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# Nuclear Magnetic Resonance Investigation of $^{15}$N-Labeled Histidine in Aqueous Solution

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**Abstract:** The pH dependence of $^{15}$N and $^{13}$C resonances of histidine, 95% $^{15}$N isotopically enriched in both imidazole nitrogens is studied. From the various couplings between the $^{13}$C, $^{15}$N, and $^{1}$H nuclei a quantitative description of the tautomeric equilibrium of the deprotonated imidazole ring is possible. The chemical shift data and the coupling constants indicate an interaction of the $\alpha$-amino group and the lone electron pair of the imidazole $\pi$ nitrogen in the pH range of 6.2-9.3. Owing to this interaction the conformation of the whole molecule can be determined from the couplings. It can be shown that also the tautomeric equilibrium of the deprotonated imidazole is influenced by this interaction. The analysis of the pH dependence of the spin-lattice relaxation times $T_1$ and the NOE values reveals that histidine is associating around the pH value of the imidazole p$K$ by forming possibly a dimeric structure.

In the last few years $^{15}$N NMR spectroscopy has become a useful tool to investigate problems connected with the nitrogen atom in several organic molecules.[1-8] The low relative sensitivity compared to the $^{1}$H NMR spectroscopy and the low natural abundance of the $^{15}$N isotope can be overcome by NMR pulse techniques and/or large volume probes. The advantages of the method—large scale of chemical shifts, high sensitivity of the $^{15}$N resonance to electronic and environmental effects—are promising also for the application in studying biological substances,[9-22] since nitrogen in the corresponding compounds is involved in many biological processes. For the investigation of biological molecules enrichment of the $^{15}$N isotope seems necessary since in most cases there is not much material available.

In this paper we want to describe the behavior of histidine in aqueous solution using nitrogen-15 and carbon-13 NMR. Histidine is known to be part of the active site of many enzymes.[23] The imidazole ring often plays a role in the catalytic function of these enzymes. It was the aim of our investigation to study the structural features of the imidazole ring of histidine and its interaction with the solvent water.

## Experimental Section

D,L-Histidine, $^{15}$N isotope enriched in both nitrogen atoms of the imidazole ring (~95% $^{15}$N), was purchased from Rohstoff-Einfuhr-GmbH, Düsseldorf, Germany.

NMR measurements were performed using 2 mL of an 0.2 M solution. For the determination of relaxation times $T_1$ and of NOE values, solutions free of paramagnetic impurities are needed. To remove traces of paramagnetic ions we have used the following procedures. D,L-Histidine was dissolved in an alkaline solution of doubly distilled $H_2O$ and given to a small column of activated Chelex 100 (Biorad Laboratories, Richmond, Calif.). The elution from the column was carried out with doubly distilled $H_2O$ and the amino acid lyophilized. After lyophilization the amino acid was again dissolved in $H_2O$ which was extracted five times with a solution of dithizone in $CCl_4$ according to Pearson et al.[24] This solution was always freshly made and had a basic pH value due to the purification procedure which was carried out at high pH values. Therefore only the adjustment with a concentrated HCl solution to lower pH values was necessary.

All glassware, tubes, and plugs used in these procedures were soaked overnight in an alkaline solution of EDTA and rinsed thoroughly with doubly distilled water.

pH values were measured directly in the 10-mm NMR sample tube (Wilmad Glass Co., Buena, N.J.) using a special combined electrode (Ingold, Frankfurt, Germany) and a Radiometer pH meter (Model PHM 26).

All $^{15}$N NMR measurements were performed on a Bruker HFX 90 at 9.12 MHz with Fourier transform mode employing a deuterium lock device. The deuterium signal was provided by $D_2O$ in a coaxial capillary inside the 10-mm NMR sample tube.

The temperature was maintained with a Bruker temperature control unit BST 100/700 and determined to be 38 ± 2 °C. $^{13}$C NMR measurements were carried out with a Bruker WH 270 at 67.89 MHz. The $^{15}$N NMR relaxation times $T_1$ were determined by the inversion recovery method ($180°-\tau-90°-5T_1$). Usually five scans were used to increase the signal-to-noise ratio. The $T_1$ values were calculated using a three-parameter nonlinear least-squares program according to the equation $M(\tau) = M_0(1 - ce^{-\tau/T_1})$.[25] The signal amplitudes were used as a probe for the magnetization $M(\tau)$. The NOE values were obtained from a comparison of spectra with and without proton broad band decoupling. The ratio of the integrated areas under the peaks was used to determine the NOE. Titration curves were calculated and fitted using the pH dependence of the chemical shift values ($\delta$) or the coupling constants ($J$) according to the Henderson–Hasselbalch equation:

$$\delta_{obsd} = \delta_{min} + \sum_i \Delta\delta_i \frac{10^{pH-pK_i}}{1 + 10^{pH-pK_i}} \quad (1)$$

or

$$J_{obsd} = J_{min} + \sum_i \Delta J_i \frac{10^{pH-pK_i}}{1 + 10^{pH-pK_i}} \quad (2)$$

where $\delta_{min}$ = minimum value of the chemical shift, $\Delta\delta_i$ = difference

