TABLE VII[a]

SOME SKELETAL VIBRATIONS OF GLYCINE AND β-ALANINE

Substance	Solvent	Form	ν_{C-N}	ν_{C-C}	$\delta(<OCO)$	$\delta(<CCO)$
Glycine	H_2O	Anion	?	896(6)		512(3b)
		Dipolar ion	1031(3)	896(7)	667(1b)	511(3b)
		Cation	1043(4)	871(7)	657(vw)	503(2b)
	D_2O	Anion	?	834(2)?	650(vw)	482(vw)
		Dipolar ion	1000(2)	840(3)	640(1)	500(1b)
		Cation	1005(4)	774(4b)	640(vw)	490(vw)
β-Alanine	H_2O	Anion	1071(3b)?	876(4), 933(5)		
		Dipolar ion	1040(4)	866(8), 928(7)		
		Cation	1040(4)	817(7), 864(8), 906(3), 926(3)		
	D_2O	Anion	1033(1)?	900(5)		
		Dipolar ion	1023(3)	850(5), 890(6)		
		Cation	1019(4)	810(6), 845(5)		

[a] The frequencies labelled ν_{C-N} and ν_{C-C} are not to be ascribed uniquely to particular bonds; both probably involve the whole molecular skeleton. Detailed assignments cannot yet be made; see text.

these configurations is stabilized and the possibility of transition to the other is blocked.

Comparison with Infrared Data.—Our studies are closely related to the infrared measurements of Lenormant.[8b] His measurements in general give results quite concordant with ours. He has recorded a number of lines between 2600 and 2900 in the glycine spectrum which are not observed under our conditions in the Raman spectrum. These may be due to harmonics or combination frequencies. He reports a C–H stretching frequency at 2940 which is unchanged by deuteration, whereas in the Raman spectrum the C–H stretching lines of glycine occur at 2970 and 3010. The reason for this difference is not clear, but similar differences between infrared and Raman spectra are observed frequently in this region of the spectrum. The general agreement between Lenormant's measurements and ours may be regarded as very satisfactory.

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[CONTRIBUTION FROM THE BIOLOGICAL LABORATORIES, HARVARD UNIVERSITY]

Raman Spectra of Amino Acids and Related Compounds. X. The Raman Spectra of Certain Peptides and of Lysozyme[1-3]

BY DAVID GARFINKEL AND JOHN T. EDSALL[4]

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Raman spectra are reported for asparagine, glycylglycine, glycyl-DL-serine, glycyl-DL-valine, L-alanyl-L-alanine and D-alanyl-L-alanine. Glycylglycine was studied as the dipolar ion, anion and cation; the other compounds as the dipolar ion and cation only, except that asparagine was observed only as the cation in acid solution. Spectra of polylysine and of lysozyme from egg white are also reported. The carbonyl stretching frequency of the un-ionized –COOH group is weak or missing in the cationic forms of most of the peptides studied. The C–H stretching and deformation frequencies observed in the constituent amino acids are generally present without much change in the peptides, as are some other vibrations associated with the side chains. The skeletal frequencies characteristic of the free amino acids are missing or markedly displaced. The Raman spectra of LL- and DL-alanylalanine in solution are much more nearly alike than the infrared spectra of the same substances in the crystalline state, as determined by Ellenbogen. The Raman spectrum of lysozyme is faint, but some characteristic frequencies can be clearly identified. The spectrum of lysozyme is also faint, but reveals a number of frequencies not recorded in the infrared spectra reported earlier by other workers.

In the preceding papers[3] of this series the Raman spectra of free amino acids have been described. Peptides and proteins can also be studied by this technique, although the difficulty of recording the spectra photographically increases with increasing size of the molecule, since this increases the Rayleigh scattering, while the Raman scattering is roughly proportional to the mass of molecule per unit volume. This paper reports studies on several dipeptides, a polypeptide and a protein. Whenever it seems profitable, comparison is made with infrared spectra obtained by other workers on the same

(1) For the preceding paper of this series, see M. Takeda, *et al.*, THIS JOURNAL, **80**, 3813 (1958).

