cysteine hydrochloride into 5-ml volumetric flasks and then sealing each flask with a rubber stopper. Additions of KOH solution and withdrawals of samples were then made with syringes fitted with No. 24 needles. Solutions were deoxygenated by bubbling nitrogen through them briefly, before these operations were carried out. The air in the Raman spectrophotometer cell was washed out with a stream of nitrogen; the cell was then capped with a rubber stopper, and the solution under study was transferred by syringe from the stoppered volumetric flask to the cell. After the spectrum had been recorded the contents of the cell were emptied quickly into the beaker of a Beckman model G pH meter, and the pH was quickly read. Control studies indicated that no significant amounts of fluorescent impurities were given off by the rubber stoppers to the solutions.

Thioglycolic acid (Eastman Organic Chemicals, Rochester, N.Y., technical grade) was purified by distillation, the middle portion of the distillate being used for the spectroscopic studies. Preparations made from material that had initially been more highly purified gave identical Raman spectra. The preparation of the solutions for the spectroscopic studies and pH measurements was identical with that described for cysteine, except that clarification with Norite was not necessary.

The Raman spectra were obtained on a Cary Raman Spectrophotometer, Model 81 (Applied Physics Corporation, Monrovia, Calif.), which has been employed in other studies reported from this laboratory (Martin *et al.*, 1959; Martin, 1959; Martin and Edsall, 1958). The exciting line of the mercury arc was in all cases the blue line at 4358 A (22,938 cm^{-1}). Lines of shorter wave length were removed by a filter consisting of an isopropanol solution of *o*-nitrotoluene (40 ml/liter), and ethyl violet (0.55 g/liter) was also added to reduce the background at wave lengths above 4358 A. The only mercury line that falls in the same range as the Raman spectra is the moderately strong line at 4916 A (20,336 cm^{-1}). This line serves as a convenient marker to calibrate the frequency shifts of the Raman bands. It lies very close to the Raman S-H stretching frequency (2580 cm^{-1} from the exciting mercury line), but the two lines are clearly resolved.

The recorded intensities in the tables represent the relative heights of the Raman bands above the baseline. When different solutions were being compared, the intensities of the Raman bands were compared by multiplying by an appropriate factor, to allow for the relative concentrations in the different solutions and for the sensitivity settings on the Raman spectrophotometer. In many runs the intensity of an attenuated beam from the light incident on the Raman tube was recorded, in the hope that this would provide a basis for recording the intensity of the Raman lines relative to the incident intensity. However, the values so obtained were found to be somewhat inconstant from day to day, and even from run to run, perhaps owing in part to differences in background fluorescence. Therefore, to determine the intensity of the S-H frequency as a function of pH, it was found preferable to employ another line in the spectrum, with an intensity assumed to be independent of pH, as an internal standard (see below under Results).

Polarization measurements were made by surrounding the Raman tube with a cylinder of polaroid. Two such cylinders were available, one transmitting light polarized parallel, the other perpendicular, to the axis of the Raman tube. This arrangement corresponds to the first of the two methods described by Edsall and Wilson (1938) for polarization measurements. Ideally all the incident light should enter the Raman tube perpendicularly to its long axis, if true depolarization ratios (ρ) for the Raman lines are to be determined. This requirement is, of course, not fulfilled in practice with the present apparatus. The quantity measured in practice is the ratio R:

$$R = I \text{ parallel}/I \text{ perpendicular}$$

determined, for a given line, from the ratio of the measured intensities (I) when the electric vector of the incident light is, respectively, parallel or perpendicular to the axis of the Raman tube. Rank and Kagarise (1950) have concluded that a calibration curve plotting the observed ratio, R, against the true value of ρ, should be a straight line of the form:

$$R = \rho \operatorname{Cos} \theta + \operatorname{Sin} \theta \qquad (1)$$

where θ is the average angle between the direction of the incident light and the plane perpendicular to the axis of the Raman tube.

