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Raman Spectra of Amino Acids and Related Compounds. VIII. Raman and Infrared Spectra of Imidazole, 4-Methylimidazole and Histidine[1-3]

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Raman spectra have been determined for imidazole and its hydrochloride in aqueous solution and infrared spectra for these compounds in the solid state; also Raman spectra for N-deuterated imidazole and its deuterochloride, dissolved in deuterium oxide. Studies on 4-methylimidazole and its hydrochloride, and on histidine in three different states of ionization, also have been carried out. Attachment of a proton to basic imidazole produces far-reaching alterations in the spectra, corresponding to marked changes of bond character which are to be expected in the various bonds of the five-membered ring; tentative assignments of some of the frequencies are suggested. The changes associated with ionization of the imidazole group in 4-methylimidazole and histidine are also very marked. The histidine spectra can be largely interpreted in terms of vibrations associated either with the side chain or with the imidazole ring. Some of the frequencies which are altered on ionization of the latter may prove to be useful indicators of the state of the imidazole group in histidine peptides and in proteins.

The imidazole group of histidine is biologically of great importance. There are numerous recent indications of its significance in enzyme action[6]; it plays an important part in the binding of metallic ions to proteins[7]; and it ionizes in the physiological *p*H range, thus playing a major part in the buffer action of proteins.

In this paper we report studies of the Raman spectra of histidine as the doubly and singly charged cation and as the anion. The isoelectric amino acid proved too insoluble to study in aqueous solution. We have also studied imidazole and 4-methylimidazole (the simplest model compound for the substituted imidazole ring of histidine), both as the free bases and as the imidazolium ions which are their conjugate acids. The effects of deuterium substitution on the nitrogen atoms of imidazole have also been studied. Infrared spectra of imidazole in both ionization forms, with and without deuterium substitution on the =NH groups, have been obtained. Marked changes in spectrum have been found when a basic imidazole group is converted to an imidazolium ion.

The Raman spectrum of imidazole previously has been determined for the crystalline state[8] as well as in aqueous solution,[9] but in neither case is the spectrum complete.

Experimental

Materials.—Imidazole, as obtained (from the Edcan Laboratories, Norwalk, Conn.) was found to be fluorescent. It was purified by air-entrainment sublimation,[10] which permitted determination of the spectrum of imidazolium chloride. The resultant product, however, still contained too much fluorescent impurity to permit study of the free base. Additional samples were purified by vacuum sublimation and stored in the dark, and all other spectra reported are based on study of these samples.

N-Deuterated imidazole was prepared from the vacuum-sublimed material by dissolving 2 g. of imidazole in 10.4 g. of D_2O and evaporating to dryness *in vacuo* over fresh $CaCl_2$. The dry solid was again dissolved in D_2O for the study of the Raman spectrum.

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(1) For paper VII of this series see J. T. Edsall, J. W. Otvos and A. Rich, THIS JOURNAL, **72**, 474 (1950).

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

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

(4) Predoctoral fellow of the National Science Foundation 1953–1954.

(5) To whom inquiries concerning this paper should be addressed.

(6) See for instance, F. H. Westheimer, *Proc. Nat. Acad. Sci.*, **43**, 969 (1957).

(7) F. R. N. Gurd and P. E. Wilcox, *Advances in Protein Chem.*, **11**, 312 (1956).

(8) K. W. F. Kohlrausch and R. Seka, *Ber.*, **71**, 985, 1551 (1938).

(9) G. Felsenfeld, Senior Honors Thesis in Biochemical Sciences, Harvard College, 1951.

(10) R. W. Tipson, in Vol. V, Techniques of Organic Chemistry, ed. by A. Weissberger, Interscience Publishers, New York, N. Y., 1951.

A supply of 4-methylimidazole was synthesized by Mr. Charles Lipson and Dr. Yasuhiko Nozaki,[11] and was purified in the same manner as imidazole.

Histidine monohydrochloride was obtained from the California Foundation for Biochemical Research and found free of extraneous ninhydrin-reactive material by paper chromatography.

Deuterium oxide was obtained from the Stuart Oxygen Co., San Francisco, on allocation from the Atomic Energy Commission.