(2) Taken in part from the Ph.D. thesis of David Garfinkel, Graduate School of Arts and Sciences, Harvard University, 1955.

(3) The work reported here was supported by a grant from the National Science Foundation (NSF-G621) and by a special fellowship granted to one of us (J.T.E.) by the John Simon Guggenheim Memorial Foundation.

(4) To whom inquiries concerning this paper should be sent.

substances. The tripeptide glutathione, which was also studied, is described in a separate communication, owing to the special problems associated with it; however, the findings will be referred to here when necessary. Asparagine, an amino acid amide, is included with the peptides, since the amide group is closely related to the peptide group.

Experimental

The experimental technique has been described.[5] An optical anomaly in one of the three prisms in the apparatus used partially obscured the region from 1600 to 1700 cm.$^{-1}$ in nearly all the spectra reported here, and they should accordingly be considered incomplete in this region. Replacement of the defective prism has since corrected the anomaly.

Raman intensities have been estimated from the photographs and are designated 1, 2, 3 .... in order of increasing intensity, except that the faintest lines are designated weak (w) or very weak (vw). Broad lines are designated by (b) or (vb), respectively.

Infrared intensities are described as strong (s), medium (m) or weak (w). No attempt has been made to correct the Raman intensities for differences in concentration. Concentrations are reported in weight per volume, based on the weight of the isoelectric compound.

Materials.—All the peptides studied required treatment with charcoal (Norit) at slightly acid *p*H before satisfactory spectra could be obtained. This treatment removes dust particles, and also apparently removes some fluorescent impurities.

Asparagine (Hoffman-LaRoche, Nutley, N. J.) was studied in 29% solution as the hydrochloride.

Isoelectric glycylglycine (from the California Foundation for Biochemical Research, Los Angeles, California) was prepared in 16% solution by filtration with charcoal through Whatman No. 4 filter paper. To prepare the cation (as the hydrochloride) HCl was added to *p*H 1 or below. To prepare the anion (as the sodium salt) NaOH was added to *p*H 11–12.

Glycylserine and glycylvaline were obtained from the California Foundation for Biochemical Research and prepared as 10 and 18% isoelectric solutions, which were subsequently converted to the hydrochlorides by adding excess HCl. The spectra of glycylserine were incomplete.

The Raman spectra of the alanylalanines, as given in Table V, were determined in connection with the study of their infrared spectra by Ellenbogen[6]; the samples used for the infrared spectra were kindly provided by Dr. Ellenbogen. D-Alanyl-L-alanine, which was available only in a very small quantity, was prepared as a 5% isoelectric solution. After the spectrum of the isoelectric peptide had been obtained, it was converted to the hydrochloride by adding excess HCl directly to the solution. L-Alanyl-L-alanine was handled in the same way, but at an initial concentration of 25%.

The only polypeptide available to us for examination was polylysine. A sample of average chain length 16 was kindly furnished by Dr. A. Kameraad of the Kremers-Urban Co. A 20% solution was prepared and found to be fluorescent. The fluorescence was not entirely quenched by making the solution 7% in KI. The sample was accordingly treated with Amberlite XE-67 on the iodide cycle, in a manner similar to the treatment of lysine.[7] This procedure may remove picrate ion which may remain in traces after its use in isolating the lysine used in preparing the polylysine. A 12% solution was obtained yielding a fairly good spectrum.