We have calibrated the polarization measurements by determining R for the bands in the Raman spectra of several pure liquids for which absolute values of ρ are known. The substances chosen were carbon tetrachloride, benzene, toluene, ethanol, acetic acid, acetone, and chloroform, all of which have been carefully studied by Simons (1935). Our R values were plotted against the ρ values of Simons; the resulting diagram showed considerable scatter,[1] but the data could be roughly described by a straight line, with the equation

$$R = 0.954\rho + 0.181 \qquad (2)$$

[1] Part of this scatter may be due to errors in some of Simons' values. Although his work was certainly done with great care, his spectra were of necessity photographically recorded, and the uncertainty in his results must have been appreciable.

This equation is, of course, empirical; it does not fit equation (1), since the slope term (0.954) would give $\theta = 17°$ and the intercept (0.181) would give $\theta = 10°$. However, we have used equation (2) to convert our observed R values to ρ values for the cysteine and thioglycolic acid spectra. The ρ values so obtained must naturally be regarded as only approximate, but they do afford valuable evidence concerning the frequencies involved. If the ρ value is below 0.6, we infer that the band in question is probably polarized; if it is above 0.75, the band may be depolarized, and it is almost certainly depolarized if $\rho > 0.85$. Some of the values in our tables are above the theoretical maximum of 6/7. Such higher values are, of course, without specific physical meaning and are simply to be taken as an indication that the line in question is depolarized.

Results and Discussion

Spectra of Thioglycolic Acid.—Spectra were determined on the pure acid, on a 4 M solution of the acid in water, and on 4 M solutions of the monopotassium salt at pH 7.3 and of the dipotassium salt at pH above 13. The results are recorded in Table I.

A full interpretation of the spectra cannot be offered here, but we note some major points. The observed intensities for the pure acid have been corrected in Table I by dividing them all by a factor equal to the concentration ratio of $HS \cdot CH_2 \cdot COOH$ in the pure acid to that in the 4 M solution. Nevertheless, the recorded intensity values in Table I are consistently higher, by a factor of the order of 2, for the pure acid than for the aqueous solution. This difference appears to be real; it is certainly greater than can be accounted for by the uncertainties in the calibration of the absolute intensity measurements. It may be largely accounted for by difference in the refractive indices of the two liquids.

The C=O stretching frequency in the pure acid is 1680 cm^{-1}; in the aqueous solution it is 1715. The molecules of the pure acid are undoubtedly associated as dimers, through hydrogen bonding of the carboxyl groups; in the aqueous solution the carboxyl groups are hydrogen bonded to water. The shift of 35 cm^{-1} (1715–1680) is considerably less than the corresponding shift in acetic acid, from about 1660 in the pure acid to 1720 in the aqueous solution (Edsall, 1936).

The apparently depolarized CH_2 bending frequency at 1395–1400, clearly visible in the un-ionized acid, is of course present in all states of ionization. Upon ionization of the carboxyl group it is partially masked by the very intense symmetrical stretching frequency of the ionized carboxyl; the asymmetrical stretching frequency of the latter (1550–1575) also appears. Other changes associated with the ionization of the carboxyl group are relatively minor.

The changes accompanying ionization of the sulfhydryl group are more numerous and striking. Most obvious, of course, is the disappearance of the S-H stretching vibration at 2580. A large group of the lower frequency vibrations, near 590, 700, 820, 925, and 1230, while altering little in frequency, become far more intense when the sulfhydryl group ionizes. Several of them, but not all, also show a marked rise in the depolarization factor ρ; this is also true of the line at 435, although its intensity decreases on ionization of the sulfhydryl group.

The change in character of the spectra on ionization of the sulfhydryl group is further illustrated by Figure 1, which was taken from a different set of spectra from those recorded in Table I. The amplification factor for the recording at pH 11.40 was somewhat higher than at pH 6.22; this can be allowed for by noting that the strong band at 1400 cm^{-1} should have the same intensity in both. Even when this correction is made, however, the great increase in intensity of most of the lines on the right-hand side of the figure at pH 11.4 is clearly apparent. The upward displacement of the baseline is very marked in this region, as is often the case for alkaline solutions, which are generally much more difficult to clarify than acid or neutral solutions. The height of the Raman peaks above