Apparatus.—All Raman spectra here reported were obtained with apparatus made by the Applied Research Laboratories, Glendale, California.[12] The instrument used by us was equipped only for photographic recording of the spectra. It has an aperture of f/3 and a dispersion of 80 cm.$^{-1}$ per mm. at the 4358 Å. (22938 cm.$^{-1}$) mercury line, which was used as the exciting line and isolated with an ethanol filter solution containing 3% *p*-nitrotoluene, 2% $CoCl_2$ and 1:16,000 Rhodamine 5GDN Extra (du Pont). Photographs of the spectra were taken with film strips coated with Eastman Tri-Pan-X or 103a-O emulsion (the latter is preferable) and the positions of the lines then measured with a Mann comparator. The relation between distance on the film and wave number was calibrated using an argon spectrum, distances being referred to one of the weaker mercury lines (23039 or 24335 cm.$^{-1}$). The precision of the measurements permitted reading the positions of the sharper Raman lines to 1 or 2 cm.$^{-1}$, but allowing for the error in reading the reference mercury line the uncertainty in the reported frequencies is probably at least ±3 cm.$^{-1}$. For broad Raman lines it may be three or four times as great as this. Intensities of the Raman lines were estimated visually. Raman polarization studies were carried out by the methods of Edsall and Wilson[13]: a cylinder of Polaroid polarizing film was wrapped around the Raman tube so that the electric vector of the transmitted light was first parallel to and then perpendicular to the axis of the tube, and the intensities of the Raman lines in the two photographs were compared, using comparable exposures for both orientations of the Polaroid film sheet. Since the intensity estimates were not quantitative, it was possible only to specify that certain lines were definitely or probably polarized.

Infrared spectra were determined with a Perkin-Elmer model 21 spectrophotometer to a lower limit of 625 cm.$^{-1}$, using the KCl pellet or pressed-disk technique.[14] This yielded satisfactory results for the basic imidazole, and rather less satisfactory results for the imidazolium salts, which are highly hygroscopic.

Preparation of Solutions.—All Raman spectra were determined in aqueous solution. Solutions at alkaline *p*H were clarified by passage through a Millipore filter.[15] Solutions at acid *p*H were filtered with charcoal, either through a Millipore filter or through Whatman No. 4 paper. Precautions were taken to minimize exposure to air of solutions containing deuterium. Concentrations of the solutions used are recorded below as g. per 100 cc. solution, the weight referring to the free base, or to the monohydrochloride in the case of histidine.

Imidazole was studied in solutions of 20 to 28% concentration; imidazolium chloride, in solutions of 22 and 42% concentration.

Deuterated imidazole was studied in 33% solution, with 3% KI as fluorescence quencher. Deuterated imidazolium chloride was prepared from N-deuterated imidazole by treatment with DCl generated by the method of Brown and Groot[16] from the interaction of benzoyl chloride with D_2O. It was studied at 31% and at 16% concentration.

4-Methylimidazole was studied in 20% solution; the imidazolium ion derived from it was studied in 25% solution.

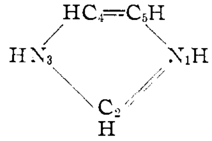
Histidine dihydrochloride was studied in 31% concentration, the monohydrochloride in 18% solution and the sodium salt in 16% solution with 1.6% KI present as fluorescence quencher.

Experimental Results

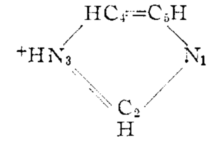
The Raman and infrared spectra of imidazole and imidazolium ion and their deuterated counterparts are presented in Table I. Examination of this table shows that the effects of ionization on the spectra are much greater than those of deuteration, particularly for the Raman spectra. The effects of ionization on the Raman spectra of 4-methylimidazole and histidine are also far-reaching, as shown in Table II. The infrared spectrum of histidine monohydrochloride has been determined[17] and will not be specifically considered here.

Discussion

Imidazolium ion may be regarded as a resonance hybrid with two major contributing structures A and B. These are obviously completely equivalent. There are also minor but significant contributions from other forms. Consideration of all of them, however, confirms the equivalence of the bonds $C_2–N_1$ and $C_2–N_3$; also of the bonds $C_4–N_3$ and $C_5–N_1$. Hence the entire ion, containing 10 atomic nuclei, should possess the symmetry C_{2v}, with one plane of symmetry in the plane of the molecule, and another, perpendicular to it, passing through C_2 and bisecting the $C_4–C_5$ bond. The approximate interatomic distances and bond angles can be inferred from the X-ray diffraction studies of Donohue, *et al.*,[18] on histidine hydrochloride monohydrate, in which the imidazole group is in the imidazolium ion form, and are shown in Fig. 1, which is a somewhat simplified version of the findings of Donohue, *et al.*, omitting the side chain which is present in histidine.