Lysozyme was prepared from the whites of four dozen fresh eggs by the procedure of Alderton and Fevold[8] and recrystallized three times. The crystals were then washed free of inorganic ions (such as bicarbonate) which might interfere, by sedimentation in 0.0001 *N* NaOH containing 5% NaCl. In the course of checking this procedure, the value of $E_{1\%}$ of lysozyme at 280 mμ was determined as 21.2. The lysozyme crystals were dissolved by addition of a minimal amount of dilute HCl, lyophilized and dissolved to yield an 18% solution at approximately neutral *p*H. This proved to be supersaturated, most of the lysozyme crystallizing out. A 7% solution was stable and was clarified by centrifugation at 20,000 r.p.m. in a Spinco Model L preparative centrifuge. Several photographs of the Raman spectrum were taken (with the 4358 Å. exciting line blocked out by a piece of black tape to avoid extreme overexposure of the film in this region), and then the solution was made 1.5% in KI to quench any fluorescence present. This yielded spectra which could be read, although most of the lines were rather faint and broad.

After the required photographs had been taken, it was found that some of the lysozyme had crystallized out in a form identical with that illustrated by Alderton and Fevold[8] for lysozyme hydro*bromide*. The crystals could not be recovered intact. The remaining solution, when placed in the cold, did crystallize in the form found by Alderton and Fevold for lysozyme hydroiodide.

Results

The Raman spectra of glycylglycine are given in Table I with the infrared spectrum[9,10] included for comparison.

The line assignments are based largely on previous work on this compound.[11–13] There is some disagreement regarding the line at 1012 cm.$^{-1}$, which Blout and Linsley attributed to a motion of the diglycyl unit, and which Asai, *et al.*, failed to find in the infrared spectra of polyalanines, but which Ellenbogen did find in polyalanines. The fact that such a line occurs in the Raman spectrum of L-alanyl-L-alanine (see below) indicates that the motion involved may not be limited to polyglycines. The line at 1027 cm.$^{-1}$ in the sodium salt may correspond to the same mode of vibration. A satisfactory explanation of the other differences involving the skeletal frequencies is not available. Differences in CH skeletal and deformation lines will be discussed below.

The Raman spectra of asparagine hydrochloride and of glycylserine and glycylvaline are shown in Table II. It is noteworthy that the lines due to CH motions in the spectra of glycylserine and glycylvaline closely resemble those of serine and valine, respectively. The resemblance between the Raman spectra of asparagine and of aspartic acid[14] is slight, much less than that between acetic acid and acetamide, or propionic acid and propionamide.[15] A possible explanation is that the presence of the amide group may change the preferred orientation of the molecule in solution.

The Raman spectra of L-alanyl-L-alanine and D-alanyl-L-alanine (which will be referred to, for brevity, as LL- and DL-alanylalanine, respectively) are shown in Table III. Also included are the infrared spectra determined by Ellenbogen.[6] The consistent differences in intensity between the Raman spectra of the LL- and DL-forms are presumably due to the fact that six times as much of the LL compound as of the DL was available for examination. There is no reason to believe that one of these yields spectra intrinsically more intense than the other.

(5) D. Garfinkel and J. T. Edsall, THIS JOURNAL, **80**, 3807 (1958).

(6) E. Ellenbogen, *ibid.*, **78**, 369 (1956).

(7) D. Garfinkel, *ibid.*, **80**, 3827 (1958).

(8) G. Alderton and H. L. Fevold, *J. Biol. Chem.*, **164**, 1 (1946).

(9) E. R. Blout and S. G. Linsley, THIS JOURNAL, **74**, 1946 (1952).

(10) The spectrum of glycylglycine is given as a curve and the numerical values quoted are accurate only to the nearest 10 wave numbers.

(11) J. T. Edsall, J. W. Otvos and A. Rich, *ibid.*, **72**, 474 (1950).

(12) H. Lenormant, *Compt. rend. acad. sci.*, **234**, 2057 (1952).

(13) N. B. Abbot and E. J. Ambrose, *Proc. Roy. Soc.* (*London*), **A219**, 17 (1953).

(14) J. T. Edsall, *J. Chem. Phys.*, **5**, 508 (1937).