Table I

Raman Spectra of Thioglycolic Acid and the Ions Derived from It

	$HS \cdot CH_2 \cdot COOH$ Pure Acid[a]			$HS \cdot CH_2 \cdot COOH$ 4 M Acid (pH <1)			$HS \cdot CH_2 \cdot COO^-$ pH 7.31			$^-S \cdot CH_2 \cdot COO^-$ pH >13		
$\Delta\nu$ (cm^{-1})	I	ρ		$\Delta\nu$	I	ρ	$\Delta\nu$	I	ρ	$\Delta\nu$	I	ρ
415	31	0.47		435	24	0.55	435	19	0.41	435	12	0.70
575	28	0.29		585	13	0.21	590	9	0.16	595	39	0.46
670	40	0.27		685	16	0.28	710	9	0.31	700	40	0.50
765	42	0.37		775	25	0.31	770	24	0.31	785	24	0.49
815	44	0.42		825	24	0.36	820	11	0.31	815	33	0.60
900	30	0.28		905	18	0.17	925	12	0.25	935	38	0.22
985	18	0.52		1000	8	0.53	985	4				
1150	11	0.90		1150	6	0.94	1160	6	0.77	1130	9	0.86
1240	13	0.78					1240	4		1220	15	0.33
1395	28	0.88		1400	13	0.82	1390	42	0.45	1380	43	0.48
										1425	12	0.92
							1575	8	0.98	1550	8	0.95
1680[b]	21	0.16		1715	25	0.30	1650	5		1645	15	0.91
2570	205	0.25		2580	110	0.26	2580	83	0.27			
2930	105	0.14		2910	60	0.19	2940	50	0.15	2925	54	0.28
3000	36	0.72		2975	16	0.84	2975	19	0.87	2945	44	0.75

$\Delta\nu$ denotes Raman frequency shift (cm^{-1}); I denotes intensity, calculated from height of peak; ρ is depolarization factor.

[a] Pure thioglycolic acid also gives the following frequency shifts, not observed in the aqueous solutions: 220 cm^{-1}, I = 28, ρ = 0.70; 1180 cm^{-1}, I = 11, ρ = 0.90; 1290 cm^{-1}, I = 8, ρ = 0.52. [b] The 1680 peak in the pure acid is very broad, extending from about 1650 to 1725 cm^{-1}; the peak near 1650 at pH 7.32 is also broad, and is at least in part due to water.

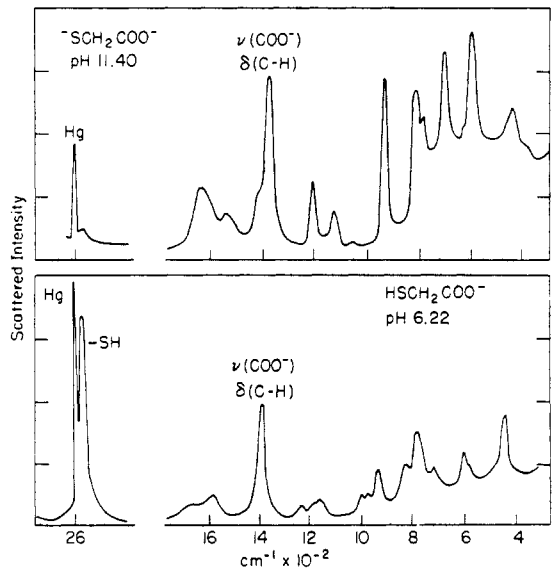

FIG. 1.—Raman spectra of the ions derived from thioglycolic acid at the indicated pH values. The peak marked Hg is the mercury line at 4916 A (20336 cm^{-1}), which closely adjoins the S-H stretching frequency. The symbol ν denotes a stretching frequency; δ (C-H) denotes the bending frequency of the methylene group.

the baseline, however, remains essentially constant for different preparations of solutions of the same composition, even though the baseline may be different from one preparation to another, depending on the amount of small scattering particles present.