A



B

The ion, with $n = 10$ atomic nuclei, should give 24 normal vibrations $(3n - 6)$. Symmetry considerations permit us to divide these 24 normal vibrations into 4 groups,[19] 9 vibrations of class A_1 (symmetrical to both planes of symmetry), 3 of class A_2 (antisymmetric to both planes), 8 of class B_1 (symmetric to the plane of the molecule) and 4 of class B_2 (antisymmetric to the plane of the molecule). The A_1 vibrations yield polarized Raman lines; the A_2 vibrations are active in the Raman spectrum but not in the infrared. Accordingly it should be possible to identify the 12 normal vibrations of these two classes. The symmetry coördinates for imidazolium ion have been derived by Felsenfeld.[9]

When a proton is removed from imidazolium ion, the symmetry C_{2v} is destroyed and there is a far-

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[13] J. T. Edsall and E. B. Wilson, Jr., *J. Chem. Phys.*, **6**, 124 (1938).

[14] The assistance to Dr. Robert P. Anderson is gratefully acknowledged.

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[16] H. C. Brown and C. Groot, THIS JOURNAL, **64**, 2223 (1942).

[17] L. Larsson, *Acta Chem. Scand.*, **4**, 27 (1950).

[18] J. Donohue, L. R. Lavine and J. S. Rollett, *Acta Cryst.*, **9**, 655 (1956).

[19] G. Herzberg, "Infrared and Raman Spectra of Polyatomic Molecules," D. Van Nostrand Co., New York, N. Y., 1945; E. B. Wilson, Jr., J. C. Decius and P. C. Cross, "Molecular Vibrations," McGraw-Hill Book Co., New York, N. Y., 1955.

TABLE I

RAMAN SPECTRA OF IMIDAZOLE AND THE IMIDAZOLIUM ION WITH AND WITHOUT DEUTERIUM

Imidazole			Imidazolium ion		
Raman	Infrared[a]	N-Deuterated Raman	Raman	Infrared[b]	N-Deuterated Raman
					428(vw)
			456(vw)		449(vw)
		618(1)	625(1)	632(s)	623(vw)
	658(s)			660(w)	
	740(m)				
	760(s)			764(s)	768(vw)
	827(m)		829(vw)	820(sb)	
839(vw)	841(s)				
		858(w)	874(vw)		
	893(w)			902(m)	886(vw)
913(w)		909(1)	907(2b)	917(w)	922(vw)
954(vw)	934(s)	942(w)			
			984(vw)		
1012(vw)					
1032(vw)				1043(s)	1040(vw)
1063(1b)	1058(s)	1066(3)	1056(w)P?	1060(s)	1061(vw)
				1086(w)	1082(2)P
1094(2b)	1101(w)	1104(3)	1097(5)	1100?	1103(2)P
1132(4b)	1142(w)	1132(9)	1125(1b)P	1124(w)	1130(w)
1156(4b)		1156(2)	1144(w)		1141(9)P
			1181(2b)P	1183(m)	
1203(vw)			1205(8b)P	1204(w)	
1255(7)	1264(m)	1247(5)	1254(vw)		
		1307(w)		1307(w)	
1326(5)	1324(m)	1320(8)		1322(m)	
		1332(w)			
		1355(5)			
					1370(2)P
					1392(7)P
1424(3b)			1405(vw)	1414(m)	
	1451(m)	1449(w)	1449(7b)P?	1449(m)	
1484(1b)		1479(4)			
1497(w)	1492(w)	1497(4)			
1515(w)		1509(3b)			
1530(w)	1550(w)		1533(vw)	1536(w)	1542(4)P
				1587(5)	
	1669(w)			1661(w)	1663(vw)
				2364(w)	
				2469 (strong unresolved band)[c]	
	2590(m)				
	2692(m)				
	2785(m)				
	2934(m)				
	3030(m)				3015(w)
					3078(1)
				3225	
3130(4)	3125(m)	3130(s)	3117(1b)P?		3109(w)**P?**
3159(4)		3158(s)	3161(4b)		3157(3b)not P
			3198(2)P		3181(5)P