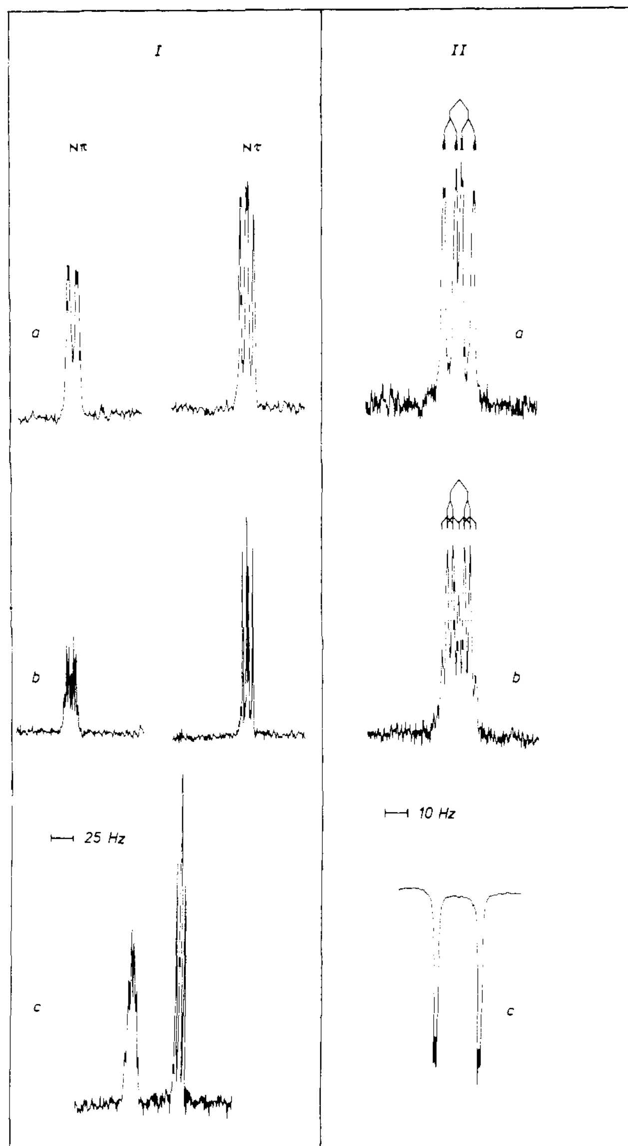



Figure 1. Typical $^{15}N$ NMR spectra of the histidine imidazole ring nitrogens in aqueous solution. I. Spectra without proton broad band decoupling showing the N-$\pi$ and N-$\tau$ resonance at different pH values. Spectra are taken after 20 000 transients (8 $\mu$s pulse width, 2 s repetition rate, 3012.05 Hz spectral width, 0.7 Hz digital resolution). (a) pH 11.05: The N-$\pi$ resonance reveals couplings to $^1H2$ (−10.3 Hz) and $^1H5$ (−2.2 Hz); the N-$\tau$ resonance is split by coupling with $^1H2$ (−8.8 Hz) and $^1H5$ (−6.6 Hz). (b) pH 6.14: The N-$\pi$ resonance exhibits couplings to $^1H2$ (−8.1 Hz), $^1H5$ (−2.6 Hz), and the $\beta$ protons (|2.6| Hz); the N-$\tau$ resonance shows the couplings to $^1H2$ (−6.6 Hz) and $^1H5$ (−5.2 Hz). (c) pH 5.01: The N-$\pi$ resonance is split by the couplings to $^1H2$ (−6.6 Hz), $^1H5$ (−2.9 Hz), and the $\beta$ protons (|2.9| Hz). Since the magnitudes of $^3J_{^{15}N_\pi-^1H5}$ and $^3J_{^{15}N_\pi-^1H\beta}$ are the same, the doublet of a doublet of a triplet which should be produced by the couplings is coalescing to the observed multiplet owing to the algebraic increase of $^2J_{^{15}N_\pi-^1H2}$. The N-$\tau$ resonance exhibits a triplet due to the same magnitude of the couplings to $^1H2$ (−4.8 Hz) and $^1H5$ (−4.8 Hz). II. $^{15}N$ NMR spectra after 9000 transients (12 $\mu$s pulse width, 5 s repetition rate, 600.24 Hz spectral width, 0.15 Hz digital resolution). (a) pH 7.83; without proton broad band decoupling; The N-$\tau$ resonance reveals a multiplet structure due to the couplings to $^1H2$ (−8.3 Hz), $^1H5$ (−5.7 Hz), and $^{15}N\pi$ (|0.6| Hz) as is shown by the splitting pattern. (b) pH 6.14, without proton broad band decoupling: The N-$\pi$ resonance is split owing to the couplings to $^1H2$ (−7.9 Hz), $^1H5$ (−2.6 Hz), and the $\beta$ protons (|2.6| Hz). The resulting multiplet structure is explained by the splitting pattern. (c) pH 1.43, with proton broad band decoupling: Owing to the NOE this spectrum reveals inverted resonances of both the N-$\pi$ and N-$\tau$ nitrogen. Both signals reveal the $^2J_{^{15}N_\pi-^{15}N_\tau}$ coupling (|0.9| Hz).