These striking spectral changes accompanying ionization of the sulfhydryl group may reflect a change in the configuration of the molecule. In the ion $^-SCH_2COO^-$ the repulsion between the two negative charges should favor an orientation of the carboxylate group that places the two oxygens, which share the charge on this group, as far as possible from the negatively charged sulfur. The favored arrangement would presumably be that in which the plane of the COO^- group is perpendicular to the plane defined by the S-C-C linkages. This arrangement would have a plane of symmetry bisecting the $-COO^-$ group, whereas the ion $HSCH_2COO^-$ would have no plane of symmetry unless the S-H bond lay in the S-C-C plane—an orientation which is relatively improbable. The formation of a plane of symmetry in the $^-SCH_2COO^-$ ion would divide the 15 fundamental vibrations of this ion into two classes—nine vibrations of class A', symmetric with respect to the plane of symmetry, and polarized; and six antisymmetric and depolarized vibrations. In Table I, fourteen Raman bands are recorded for this ion, of which seven appear to be definitely polarized ($\rho < 0.6$), five are probably depolarized ($\rho > 0.75$), and two—the bands at 435 and 820—are intermediate, with $\rho = 0.6$ to 0.7. The striking increase in ρ values for a number of the lines when the $-SH$ group ionizes may be associated with the appearance of the suggested plane of symmetry on ionization. We offer this suggestion as a tentative approach to the interpretation of the data, without attempting a detailed analysis.

The Apparent pK Value of Thioglycolic Acid.—In a series of runs in which varying amounts of KOH were added to the monopotassium salt of the acid, the intensity of the 2580 cm^{-1} line was measured as a function of pH. As an internal standard the band at 1390 was chosen for comparison, since all the evidence indicated that the intensity of this band was little affected by change of pH above pH 6. From Table I it is apparent that, at pH near 7, the ratio $I_r^\circ = I_{2580}/I_{1390} = 83/42 = 2.0$ approximately. At higher pH values this ratio (I_r) decreases as the sulfhydryl group ionizes. The ratio I_r/I_r° may be taken as a measure of α_{SH}, the fractional ionization of the sulfhydryl group, and the pK' value may thus be calculated from the relation

$$pH = pK' + \log [\alpha_{SH}/(1 - \alpha_{SH})]$$

In other runs, I_{2580} was estimated on an absolute scale by comparison with the intensity of the attenuated primary beam from the light incident upon the Raman tube.

The pK' value obtained for 2 M thioglycolic acid —10.01 is an average value from the last column of Table II—is, of course, not readily compared with values obtained at lower ionic strengths. Benesch and Benesch (1955) have reported a value of 10.31 at 25° and ionic strength not precisely defined but near 0.2. Our own value was obtained primarily to explore the use of Raman spectra in determining such constants as a preliminary to the measurements on cysteine.

TABLE II

pK' VALUES OF THE —SH GROUP IN THIOGLYCOLIC ACID (2 M)

pH	pK' External	pK' Internal
9.15	9.78	9.97
9.38	10.09	10.10
9.60	10.06	
9.90	10.00	9.95
9.95	9.97	10.06
10.30	9.97	10.01
10.50	9.89	
10.65	9.91	9.96

The values of pK' external were determined by comparison of the band at 2580 cm^{-1} with the intensity of the attenuated primary beam; values of pK' internal by comparison of the intensity of the band at 2580 with that of the band at 1390 cm^{-1} in the same spectrum. The temperature in the Raman tube was approximately 25°, but the tube was not fitted with a thermostat.

Raman Spectra and Ionization Constants of Cysteine.—Since detailed Raman spectra of cysteine have been obtained photographically for the various states of ionization (Garfinkel and Edsall, 1958), our concern here is primarily with the quantitative determination of pK' values from the Raman spectra. However, some general comment on the spectra is desirable. Figure 2, with spectra at pH 6.7 and 11.25, shows the relative breadths and intensities of the major peaks; at the lower pH the $-SH$ group is un-ionized and the $-NH_3^+$ group is positively charged, whereas at the higher pH both groups have lost their protons. Most of the Raman bands reported by Garfinkel and Edsall (1958) are clearly visible; a few of the weaker bands recorded by them are difficult to distinguish from the background by the photoelectric method, and some