[a] The infrared spectrum of deuterated basic imidazole differs from this one only in that bands at 966 cm.$^{-1}$(m), 1239(m), 1355(w) and 2093(s, broad) appear on deuteration. [b] The infrared spectrum of deuterated imidazolium chloride differs from this one only in that the bands at 1204, 1307 and 1536 cm.$^{-1}$ disappear, the one at 1061 weakens, and the strong unresolved band is resolved into 2638(s), 2778(s), 2941(s), 3076(s). [c] This band might have been resolved with a better preparation of the pellet. The imidazolium salt is very hygroscopic, and the presence of some moisture probably modified some of the frequencies, due to hydrogen bonding. Raman intensities are given as numerical values (1, 2, . . . etc.) in increasing order of intensity, except that the faintest lines are designated weak (w) or very weak (vw). Broad lines are designated b, very broad, vb. Infrared intensities are given as weak (w), medium (m) or strong (s). P indicated polarized; P?, probably polarized.

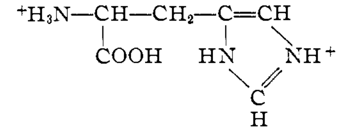
reaching alteration of bond character among all the bonds in the ring. Correspondingly there is a drastic change in the Raman spectra on ionization. X-Ray or electron diffraction data on the interatomic distances in basic imidazole are not available. On considering various possible resonance forms, and estimating the relative contributions which they may make to the actual structure, we

TABLE II

RAMAN SPECTRA OF 4-METHYLIMIDAZOLE AND HISTIDINE IN DIFFERENT STATES OF IONIZATION

4-Methylimidazolium chloride	4-Methylimidazole	Histidine dihydrochloride	Histidine monohydrochloride	Histidine Na salt	Suggested assignments
	262(1)				
270(2b)	274(1)				
	312(vw)	303(vw)		312(vw)	
339(w)	339(vw)				
		361(vw)	375(vw)		
		484(vw)			
		528(vw)	515(vw)		
			544(vw)		
601(vw)					
633(vw)	621(vw)	633(1b)	626(vw)		
658(4)	660(3)	657(w)			Methyl group motion
696(vw)		704(w)	712(vw)		
			750(vw)		
		777(w)			
			801(vw)		
	822(vw)	818(w)			
841(vw)		854(1)	845(vw)	849(vw)	
			867(w)	866(w)	
		900(vw)			
921(1b)		922(1)	918(1b)	910(w)	
	934(vw)			933(w)	
		951(vw)	957(vw)		
970(1b)	968(vw)	970(vw)		985(vw)	
1002(3)	1008(2)	993(3)	989(2)	1010(vw)	Im and ImH$^+$
	1029(vw)				
		1041(w)	1047(vw)		
		1065(vw)	1073(vw)		
1083(1b)	1081(vw)	1090(2)	1093(w)	1087(w)	Im and ImH$^+$
	1099(1)			1105(1b)	Im
	1144(w)			1148(1)	Im
	1160(w)			1165(2b)	Im
1180(3vb)		1189(4vb)	1188(3vb)		ImH$^+$ ion
1198(1b)					
	1224(4)			1233(3)	Im
1262(5)	1254(4)	1264(5)	1266(3)	1265(2b)	*Im* and ImH$^+$
	1297(5)			1285(2)	Im
	1336(w)	1322(vw)	1330(1b)	1318(2b)	Im(?)
1367(vw)		1360(2)	1353(1b)	1354(3b)	νcarboxyl (?)
1391(3)	1385(2)		1396(w)	1412(1b)	Im and $\nu(COO^-)$
	1420(2b)				ImH$^+$ (?)
1435(1vb)	1448(2b)	1433(3)	1438(1b)	1440(3b)	δ (CH_2 or CH_3)
1480(4vb)	1486(1b)	1485(6b)	1485(4b)	1488(2b)	Im and ImH$^+$
	1525(w)			1531(vw)	Im
1561(1)	1568(1)			1567(3b)	Im (?)
				1588(2b)	$\delta(NH_2)$(?)
				1609(1b)	$\delta(NH_2)$(?)
1629(2)	1629[a]	1625(4)	1625(3)	1633(1b)	Im and ImH$^+$
		1739(2vb)			$\nu(C{=}O)$, carboxyl
2769(w)					
2809(vw)					
	2850(w)	2861(vw)	2860(vw)		
2878(w)	2870(w)			2886(w)	$\nu(CH)$
2915(w)					
2939(8)	2928(4)			2934(4b)	ν(CH_2 or CH_3)
		2946(5)	2947(2b)		(Side chain)
				2961(w)	
2974(1)		2974(3b)	2974(2b)		$\nu(CH_2)$
3000(w)					
3030(vw)					
3123(w)	3126(3b)			3132(4)	$\nu(CH)$, Im
3160(4b)		3159(3b)	3160(2b)	3149(2b)	$\nu(CH)$, Im
				3302(4b)	$\nu(NH_2)$

