Table IV. ^{13}C and ^{14}N Shifts of *N*-Methylimidazole in Various Solvents

	^{13}C chemical shifts[a]				^{14}N chemical shifts	
	C-2	C-4	C-5	CH_3	N_1	N_3
CCl_4	−137.10	−119.29	−129.13	−32.66	−138.6 ± 1	−247.8 ± 3
Neat	−138.61	−121.19	−129.17	−33.17	−143.9 ± 3.3	
Acetone	−138.49	−120.79	−129.52	−33.14	−140.5 ± 1	−233.9 ± 1
D_2O	−140.63	−123.57	−130.12	−35.32	−146.9 ± 1	

[a] ^{13}C shift referred to TMS.

respectively.[33] The effect of self-association is probably negligible, since the concentration of the solute is low (1 mol %) in polar solvents. Then it is found that the ^{14}N shifts of imidazole in acetone and DMSO (proton acceptors) are shifted downfield with respect to methanol, water, and trifluoroacetic acid (proton donors). The difference of ^{14}N shifts is 6–9 ppm over the range of the experimental error. Similarly upfield ^{14}N shifts of 6 ± 3 ppm and 7.4 ± 3–9.8 ± 5 ppm are observed in pyrazole and 1,2,4-triazole, respectively. These values are recognized as about half of the corresponding values for *N*-methyl derivatives, taking into account the argument described above.

Sometimes difficulty arises in observing ^{14}N shifts in protonated species, because a longer correlation time due to protonation tends to broaden nmr lines and make the two- and three-bond N–H couplings unobservable. Protonation shifts on imidazole and thiazole are obtained by the decoupling of one-bond N–H couplings. The upfield ^{14}N shifts are 41 ± 3 ppm and 123.1 ± 3 ppm for imidazole and thiazole, respectively. The latter is in good agreement with the similar upfield shift of protonated pyridine, 123 ± 11 ppm.[6] The ^{14}N shift of protonation on imidazole by one nucleus is 82 ppm which is twice the value 41 ppm. This is too small compared with protonated pyridine, probably because the inductive effect by protonation to one nucleus causes another shift downfield.

Inductive Effect. It should be noted that there exists another cause of displacement of the ^{14}N shift in addition to the hydrogen bond formation mentioned above. A relatively larger downfield shift (−8.3 ± 2 ppm) is denoted in aqueous solution for the ^{14}N shift of the $N-CH_3$ group of *N*-methylimidazole with respect to carbon tetrachloride solution. On the basis of molecular structure, it is not ascribed to the hydrogen bond as discussed above. One possible explanation of this is the inductive effect by the hydrogen-bond formation to tertiary nitrogen atom with water. In order to confirm this postulate, measurements of ^{13}C resonance, the shifts of which being more accurately determined than ^{14}N resonance, are performed in several solvents. The parallel relationship among displacements of ^{13}C shifts of methyl, C-3 and C-4 and the ^{14}N shift of NMe shows the presence of this mechanism (Table IV). Polarization of molecules in polar medium[34] is also responsible for the downfield ^{13}C and ^{14}N shifts, though the displacement of the former is smaller than the latter.

(33) Strictly speaking it is too straightforward to conclude simply from the results of monofunctional compounds. If a hydrogen bond is formed between $\gg$N and XH, as in eq 1, the ^{14}N shift of the N–H group is also influenced by the inductive effect.

(34) H. Saitô, Y. Tanaka, S. Nagata, and K. Nukada, *Can. J. Chem.*, in press; H. Saitô and Y. Tanaka, in preparation.

Determination of the Tautomeric Form of the Imidazole Ring of L-Histidine in Basic Solution by Carbon-13 Magnetic Resonance Spectroscopy

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Abstract: Comparison of ^{13}C chemical shift–pH profiles for imidazole, L-histidine, and 1-methyl- and 3-methylhistidine provides conclusive evidence that the 1-H tautomer is the predominant tautomeric form of the imidazole ring of histidine in basic solutions. This viewpoint is supported by theoretical calculations of ^{13}C chemical shifts based on the average energy approximation and electron densities determined by CNDO/2 MO calculations. The characteristic titration shifts for histidine and the methylhistidines are used to determine the tautomeric equilibrium of the imidazole ring in several derivatives of histidine and in polypeptides containing a histidyl residue.

The amino acid L-histidine is trifunctional since in addition to the α-amino and α-carboxyl functions, the imidazole side chain is a protonation site (pK = 6.0).[2] The protonation step for the imidazole ring involves transition from a neutral species to a cationic

(1) (a) Department of Chemistry; (b) Faculty of Pharmacy.