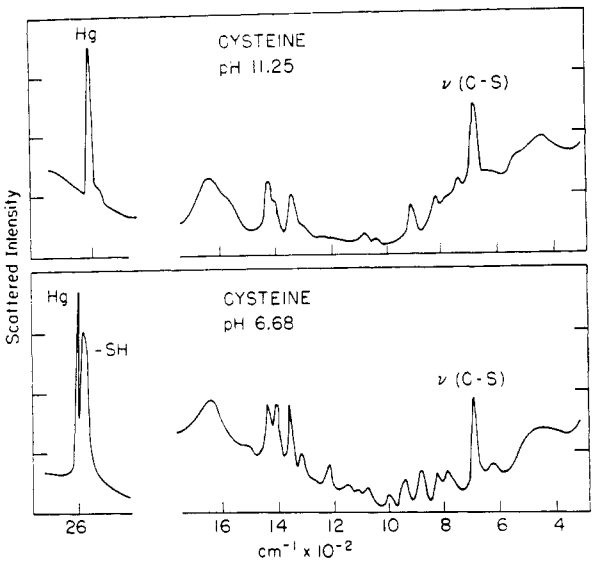

FIG. 2.—Raman spectra of cysteine as the dipolar ion (*bottom*) and as the doubly charged anion (*top*). Note that the S-H stretching frequency has not quite disappeared at pH 11.25. Compare with Figure 1.

which they reported to lie very close together are not clearly resolved in the present study. Indeed, the resolution of bands separated by only 10–15 cm^{-1}, as reported in several instances by Garfinkel and Edsall (1958), is uncertain; in such cases the photographic method, with an instrument of high dispersion and resolving power, may perhaps give better resolution than the photoelectric method. We confirm the finding that there is a marked downward displacement of the C-H stretching frequencies near 2900 cm^{-1}, when the amino and sulfhydryl groups lose their acidic protons. At pH 6.7 there is a very strong polarized band (I = 54, ρ = 0.25) at 2960 and a weaker depolarized band at 3005 (I = 31, ρ = 0.9 approx.). At pH 11.25 there is one strong band centered at 2920 (I = 31, ρ = 0.5) and a very weak band at 2875. These bands (not shown in Figure 2) correspond with the major features reported earlier, and our measurements with photoelectric recording establish this shift of the C-H stretching frequencies on a more objective basis than was possible with the earlier apparatus. Most of the shift is probably to be attributed to a loss of the proton from the charged $-NH_3^+$ group, for similar effects are observed for the C-H frequencies in glycine and β-alanine when this group loses its charge (Takeda *et al.*, 1958).[2] The ionization of the —SH group in thioglycolic acid (see Table I) causes a considerably smaller shift in the same direction.

For the study of the sulfhydryl ionization, we consider two strong bands in particular: the S-H stretching band at 2580 (ρ = 0.25) and the 680 band (ρ = 0.32–0.35 in acid and neutral solutions, rising to 0.47 at pH 10.2 and to 0.57 at pH above 13) which is usually interpreted as a C-S stretching frequency. The intensity of the latter appears to be independent of pH, even though its depolarization factor apparently varies, and it therefore serves as a convenient internal standard for determining the relative intensity of the 2580 band as it decreases with increasing pH. At pH values between 6 and 7, where we may assume the sulfhydryl group to be un-ionized, the peak height ratio, I_{2580}/I_{680}, is 1.66 ± 0.01. We denote this ratio as A_0, and the corresponding ratio at higher pH values as A. Then the fractional ionization of the S-H group, α_{SH}, may be taken[3] as:

$$\alpha_{SH} = 1 - (A/A_0) \qquad (3)$$

In Table III we list values of α_{SH} from equation (3), obtained over a range of pH values from 7.28 to 11.25. For comparison we have tabulated α_{SH}, as determined from the intensity of the band at 2580, corrected to a standard arc intensity by comparison with the attenuated beam, and also corrected for the sensitivity setting on the instrument. Although the latter method should theoretically be preferable to the use of an internal standard (the 680 band) the results obtained by this method are in practice somewhat less stable and reproducible. The two methods give results in generally close agreement at high α_{SH} values, but the discrepan-