[a] Intensity appreciable but could not be accurately determined. For other symbols see footnote to Table I. ν and δ denote stretching or bending motions, respectively, of the indicated groups. "Im" and "ImH$^+$" denote vibrations associated with the imidazole ring in the basic or the conjugate acid form, respectively. The cation of histidine dihydrochloride (writing only one resonance form for the imidazolium ring) is



The cation of the monohydrochloride is the same, except that the carboxyl group is ionized; the state of the acid and basic groups may be denoted for brevity as [^+H_3N-(COO$^-$)-ImH$^+$]. The anion of the sodium salt, in the same notation, may be written [H_2N-(COO$^-$)-Im].

tentatively suggest that the general character of the molecule may be represented by a diagram such as Fig. 2.

The C_2–N_1 and C_2–N_3 bonds are no longer equivalent, as they are in the imidazolium ion; the bond involving the nitrogen which has lost a proton must acquire a large amount of double bond character, whereas the other bond (C_2–N_1 in Fig. 2) becomes more like a single bond.

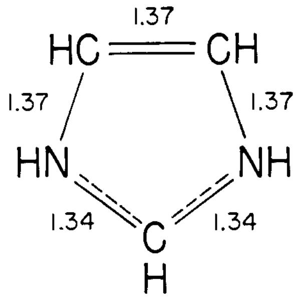


Fig. 1.—The structure of the imidazolium ion. Interatomic distances are inferred from the work of Donohue, *et al.*[18] All internal bond angles are close to 108°.

This molecule has 21 normal vibrations and only one element of symmetry (the plane of the molecule), so that its theoretical analysis would be much more difficult than that of imidazolium ion. On the average, over a long period of time, the two nitrogen atoms N_1 and N_3 must be statistically equivalent, owing to proton transfers between the basic nitrogens and the solvent. However the time interval between such transfers is almost certainly long compared to the time required for molecular vibrations to take place, which is of the order of 10^{-13} sec. Hence, during the vibrations, we must assume the C–N bonds to be non-equivalent, as indicated by Fig. 2.

On examination of the spectra in Table I, it is found that more lines are observed for each form of imidazole (with and without a proton, with and without deuterium) than the theoretically permitted ($3n - 6$) number of fundamental frequencies. Accordingly some of the observed frequencies must correspond to combination tones or overtones. A considerable number of lines in the infrared spectrum, and some in the Raman spectrum, are weak and may well not correspond to fundamental frequencies. A complete assignment of fundamental vibrations is not yet possible from the available data; but some can be distinguished. In the Raman spectrum of the imidazolium ion four lines are definitely polarized and three probably polarized, while in the deuterated imidazolium ion spectrum seven lines are definitely and one probably polarized. If we make the assumption that an intense line is probably still intense after deuteration, we may then tentatively match these two sets of lines in a table, keeping in mind the rule[19] that on deuteration a frequency may not shift upwards and may not be decreased by a factor of more than 1.36. The results are shown in Table III, and it is seen that the correlations given can account for all 9 lines of this class. In view of the number of gaps and uncertainties in this table, however, it is not yet a satisfactory basis for further calculations.

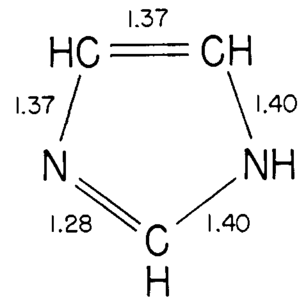
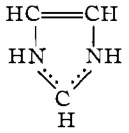
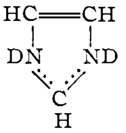


Fig. 2.—A tentative model for the structure of basic imidazole. All internal bond angles are close to 108°.