(15) K. W. F. Kohlrausch and A. Pongratz, *Z. physik. Chem.*, **B27**, 176 (1934).

TABLE I

RAMAN SPECTRA OF GLYCYLGLYCINE

For the notation used for intensities, see reference 5.

Hydrochloride	Isoelectric	Na salt	Infrared	Assignments
387(w)	390(w)	396(vw)		Skeletal deformation??
		511(w)		
		657(vw)		
			700(m)	
834(vw)				
873(5)	881(3)	873(3b)		Skeletal vibration?
898(1b)				
	917(4)	917(5)	910(m)	Peptide bond group motion?
			930(s)	
974(vw)	966(vw)	972(vw)	970(w)	
1012(2)	1012(1)		1015(m)	Diglycyl unit skeletal motion
		1027(4)		
1039(1)	1042(vw)			
		1077(vw)		NH_2 motion??
1094(w)				
		1119(w)	1110(w)	
1135(w)			1140(w)	
		1168(w)	1170(m)	
		1227(1b)		
1243(1b)	1250(vw)		1240(m)	NH_3^+ deformation?
1274(4b)	1276(3b)	1263(3vb)	1280(m)	Peptide bond group motion?
1295(vw)			1305(w)	
	1317(2b)	1315(3)	1315(m)	COO^- motion?
			1340(m)	
1395(3)	1393(6)	1391(6)	1400(s)	Includes COO^- symm. stretching
1413(4b)				
	1426(w)	1425(5)		
1440(4)	1144(1)	1448(w)	1440(w)	CH_2 deformation, in part
			1480(s)	
			1540(s)	NH_3^+ deformation?
	1566(w)		1575(m)	COO^- asymm. stretching
	1602(w)		1608(m)	NH_3^+ deformation?
	1639(w)		1630(m)	NH_3^+ deformation??
			1655(m)	
1692(w)	1688(2)		1680(s)	Peptide group motion
1732(1vb)				CO in COOH
			2080(w)	
			2610(m)	
			2720(m)	
		2858(vw)	2840(m)	
2878(w)	2883(vw)			
		2911(1)		
2949(6)	2949(5)	2939(7)	2930(m)	
2973(6)	2979(5)	2978(3b)		
3003(1b)	3009(w)			
3015(1b)	3027(w)			
			3070(s, broad)	(NH_3^+ stretching)
			3290(s)	(NH stretching in CO-NH)
		3320(5)		(NH_2 stretching symm.
		3364(?)		asymm.)

The differences observed in the infrared spectra are comparable to those seen in other such sets of peptides which are not mirror images.[16] The differences between the Raman spectra of the LL- and DL-forms are slight by comparison; they are also slight compared to the differences between the D- and *meso*-tartaric acids studied by Edsall.[14] Such differences as do appear in the Raman spectra are primarily in frequencies due to skeletal motions (discounting those differences which might be due to there being so much less of the DL-form available, so that the fainter lines in its spectrum might not be observed). In particular, the behavior of the line at 1007 cm.$^{-1}$ cannot be considered as support

(16) M. C. Otey and J. P. Greenstein, *Arch. Biochem. Biophys.*, **53**, 501 (1954).

TABLE II

RAMAN SPECTRA OF ASPARAGINE HYDROCHLORIDE, GLYCYL-DL-SERINE AND GLYCYL-DL-VALINE

Asparagine hydrochloride: 370(vw), 520(vw), 667(vw), 740(vw), 804(3), 874(w), 916(vw), 988(vw), 1098(vw), 1127(vw), 1226(vw), 1294(vw), 1340(w), 1423(2vb), 1734(1b), 2914(vw), 2944(4b), 2968(2vb)

Glycylserine hydrochloride: 379(vw), 481(vw), 521(vw), 609(vw), 787(1b), 852(w), 915(vw), 954(3), 1051(vw), 1135(vw), 1285(2vb), 1342(w), 1370(1b), 1399(vw), 1438(2), 1459(2), 1687(w), 2846(vw), 2910(1), 2968(3b), 3013(w)