TABLE III

FRACTIONAL IONIZATION (α_{SH}) OF THE SULFHYDRYL GROUP IN CYSTEINE AND COMPUTATION OF pM VALUES

pH	A = I_{2580}/I_{680}	α_{int} = 1 − (A/A_0)	$\alpha_{Ext.\ Std.}$	pM_{int}	$pM_{Ext.\ Std.}$
7.28	1.57	0.05	0.13	8.58	8.17
7.39	1.52	0.09		8.42	
7.70	1.49	0.13		8.53	
7.81	1.29	0.22	0.13	8.36	8.63
8.11	1.31	0.23		8.63	
8.32	0.99	0.40	0.39	8.50	8.51
8.73	0.93	0.46		8.80	
8.87	0.70	0.58	0.57	8.73	8.75
9.01	0.72	0.58	0.65	8.87	8.74
9.19	0.70	0.58		9.03	
9.40	0.57	0.66	0.72	9.11	8.99
9.42	0.50	0.70	0.70	9.05	9.04
9.80	0.46	0.72		9.32	
10.09	0.40	0.76		9.59	
10.31	0.32	0.81		9.68	
10.62	0.24	0.85	0.87	9.87	9.79
10.69	0.22	0.87	0.87	9.86	9.85
10.81	0.16	0.91	0.92	9.83	9.75
10.94	0.13	0.92	0.94	9.88	9.77
11.25	0.08	0.95	0.96	9.94	9.87

Total concentration of cysteine in all forms was 1 M in all solutions. Temperature was approximately 25°, but Raman tube was not fitted with a thermostat.

The value of $A_0 = I_{2580}/I_{680}$ in spectra taken at pH values between 6 and 7, is 1.66 ± 0.01. α_{int} denotes α_{SH} calculated from the ratio A/A_0; $\alpha_{Ext.Std.}$ denotes α_{SH} calculated from the height of the peak at 2580 cm^{-1} corrected to a standard arc intensity and sensitivity setting. The function pM is defined in the text. Values of pM_{int} are calculated from α_{int}; values of $pM_{Ext.\ Std.}$ from the values of $\alpha_{Ext.\ Std}$

[2] These effects in cysteine have also been noted by Evans and Ellman (1959). Further studies of the effect of ionization of the amino group on the C-H stretching frequencies in amino acids, aliphatic diamines, and related compounds have been carried out by S. A. S. Ghazanfar and J. T. Edsall, and will be reported later.

[3] We here make the assumption that the intensity of the band at 2580 depends only on the concentration of unionized sulfhydryl groups in the solution, and is not influenced by the presence or absence of a positive charge on the neighboring amino group. This assumption of course remains unproved; it is analogous to the assumptions commonly made in deducing pK values from ultraviolet absorption spectra. See the discussion of the microscopic pk values below.

cies are more marked at the low α_{SH} values. This is not surprising since, when α_{SH} is small, I_{2580} differs little from the maximum value it attains at pH below 7. Hence in this region a small error in measuring I_{2580}, and its ratio to I_{680}, leads to a relatively large error in α_{SH} (see equation 3).

Some calculations were made by comparing the areas, rather than the heights, of the peaks at 2580 and 680. The resulting α_{SH} values differed little from those derived by the simpler method of comparing heights. The ratio of height to area, for a peak corresponding to a given type of vibration, should be essentially constant, so that this agreement is probably to be expected.

It may appear surprising that the use of an internal standard for calibration should give such consistent results. As we have pointed out above, the point-group symmetry of thioglycolate may alter when the sulfhydryl group acquires a negative charge. Certainly the intensities of many of the Raman bands of lower frequency change drastically when this occurs. Nevertheless the bands at 1390 in thioglycolic acid, and at 680 in cysteine, appear essentially unchanged in intensity when the sulfhydryl ionization occurs. The apparent change in the depolarization value of the latter frequency at high pH values is indeed puzzling, and we can at present only record as an empirical fact the generally good correlation between the values of α_{SH} obtained by the two methods of calibration.