TABLE III

IMIDAZOLIUM ION: FREQUENCIES OF CLASS A_1[a]

	
1056(w)	Unknown
1125(1b)	1082(2b)
1181(2b)	1103(2b)
1205(8b)	1141(9)
Unknown	1370(2)
1449(7b)	1392(7)
Unknown	1542(1)
3117(1b)	3109(w)
3198(2)	3181(5)

[a] Polarization studies indicate that all these lines are definitely or probably polarized.

There should be three frequencies of class A_2 present in the Raman spectrum but not in the infrared. There are 6 lines in the imidazolium chloride spectrum that meet this criterion (at 874, 984, 1097, 1144 and 1254 cm.$^{-1}$ and perhaps at 456 cm.$^{-1}$). The line at 1097 cm.$^{-1}$ is intense enough so that it must be a fundamental, presumably of this class. There is no basis, however, for selecting two out of the other five lines, all of which are faint, and in the deuterated imidazolium spectrum the lines at 1130 and 866 cm.$^{-1}$ are the only ones that seem to fall into this class. The A_2 class is therefore not satisfactorily determined on the basis of the present data, and there is no basis for identification of the B class lines, except that the line at 3161 cm.$^{-1}$ is probably a B_1 vibration. From its frequency, this line must be due to a CH or NH stretching motion, which must be in the plane of the molecule and therefore symmetrical to it. Since it is not polarized, we know it is not symmetrical to the other plane of symmetry. Since it is unaltered by deuteration or ionization on the nitrogen, it must be a C–H frequency.

Examination of the frequencies due primarily to the stretching of CH or NH bonds (above 2000 cm.$^{-1}$) shows that there is still H present in the deuterated molecules. Presumably we have exchanged only those hydrogen atoms bound to nitrogen by this method of preparation. Study of a completely deuterated imidazole would undoubtedly yield much important information.

One real difficulty here is the fact that imidazole is capable of hydrogen-bonding to the solvent used, and this may cause change in its vibrational behavior. The infrared spectrum of imidazole dissolved in dioxane was determined and found to show marked differences from that of solid imidazole, perhaps due to hydrogen bonding.

Some specific line assignments may be suggested: the Raman lines at 3130 and 3159 cm.$^{-1}$ in the spectrum of imidazole are unchanged on deuteration and therefore due to CH stretching motions alone. This leaves no line obviously due to NH stretching. A possible N–D stretching line is present at 2093 cm.$^{-1}$ in the infrared spectrum of deuterated imidazole. This is of slightly lower frequency than the lines near 2180 cm.$^{-1}$ found by Edsall and Scheinberg[20] in simple amine salts containing the $-ND_3^+$ group. The slight effect of deuteration on the lines above 3000 cm.$^{-1}$ in the imidazolium chloride spectrum suggests that these, also, are due primarily to CH motions.

On examining the Raman spectra of 4-methylimidazole and the conjugate imidazolium ion (Table II), we find changes on ionization nearly as extensive as with imidazole itself. Tentative assignments of some of the observed frequencies are indicated on the right hand side of Table II. Kohlrausch and Seka[8] have determined the Raman spectra of 1-methyl- and 2-methylimidazoles (the former as the pure liquid base, the latter in crystalline form). The spectra show some general resemblances to that reported in Table II for 4-methylimidazole in water solution; but they are markedly different in detail.

The spectra of histidine, in several ionic forms studied (Table II), show close relations to those of 4-methylimidazole and its conjugate acid. In making comparisons, it should be noted that the imidazole ring of histidine is positively charged in both the hydrochlorides studied; only in the sodium salt is it uncharged, and here the amino group of the side chain is uncharged also. Certain frequencies of the histidine spectrum must arise from this side chain, as indicated in the suggested assignments listed in the right-hand column of Table II. To a large extent, however, the spectrum of histidine corresponds with that of 4-methylimidazole. Certain frequencies, such as those near 1000, 1090, 1260, 1485 and 1630, appear to be present whether the imidazole ring is in the basic or in the conjugate acid (imidazolium) form. Some, like those near 1100, 1145, 1160, 1230, and 1290, appear to be characteristic of the basic form of the imidazole group in both 4-methylimidazole and histidine. The strong line near 1185 appears to be associated with the imidazolium form of the ring structure. Whether such correlations will be found consistently in histidine peptides or in proteins is still unknown. If so, the use of Raman or infrared spectra may furnish a powerful tool for studying the state of ionization of the histidyl residues in these more complex structures.

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(20) J. T. Edsall and H. Scheinberg, *J. Chem. Phys.*, **8**, 520 (1940).