(2) H. A. Sober, "Handbook of Biochemistry," 2nd ed, Chemical Rubber Publishing Co., Cleveland, Ohio, 1970, p J-198.

species (Figure 1). The neutral ring can exist in two tautomeric structures: either the illustrated (Figure 1) 1-H tautomer or the 3-H tautomer.[3] It has never been definitely established which tautomeric form is the more stable.[4] We present here evidence which conclusively establishes that the 1-H tautomer is the more stable tautomer in basic solutions.

Results and Discussion

Our interest in this problem was prompted during a more general investigation of the pH dependence of ^{13}C chemical shifts of amino acids.[5] It was noted that upon protonation of the imidazole ring of histidine the C(4) ^{13}C resonance of histidine is shifted to high field by 4.8 ppm while the C(5) resonance is shifted to low field by 0.7 ppm (Table I). By contrast, the equivalent

Table I. Experimental and Calculated ^{13}C Chemical Shifts of Imidazole Carbons in Imidazole and Histidine in the pH Region of Imidazole Ring Protonation

(a) Experimental ^{13}C Chemical Shifts[a]

	Imidazole			Histidine		
Carbon	Neutral	Cation	Δ[b]	Neutral	Cation	Δ
C(2)	+7.5	+5.1	−2.4	+8.3	+5.9	−2.4
C(4)	−6.6	−9.3	−2.7	+3.9	−0.9	−4.8
C(5)	−6.6	−9.3	−2.7	−11.1	−10.4	+0.7

(b) Calculated ^{13}C Chemical Shifts[a]

	Neutral						
Imidazole	1-H[c]	3-H[d]	Av	Cation	Δ1-H	Δ3-H	ΔAv
C(2)	+8.5	+8.5	+8.5	+6.3	−2.2	−2.2	−2.2
C(4)	−1.5	−7.6	−4.6	−6.9	−5.4	+0.7	−2.3
C(5)	−7.6	−1.5	−4.6	−6.9	+0.7	−5.4	−2.3

	Neutral				
Histidine	1-H	3-H	Cation	Δ1-H	Δ3-H
C(2)	+8.7	+9.3	+6.9	−1.8	−2.4
C(4)	+19.0	+13.2	+14.2	−4.2	+1.6
C(5)	−8.8	−2.6	−9.6	−0.8	−7.0

[a] Relative to (internal) $^{13}C_6H_6$. Low-field shifts are positive. [b] Change in chemical shift on going from neutral imidazole ring to cationic imidazole ring. [c] 1-H tautomer. [d] 3-H tautomer.

C(4) and C(5) resonances in imidazole both shift to high field by 2.7 ppm upon ring protonation to yield the cationic form. In imidazole, C(4) and C(5) are magnetically equivalent in the cation due to the C_{2v} symmetry of this species; the equivalence of these carbons in the neutral species arises from rapid (on the nmr time scale) tautomeric equilibrium between 1-H and 3-H forms.

(3) Throughout this paper the imidazole ring of histidine is labeled according to IUPAC nomenclature (see Figure 1) rather than the common biochemical nomenclature (K. Hoffman, "Imidazole and Its Derivatives," Part I, Interscience, New York, N. Y., 1953, p 188) in which N(1) and N(3) as well as C(4) and C(5) are reversed. Thus, what we refer to as the 1-H tautomer would be the 3-H tautomer according to biochemical nomenclature.

(4) D. J. Patel, C. K. Woodward, and F. A. Bovey, *Proc. Nat. Acad. Sci. U. S.*, **69**, 599 (1972).

(5) While in this paper we focus only on the effect of the imidazole protonation step in histidine upon the ^{13}C shifts of the imidazole ring carbons, it should be noted that the entire ^{13}C chemical shift *vs.* pH profile for each carbon of histidine displays three distinct titrations corresponding to the trifunctionality of this amino acid. This sensitivity in the ^{13}C shifts of all carbons of an amino acid to the protonation state of the amino acid has been reported by several groups: M. H. Freedman, J. S. Cohen, and I. M. Chaiken *Biochem. Biophys. Res. Commun.*, **42**, 1148 (1971); F. R. N. Gurd, P. J. Lawson, D. W. Cochran, and E. Wenkert, *J. Biol. Chem.*, **246**, 3725 (1971); G. Jung, E. Breitmaier, and W. Voelter, *Eur. J. Biochem.*, **24**, 438 (1972). A full theoretical account of the pH-dependent ^{13}C shifts in histidine and other amino acids will be the subject of a forthcoming article.