Calculation of the Microscopic Ionization Constants of Cysteine.—The overlapping of the ionizations of the —SH and $-NH_3^+$ groups in cysteine requires that the process be described in terms of four microscopic ionization constants. Denoting the carboxyl group by the symbol 1, the sulfhydryl by 2, and the ammonium group by 3, the ionization scheme for the two latter groups may be written:

$$\left(R^{-SH}_{-NH_3^+}\right) \underset{k'_{13}}{\overset{k'_{12}}{\rightleftharpoons}} \left(R^{-S^-}_{-NH_3^+}\right) \underset{k'_{132}}{\overset{k'_{123}}{\rightleftharpoons}} \left(R^{-S^-}_{-NH_2}\right) \qquad \left(R^{-SH}_{-NH_3^+}\right) \underset{k'_{13}}{\overset{k'_{12}}{\rightleftharpoons}} \left(R^{-SH}_{-NH_2}\right) \underset{k'_{132}}{\overset{k'_{123}}{\rightleftharpoons}} \left(R^{-S^-}_{-NH_2}\right)$$

The last numeral in the subscript of each k′ value denotes the ionizing group; the preceding numerals denote the acidic groups that have already lost their protons before the ionization in question takes place (Edsall and Wyman, 1958). In terms of the molecular species given in the scheme above, the value of α_{SH} may be written:

$$SH = \frac{\left(R^{-S^-}_{-NH_3^+}\right) + \left(R^{-S^-}_{NH_2}\right)}{\text{(total conc. of cysteine in all forms)}} \qquad (4)$$

We define the function pM_{SH} by the equation:

$$pH = pM_{SH} + \log \frac{\alpha_{SH}}{1 - \alpha_{SH}} \qquad (5)$$

It is readily shown (Edsall *et al.*, 1958; Martin *et al.*, 1958) that pM_{SH} is related to the microscopic constants in the ionization scheme above by the equation:

$$pM_{SH} = -\log \frac{k'_{12}(H^+) + k'_{13}k'_{132}}{(H^+) + k'_{13}} \qquad (6)$$

As $\alpha_{SH} \to 0$, (H^+) becomes very large with respect to all the k′ values, and pM_{SH} approaches pk'_{12} as a limit; as $\alpha_{SH} \to 1$, (H^+) becomes very small with respect to all the k′ values, and pM_{SH} approaches pk'_{132} as a limit. A plot of pM_{SH} as a function of α_{SH} is given in Figure 3; the data for α_{SH} calculated by equation (3) and listed in the column headed α_{int} in Table III are employed. Although the scatter of the points is greater than for the corresponding plot (Edsall *et al.*, 1958, p. 515) based on the ultraviolet absorption data of Benesch and Benesch (1955), the limiting extrapolation of pM_{SH}, to give pk'_{12} at $\alpha = 0$ and pk'_{132} at $\alpha = 1$, may still be made with considerable confidence. Given these two constants, the other two microscopic constants, pk'_{13} and pk'_{123}, may be calculated from the data by methods previously described (Edsall *et al.*, 1958; Martin *et al.*, 1958).[4]

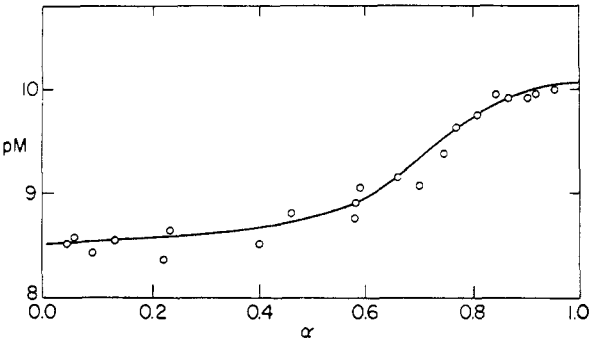

FIG. 3.—The function pM_{SH} (equation 6) in its dependence upon α_{SH} for cysteine.

The resulting values are, for the —SH group, $pk'_{12} = 8.50$, $pk'_{132} = 10.00$; for the $-NH_3^+$ group, $pk'_{13} = 8.85$, $pk'_{123} = 10.35$, with estimated probable error of ± 0.05. These values are remarkably close to those reported by Benesch and Benesch (1955) at ionic strength near 0.2, which were 8.53, 10.03, 8.86, and 10.36, respectively, for the four constants listed above. The agreement, however, cannot be considered particularly significant, since the cysteine concentration and the ionic strengths were much higher in our studies.