Glycylserine (isoelectric): 519(vw), 563(w), 765(vw), 785 (w), 825(vw), 853(w), 876(vw), 906(vw), 949(2), 1010(vw), 1145(vw), 1222(vw), 1271(1vb), 1320(vw), 1395(2vb), 1438 and 1459 (these lines are too faint to measure accurately, but visually these are in the same position as in the hydrochloride, and it has been assumed that the actual positions are also the same), 1675(vw), 2912(1b), 2935(1), 2970(3b)

Glycylvaline hydrochloride: 497(vw), 556(vw), 596(vw), 742(w), 837(w), 928(1b), 985(vw), 1023(vw), 1113(w), 1173(vw), 1266(w), 1365(w), 1399(w), 1442(3), 1468(2), 1687(vw), 2886(2), 2911(2b), 2947(3), 2981(5b)

Glycylvaline (isoelectric): 839(vw), 936(vw), 984(vw), 1110(vw), 1259(w), 1308(w), 1342(w), 1398(2vb), 1444(3), 1467(3), 1684(w), 2881(2), 2907(w), 2945(2), 2978(4)

for Ellenbogen's[6] hypothesis that this line may be missing from the infrared spectra of polyalanines because of racemization. In contrast with the Raman spectra, the differences between the infrared spectra seem to involve all types of motions. It is suggested that the differences among the infrared spectra are due more to intermolecular interactions in the closely packed structure of the crystals than to differences in the characteristic vibrations of the molecules themselves.

In alanylalanine, as with the other peptides examined, the CH vibrations (stretching and deformation) of alanine seem to be present nearly unchanged.

The only polypeptide available to us for examination was polylysine (degree of polymerization 16). The spectrum that was obtained, after long exposure, is given in Table IV.

The assignments of 1403 as due to symmetrical $-COO^-$ stretching, of 1442 as due to CH_2 deformations, and of the lines above 2870 to C–H stretching, appear to be clear. One of the most striking general characteristics of this spectrum is its extreme faintness. If the polylysine were hydrolyzed and the Raman spectrum of the hydrolyzate determined, the spectrum would be far more intense.

On comparing this spectrum with that of lysine,[7] it is found that some, but not all, of the lysine CH lines are present (both stretching and deformation), as well as some of the skeletal frequencies. Two frequencies often associated with a "polypeptide backbone," at 1163 and 718 cm.$^{-1}$, are also present.

Lysozyme.—The infrared spectra of many proteins have been extensively studied, but the Raman spectrum of a protein has thus far not been determined, largely owing to the high intensity of the Rayleigh scattering. Lysozyme was selected for study because it is a small protein and can be readily prepared pure and colorless.

The Raman spectrum of lysozyme (determined

TABLE III

SPECTRA OF ALANYLALANINES

For the meaning of the symbols in parentheses, denoting intensities, see Reference 5.