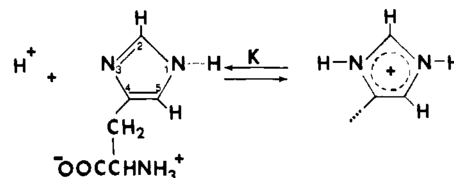

Figure 1. Protonation of the imidazole ring of the 1-H tautomer of L-histidine (pK = 6.0). The imidazole ring is numbered according to the IUPAC convention rather than common biochemical usage.

However, the C(2) carbons of histidine and imidazole show identical titration shifts of −2.4 ppm (similar ^{13}C titration shifts have been previously reported for imidazole[6]).

In view of the surprising differences for C(4) and C(5) for these closely related compounds, it was decided to carry out theoretical calculations of ^{13}C chemical shifts for imidazole and histidine in an attempt to interpret the observed ^{13}C chemical shift–pH profiles. Chemical shifts have been calculated using equations developed by Grant, *et al.*, for estimation of diamagnetic[7] and paramagnetic[8] contributions to ^{13}C chemical shifts from electron densities estimated by all valence electron molecular orbital calculations. Electron densities necessary for the computation of chemical shifts were determined by CNDO/2 molecular orbital calculations.[9] Following the suggestion of Grant[8] and Adam,[10] ΔE values for paramagnetic contributions were estimated from the difference in average energies of filled and unfilled molecular orbitals. Calculated chemical shifts are expressed relative to benzene with ΔE for benzene scaled to 10 eV. Calculations for histidine were carried out assuming the same ΔE values for ring carbons as used for imidazole. Chemical shifts were averaged for all three rotational isomers about the C(α)–C(β) bond.

The results of the calculations for the ring carbons of imidazole and histidine are summarized in Table I. The theoretical calculations accurately predict the ^{13}C chemical shifts for imidazole when the calculated ^{13}C chemical shifts for C(4) and C(5) in the neutral form are averaged.[11] However, without averaging, the calculated protonation chemical shifts for the ring carbons of the 1-H tautomer of imidazole are nearly identical with the experimental chemical shifts for the corresponding ring carbons in histidine (calculated C(4) and C(5) titration shifts are reversed for the 3-H tautomer). Similarly, the calculated chemical shifts for the 1-H tautomer of histidine are in much better agreement with experimental data than the calculated shifts for the 3-H tautomer, although the agreement is not as good as for imidazole.[12] Thus the calculations provide a rationalization for the ^{13}C chemical shift titration curves for histidine upon imidazole ring protonation in terms

(6) R. J. Pugmire and D. M. Grant, *J. Amer. Chem. Soc.*, **90**, 4232 (1968).

(7) B. V. Cheney and D. M. Grant, *ibid.*, **89**, 5319 (1967).

(8) R. J. Pugmire and D. M. Grant, *ibid.*, **90**, 697 (1968).

(9) J. A. Pople and G. A. Segal, *J. Chem. Phys.*, **44**, 3289 (1966).

(10) W. Adam, A. Grimison, and G. Rodriguez, *ibid.*, **50**, 645 (1969).

(11) Reasonable agreement between experimental and calculated ^{13}C chemical shifts of imidazole has previously been reported (ref 6 and 10).

(12) As previously discussed (R. J. Pugmire, D. M. Grant, M. J. Robins, and R. K. Robins, *J. Amer. Chem. Soc.*, **91**, 6371 (1969)), this type of calculation tends to overestimate ^{13}C chemical shifts of substituted ring carbons.

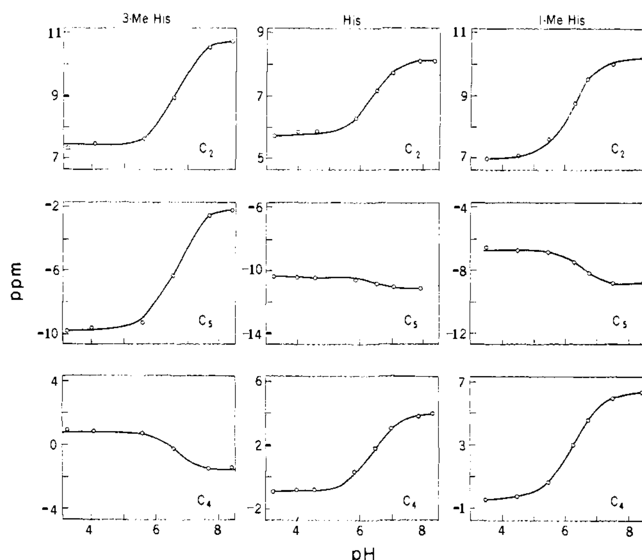

Figure 2. ^{13}C chemical shift–pH profiles of the imidazole ring carbons of 1-methyl-L-histidine, L-histidine, and 3-methyl-L-histidine in the pH region 3–8.5. Chemical shifts are relative to $^{13}C_6H_6$ with low-field shifts positive.