The results of Evans and Ellman (1959) were similar to, and entirely compatible with, our findings. They used 2.65 M cysteine solutions, whereas ours were 1 M, so that the activity coefficient corrections for their work are even more serious than for ours. They were troubled by oxidation and the formation of insoluble material (presumably cystine); the precautions we have described in the experimental section of this paper virtually eliminated this difficulty.

Obviously Raman spectroscopy is a more laborious and at present somewhat less accurate method for determining ionization constants than some others that are available. However, it has the advantage that a number of bands in the Raman spectrum can be clearly correlated with the vibrations of particular groups, so that different ionizing groups often can be discriminated from one another

[4] Since $pk'_{12} + pk'_{123} = pk'_{13} + pk'_{132}$, only three of the constants are independent.

more unequivocally than in ultraviolet spectroscopy. Raman spectra require much higher concentrations of solute than infrared spectra for accurate measurement; on the other hand, Raman spectra are well adapted for aqueous solutions, since the Raman bands of water appear in only two limited regions, and the bands due to the solute appear clearly everywhere else. The sulfhydryl group, which we have studied in this paper, is particularly suitable for such study, since the S-H stretching frequency at 2580 is a very strong line in the Raman spectrum, although relatively weak in the infrared.

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Hydroxyl Group Catalysis. II. The Reactivity of the Hydroxyl Group of Serine. The Nucleophilicity of Alcohols and the Ease of Hydrolysis of Their Acetyl Esters as Related to Their pKa′*

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Since the hydroxyl group of peptide-bound serine is purported to be the nucleophilic center of many esteratic enzymes, it is important to ascertain if this group possesses special nucleophilic properties toward the ester bond. We have shown that in a suitable model, *N*-acetylserinamide, it does not. The second-order rate constant (k_T) for the reaction of $ArO^{\ominus}$ and $AlkO^{\ominus}$ with *p*-nitrophenyl acetate and the second-order rate constants (k_{OH}) for the alkaline hydrolysis of $ArOCOCH_3$ and $AlkOCOCH_3$ can be correlated with the pKa′ of ArOH or AlkOH *via* the expressions $\log k_T = 0.76\ pKa' - 6.3$ and $\log k_{OH} = -0.26\ pKa' + 2.56$. The pKa′ of *N*-acetylserinamide has been determined conductometrically (13.60 ± 0.05) and has been found to correlate the $\log k_T$ and the $\log k_{OH}$ values for *N*-acetylserinamide anion and *N,O*-diacetylserinamide respectively. To dispel misconceptions on the mechanism of the reaction of *N*-acetylserinamide anion with *p*-nitrophenyl acetate we have determined the second-order rate constant for the reaction of $CF_3CH_2O^{\ominus}$ with *p*-nitrophenyl acetate.

In the sequence (1) both $\log k_T$ and $\log k_{OH}$ should be linear functions of the pKa′ of ROH. Thus, the log of the second-order rate constants for the displacement of *p*-nitrophenol from *p*-nitrophenyl acetate by a series of 4(5)-substituted imidazoles was shown to be a linear function of the pKa′ of the imidazoles employed (Bruice and Lapinski, 1958). Inferences are that this would be

$$ROH \overset{Ka'}{\rightleftharpoons} RO^{\ominus} + H^{+}$$

$$RO^{\ominus} + CH_3\overset{O}{\overset{\|}{C}}-OR' \xrightarrow{k_T} CH_3-\overset{O}{\overset{\|}{C}}-OR + \overset{\ominus}{O}R' \qquad (1)$$

$$CH_3-\overset{O}{\overset{\|}{C}}-OR \xrightarrow[OH^{\ominus}]{k_{OH}} CH_3COO + R\overset{\ominus}{O}$$

so also for other series of nucleophiles, including aryl and alkoxides (Bruice and Lapinski, 1958; Jencks and Carrioulo, 1960). The correlation of $\log k_{OH}$ to the pKa′ of ROH is self-evident in the success of the Hammett $\rho\sigma$ relationship (Hammett, 1940) in correlating both pKa′ of ROH and k_{OH} (Ballinger and Long, 1960; Taft, 1956).

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