D-Alanyl-L alanine Raman spectra Iso-electric	D-Alanyl-L alanine Raman spectra Hydro-chloride	D-Alanyl-L alanine Infrared spectrum[6]	L-Alanyl-L-alanine Raman spectra Iso-electric	L-Alanyl-L-alanine Raman spectra Hydro-chloride	L-Alanyl-L-alanine Infrared spectrum[6]	Assignments
				305(vw)		
			337(w)	334(vw)		Skeletal deformation??
369(w)	358(vw)		355(w)			
			385(w)			
422(vw)						
458(w)			447(1b)			
	477(vw)					
				527(vw)		
563(vw)	554(vw)		547(1)			
586(vw)	595(vw)		580(1b)			
			660(w)			
	678(0)	688(m)			680(w)	(CH, NH rock)
			701(vw)	706(vw)		
		721(w)			732(m)	(CH, NH rock)
742(vw)	756(vw)	760(w)		750(vw)		
			774(1vb)			
					802(m)	
824(vw)	826(w)		821(vw)	831(w)		
		854(m)	853(w)		854(w)	
878(4)	871(3)	885(w)	877(5)	874(4)	886(w)	Skeletal stretching?
	899(vw)					
919(vw)	925(w)	917(w)	923(2b)	924(w)	912(w)	(CH, CH_3 rock) Peptide bond group complex motion?
948(vw)		946(m)			942(w)	
			953(2)	952(w)	956(m)	CH, CH_3 rocking
994(vw)	999(w)	993(w)	993(vw)			
1007(w)			1008(2)	1004(1b)	1008(w)	CNC stretching, skeletal motion?
		1025(w)				
1051(w)	1039(vw)		1043(1)	1050(w)	1046(m)	
		1063(w)	1067(vw)		1075(w)	
1100(1b)	1095(w)		1098(3)		1094(w)	
	1114(w)	1111(m)	1112(2)	1107(2b)		
		1117(m)			1117(m)	
					1130(w)	
		1147(w)			1154(s)	
1172(w)	1169(w)		1167(w)	1171(1)		
1214(1)		1216(w)				
	1225(vw)	1232(w)		1233(vw)	1235(m)	
1270(3b)	1267(1)		1271(4b)	1270(3b)	1262(s)	Peptide bond group motion
	1288(vw)	1280(w)			1287(s)	(CN stretch and/or NH deformation)
1311(w)		1315(w)	1320(2)		1322(w)	
1337(1)	1337(1)	1333(m)	1338(2)	1339(2b)	1341(w)	
		1361(s)				
1367(1)		1373(s)	1369(3)	1374(1)	1375(m)	
		1384(s)				
1400(3)	1400(1b)	1414(s)	1402(5)	1402(3)	1407(s)	COO^- symm. stretching (CH deformation)
1455(4)	1454(3)	1450(s)	1456(6)	1456(4)	1460(s)	CH_3 deformation (as in alanine)
		1517(s)			1528(w)	(νCH and/or δNH)
		1565(s)			1555(s)	(νCO and νCH from peptide) COO^- symm. stretching
					1610(s)	$\delta(NH_3^+)$?
		1625(s)				Water band
1675(w)	1678(vw)	1670(s)	1674(w)		1685(s)	Peptide bond group motion, possibly NH_3^+ deformation (νCO, H-bonded)
		2082(w)				νNH from NH_3
		2515(m)			2520(w)	
		2597(m)				NH_3^+–COO^- motions
					2628(w)	
		2724(m)	2726(w)		2732(w)	
			2758(vw)	2753(vw)		
		2787(w)				

TABLE III (*Continued*)

D-Alanyl-L-alanine Raman spectra Iso-electric	Hydro-chloride	Infrared spectrum[8]	L-Alanyl-L-alanine Raman spectra Iso-electric	Hydro-chloride	Infrared spectrum[8]	Assignments
		2835(w)		2835(vw)		
2885(2)	2891(2)		2889(5)	2894(3)		CH stretching, from alanine
		2927(s)			2932(w)	(CH stretching)
2944(5)	2945(5)		2947(7)	2948(6)		CH stretching, from alanine
		2979(s)			2967(s)	(CH stretching)
2993(3b)	2998(3b)	2996(w)	2997(5b)	3005(4b)		CH stretching, from alanine
					3049(s)	(νNH, H-bonded (partly))
		3177(m)				
					3208(m)	(νNH, H-bonded (partly))
		3355(s)				

after long exposures) is presented in Table V, with the infrared spectrum[17] for comparison.