of the predominance of the 1-H tautomer in basic solutions. The calculations can also be used to rationalize the difference in protonation shifts for C(4) and C(5) in histidine in terms of differences in electron densities; in imidazole the calculated C(4) valence electron density decreases by only 0.028 on going from the 1-H tautomeric form to the protonated form while C(5) decreases by 0.050.[13] Similar trends are noted for histidine.

While these calculations indicated that the illustrated tautomeric form of histidine (Figure 1) is the predominant form in basic solution, they could not be regarded as proof of this contention, especially in view of the approximations involved in the theory. In particular, the use of the average energy approximation for ^{13}C chemical shift calculations has been seriously criticized because the choice of ΔE values is arbitrary.[14] In addition, the validity of the calculations depends upon the accuracy of electron densities determined by an approximate molecular orbital calculation. Consequently, the ^{13}C chemical shift titration curves for 1-methyl- and 3-methylhistidine were determined in an attempt to obtain experimental confirmation that the 1-H tautomer is the stable tautomeric form of histidine in basic solutions. The N–CH_3 bond should be fixed, eliminating tautomerism. The electronic similarity between the N–H and N–CH_3 bonds should give rise to similar titration curves for histidine and for the methylhistidine derivative that corresponds to the major tautomer in basic solutions. Results for the imidazole ring titration of the two methylhistidines and histidine are illustrated in Figure 2. The titration chemical shifts for all of the imidazole carbons of 1-methylhistidine are identical in sign and similar in magnitude with those for histidine while the C(4) and C(5) ^{13}C chemical shift–pH profiles for 3-methylhistidine are totally inconsistent with those for histidine. Thus the titration curves for the methylhistidines provide conclusive experimental evidence for the predominance of the 1-H tautomer of histidine in basic solutions. However, this does not rule out the presence of some of the 3-H tautomer. C(4) and C(5) titration shifts for histidine are intermediate between those for the two methylhistidines (Table II). Assuming that the titration shifts for the two methylhistidines are equal to the shifts for the two tautomers of histidine, the shifts for histidine correspond to an approximate 4:1 ratio of 1-H:3-H. It should be stressed that this is only an approximate value whose validity depends upon the above assumption.

Table II. Changes in ^{13}C Chemical Shift of Imidazole Ring Carbons of Histidine Derivatives upon Protonation of the Neutral Imidazole Ring

Compound	C(2)	C(4)	C(5)	pK[a]
Histidine	−2.4[b]	−4.8	+0.7	6.2
1-Methylhistidine	−3.4	+2.3	−7.1	6.7
3-Methylhistidine	−3.5	−6.8	+2.1	6.3
Histidine methyl ester	−1.4	−4.6	+1.3	5.9
Histidylglycine	−1.7	−4.8	+0.8	6.2
N-Acetylhistidine	−2.6	−3.5	−0.9	7.3
Glycylhistidylglycine	−2.7	−4.3	−0.6	6.7
Bacitracin[c]	−2.6	−4.3	−0.6	6.9

[a] Average value of the individual carbon–pH profile pK's. [b] High field (low frequency) shifts are negative, low field shifts are positive. [c] Histidine = residue 10.

There are two obvious applications of the experimental results outlined above. Firstly, it should be possible to utilize the ^{13}C chemical shift titration curves of the imidazole ring to determine the tautomeric form of histidyl residues in polypeptides and proteins. A pattern of chemical shifts similar to that observed for histidine and 1-methylhistidine should be diagnostic for the predominance of the 1-H tautomer while a pattern similar to that for 3-methylhistidine should indicate that the 3-H form is the major tautomer. We have applied this criterion in analyzing the pH-dependent ^{13}C chemical shifts of the imidazole ring carbons in *N*-acetylhistidine (NAH), histidine methyl ester (HME), histidylglycine (HG), glycylhistidylglycine (GHG), and the cyclic dodecapeptide bacitracin (histidine = residue 10). The chemical shifts on imidazole ring protonation are given in Table II. All titration shifts are consistent with a predominance of the 1-H tautomer in basic solution. Histidine derivatives in which only the carboxyl group is blocked (HME and HG) show titration shifts for C(4) and C(5) which are very similar to those for histidine. However, all derivatives in which the primary amino function is blocked (NAH, GHG, and bacitracin) show small negative (high field) shifts for C(5) on ring protonation while the negative shift for C(4) is not as large as in histidine. This pattern is insensitive to whether or not the carboxyl function is blocked. The change in sign for C(5) and the small shift for C(4) are both consistent with a slightly smaller proportion (*ca.* 70%) of the 1-H tautomer in histidine derivatives in which the primary amino function is blocked.

The extension of this approach to histidyl derivatives

(13) The overall high-field shifts for imidazole on protonation are presumably due to the larger average excitation energy for the cation than the neutral species. This leads to a decrease in paramagnetic contributions to ^{13}C chemical shifts in the former case. The calculations suggest that the larger ΔE value is more than compensated by the large decrease in electron density at C(5) but not at C(4) where the electron density change is smaller.

(14) P. D. Ellis, G. E. Maciel, and J. W. McIver, Jr., *J. Amer. Chem. Soc.*, **94**, 4069 (1972).

in proteins might prove interesting in that histidyl residues are often involved in the binding of substrates *via* hydrogen bonding with the imidazole ring nitrogen protons or in complexing of metal ions by the imidazole nitrogen. It is possible that comparisons of titration curves for imidazole carbons of the bound and unbound peptide may provide information about the site of binding to the imidazole ring of the histidyl residue(s).

In addition, the use of *N*-methyl derivatives in combination with ^{13}C chemical shift titration curves should be an approach of general applicability to problems of tautomeric equilibrium in di- and polyfunctional nitrogen bases. Comparisons of ultraviolet spectra for nitrogen bases and their *N*-methyl derivatives have been used to elucidate problems of tautomeric equilibrium.[15] However, this approach appears to have been most successful in cases where there is a large difference in electronic character between tautomers, *e.g.*, 2-hydroxypyridine and 2-pyridone.[16] ^{13}C magnetic resonance spectroscopy should be a more sensitive probe since it reflects the electronic distributions at individual carbons. Some indication of this greater sensitivity is provided by the results we have described above and also by the investigation of Pugmire and Grant of tautomeric equilibrium in purine.[17]

In conclusion, this investigation has elucidated the predominant tautomeric form of histidine in basic solution as the 1-H form (Figure 1). It has also demonstrated that this tautomeric preference is maintained in a number of histidine derivatives including the polypeptide bacitracin. Thus it has provided a criterion for determining the preferred tautomeric form of histidyl residues in polypeptides and proteins. The technique of using *N*-methyl derivatives as model compounds for tautomers should also be applicable to other investigations of tautomeric equilibrium by ^{13}C magnetic resonance spectroscopy.

(15) A. R. Katritzky and J. M. Lagowski, *Advan. Heterocycl. Chem.*, 312 (1963).

(16) H. Specker and H. Gawrosch, *Ber. Deut. Chem. Ges.*, **75**, 1338 (1942).

(17) R. J. Pugmire and D. M. Grant, *J. Amer. Chem. Soc.*, **93**, 1880 (1971).

Experimental Section and Details of Calculations

^{13}C shifts on 10% w/v aqueous solutions of the compounds were determined on a XL-100-15 spectrometer operating in the pulsed-Fourier transform mode. Probe temperature, under proton-decoupled conditions, was 32 ± 3°. pH determinations were made on an Orion Model 801 pH meter equipped with combination electrode. Chemical shifts were measured relative to internal dioxane and converted to a ppm scale relative to internal $^{13}C_6H_6$ (using a conversion factor of 62.0 ppm) in order to be consistent with the calculations. The 1-methyl- and 3-methyl-L-histidine were obtained from Sigma Chemical Co. (St. Louis, Mo.).

^{13}C chemical shifts were calculated using a modified version of a standard CNDO/2 program.[18] The modified program computes chemical shifts directly from the density matrix. Increased running time over a normal MO calculation is typically 5–10%. Paramagnetic contributions from orbitals on the same carbon and from orbitals on the other atoms are estimated using eq 6 and 7 of ref 8. Effective nuclear charges were calculated using eq 10 of ref 8 and $\beta = 1.10$. The average excitation energy of benzene was set at 10 eV while average excitation energies of 10.08 and 10.21 eV were respectively used for neutral and cationic forms of the imidazole ring of imidazole and histidine. The latter values were obtained by estimating the difference in average energies of filled and unfilled MO's for both forms of imidazole and scaling these relative to 10 eV for benzene. In the case of histidine all C, N, and O atoms were included in the calculation of paramagnetic contributions from other atoms.

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(18) Quantum Chemistry Program Exchange, Program 141.