TABLE IV

RAMAN SPECTRUM OF POLYLYSINE; AVERAGE DEGREE OF POLYMERIZATION 16

664(vw), 718(vw), 746(vw), 877(vw), 950(vw), 1028(vw), 1052(vw), 1163(vw), 1210(vw), 1250(vw), 1319(w), 1338(1b), 1403(1b), 1442(3), 2876(2), 2932(4b), 2971(vw)

TABLE V

RAMAN SPECTRUM OF LYSOZYME

Raman	Infrared[a]	Assignments and interpretation
832(vw)		
1012(vw)		
1049(vw)		OH deformation?
1104(w)	1098(w)	
1187(vw)		
1234(w)	1242(w)	Polypeptide backbone vibration?
	1298(w)	
1339(2b)		
	1379(w)	(CH motions)
1412(1b)		COO^- symm. stretching
1451(2b)	1438(w)	CH deformations
	1526(s)	(NH deformation) "Amide II"
	1652(s)	(CO stretching) "Amide I"
1655(3 band)[b]		Water band
2850(w)		
2889(2b)		CH stretching
2941(4b)		CH stretching
2988(1b)		
	3076(m)	(Aromatic CH)?
	3448(s)	

[a] For the infrared spectrum see ref. 17. [b] There are signs of a Raman line in this region (1665 or 1680 cm.$^{-1}$), but it is impossible to verify its presence because of the intense water band.

The lysozyme spectrum is quite faint, much like that of polylysine. On visual examination, the photographs give the impression of being more complex than would be indicated by the spectrum reported. This seems to be in agreement with results of infrared studies and with the expectation[18] that there should be many faint lines present, which overlap and cannot be distinguished individually. Recent improvements in apparatus, using photoelectric recording and improved optics, suggest that it should be possible to obtain better Raman spectra of proteins in the near future. Such studies are to be undertaken shortly in this Laboratory, when an improved apparatus with photoelectric recording is available.

Discussion

No attempt at a detailed theoretical analysis of any of the spectra presented will be made. However, on examining the spectra (and also those of glutathione), several correlations may be pointed out: (1) the "sensitive frequency"[14] of the small amino acids is completely missing. (2) The carbonyl stretching frequency of the COOH group, at about 1730 cm.$^{-1}$, is weak or entirely missing in the cationic peptides, with the notable exception of glycylglycine. This may be due to an interaction of the COOH group with the peptide bond group, but the apparent disappearance of this frequency is harder to explain for glutathione,[19] where there is a carboxyl group rather far removed from the peptide bond. (3) The $-COO^-$ symmetrical stretching frequency is found at 1400 cm.$^{-1}$ instead of at 1410 cm.$^{-1}$. This displacement, however, is small, and not much beyond the experimental error. (4) The CH stretching and deformation frequencies characteristic of the constituent amino acids are usually present without much alteration in the peptides. So also are some other vibrations characteristic of the side chains alone. Skeletal frequencies of the free amino acids generally are missing or markedly displaced. (5) A line at 915 cm.$^{-1}$ is generally present, except in the $-COOH$ forms of peptides with C-terminal glycine. This may be due to a motion of the peptide bond group. (6) A line at about 1270 cm.$^{-1}$ is generally present. This may also be due to a peptide group vibration. Sutherland[20] ascribes an infrared band at 1230 cm.$^{-1}$ to a polypeptide motion; this frequency is present in the Raman spectrum of lysozyme, and the line at 1250 cm.$^{-1}$ in the Raman spectrum of polylysine may be due to the same motion. It is possible that all these are due to similar kinds of motion, the frequency depending on the number of groups involved in the vibration. (7) A line at 1670 to 1690 cm.$^{-1}$ is generally present. Owing to the optical anomaly in the instrument, which had not yet been corrected when most of these studies were carried out, the presence or absence of such a line was hard to determine. It probably is due to a peptide bond motion in which CO stretching is

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particularly prominent. (8) Although only two peptides (glycylglycine and glutathione) have been studied in alkaline solution, it seems that some of the CH stretching and deformation lines of peptides may change as much on removal of a proton from the $-NH_3^+$ group as do those of some amino acids.[7] Some changes also occur on ionization of the carboxyl group, but no general relationship is evident.

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Raman Spectra of Amino Acids and Related Compounds. XI. The Ionization of Cysteine[1-3]

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The Raman spectrum of cysteine has been determined at a series of pH values, from strongly acid solution to pH 11.9. The ionization of the sulfhydryl group has been followed by observing the intensity of the S–H stretching frequency near 2575, and the bending frequency near 875 cm.$^{-1}$, as functions of pH. At pH 9.4, these intensities are about half as great as in acid solution, indicating that the acidic ionization constant of the –SH group in the presence of an adjoining $-NH_3^+$ is not far from equal to that of the $-NH_3^+$ group in the presence of an adjoining –SH. This agrees with evidence from ultraviolet spectra and other data. A line at 1084 cm.$^{-1}$ appears to be associated with the un-ionized amino group. The ionization of the carboxyl group is attended by the disappearance of the line at 1739 and the appearance of strong lines at 1345 and 1400; these changes are characteristic of carboxylic acids. The loss of a proton from the $-NH_3^+$ group is attended by marked changes in the C–H stretching vibrations, similar to those already observed in glycine and β-alanine.

The Raman spectrum of the cysteine cation has been studied already.[6] However, a further study appeared highly desirable, for the ionization of each of the three acidic groups—carboxyl, ammonium and sulfhydryl—in the cysteine molecule is associated with significant changes in the spectrum. In particular, the intensity of the strong Raman line near 2570 cm.$^{-1}$, which represents a stretching frequency of the S–H bond, is an excellent indicator of the concentration of un-ionized sulfhydryl groups present. Since the sulfhydryl and ammonium groups ionize in the same pH range, it is important to follow spectroscopically the ionization of the individual groups in order to determine the relative concentrations of the different individual forms present.

In view of the fact that the spectra were determined photographically, the results obtained must be considered only semi-quantitative. However, in principle, this should be one of the most reliable methods available for determining the numbers of molecules in the various ionized states in aqueous solution.

Experimental

The technique for obtaining the Raman spectra has been described.[7] Solutions of cysteine (approximately 0.67 molar, in each of the various ionization states) were prepared from Mann cysteine hydrochloride (this was assayed by Dr. M. J. Hunter and found to contain at least 98% of the theoretical sulfhydryl content). In order to prevent the oxidation of cysteine to cystine, which occurs at alkaline pH and is known to be catalyzed by heavy metal ions,[8] all solutions were adjusted to the desired pH (as measured with the Beckman Model G pH meter) with 3 N KOH containing 0.01 M KCN (the Raman line of cyanide ion at 2081 cm.$^{-1}$ does not appear in any of our spectra, and neither does the strongest line of cystine.) The initial cysteine hydrochloride solutions were treated with charcoal and filtered through Whatman No. 4 paper. The pH adjustment was then made in an ice-bath. At alkaline pH the solutions promptly turned purple, but the color faded on standing and was reversibly bleached by light.

Results

The spectra obtained, together with the infrared spectrum of cysteine hydrochloride[9] for comparison, are presented in Table I. The assignments in this table are based in part on work by Edsall, Otvos and Rich[6] and Sheppard.[10]

Discussion

Cysteine has three observed ionization constants, the corresponding pK values, extrapolated to zero ionic strength, being 1.71, 8.33 and 10.78.[11] The first of these obviously is due to ionization of the carboxyl group. The assignment of the second and third is not so obvious and has caused much discussion.[12-17]

Using the notation of Hill,[18] we may set up a detailed ionization scheme for cysteine (see also ref. 17). The three ionizing groups—carboxyl (1), sulfhydryl (2) and ammonium (3)—are designated by numbers for convenience. In this no-

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(2) Taken in part from the Ph.D. thesis of David Garfinkel, Graduate School of Arts and Sciences, Harvard University, 1955.

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