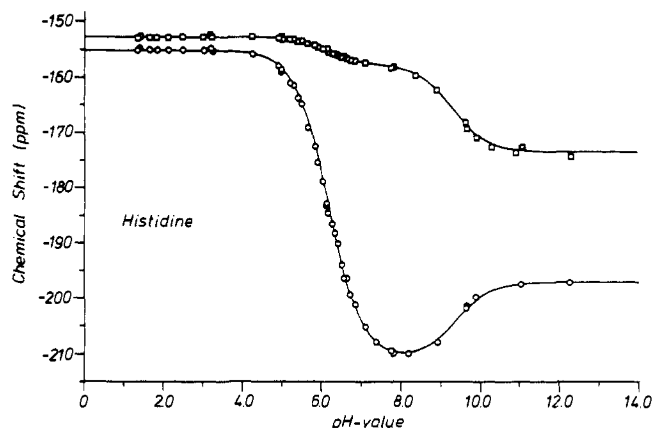



Figure 2. pH dependence of the $^{15}N$ chemical shifts of both imidazole nitrogens (N-$\pi$, ○; N-$\tau$, □). The solid lines represent the calculated titration curves using the Henderson-Hasselbalch equation.

Table I. $^{15}N$ Chemical Shifts of Imidazole Nitrogens and Ionization Constants of Histidine

| | Chemical shifts, ppm[a] | | | pK values | |
|---|---|---|---|---|---|
| | Cation[b] | Amphion | Anion | $pK_1$[c] | $pK_2$[d] |
| N-$\pi$ | −155.13 | −211.05 | −197.08 | 6.15 ± 0.007 | 9.35 ± 0.05 |
| N-$\tau$ | −152.72 | −157.73 | −173.41 | 6.19 ± 0.04 | 9.28 ± 0.02 |

[a] Negative sign means downfield from external 4 M $^{15}NH_4NO_3$ in 2 M $HNO_3$. [b] Cation represents the dicationic species of histidine as well as the monocationic species. [c] $pK_1$ is related to the ionization of the imidazole ring. [d] $pK_2$ is related to the ionization of the $\alpha$-amino group.

in the chemical shift of the resonances of the protonated and the deprotonated species, $K_i$ = equilibrium constant of the corresponding protonation process, $J_{min}$ = minimum value of the coupling constant, and $\Delta J_i$ = difference of the coupling constants of the protonated and the deprotonated species.

A nonlinear least-squares program uses $\delta_{min}$, $\Delta\delta_i$, or $J_{min}$, $\Delta J_i$ and the $pK_i$ values as adjustable parameters. It is assumed that there is rapid exchange between the ionized species resulting in one average signal and one average coupling constant.

## Results

**Chemical Shifts and pK Values.** Typical $^{15}N$ spectra of the imidazole nitrogens of D,L-histidine are shown in Figure 1. In Figure 2 the $^{15}N$ chemical shifts are plotted in dependence of pH. The corresponding values of the chemical shifts for the different ionized species of histidine and the pK values which are derived from curve-fitting procedures are listed in Table I.

**Coupling Constants.** $^{15}N$-$^1H$ coupling constants and $^{13}C$-$^{15}N$ coupling constants of the histidine imidazole ring are plotted in Figures 3 and 4 vs. pH. The titration curves are fitted using a nonlinear least-squares program. The coupling constants for the ionized species of histidine are listed in Table II. The $^{15}N$-$^{15}N$ coupling constants of the imidazole ring nitrogens are also given in Table II.

**Relaxation Times $T_1$ and NOE Values.** The $T_1$ values of the $\pi$ and the $\tau$ imidazole nitrogens are plotted in Figure 5 vs. pH. The solid line represents the calculated curve for the pH dependence of the relaxation rates as discussed below. In Table III the NOE values of both imidazole nitrogens are given for the ionized species of histidine. Although much care has been applied to get rid of paramagnetic ions, the $T_1$ values and the NOEs may be erroneous above pH 9, since histidine is a strong chelating reagent at high pH values.

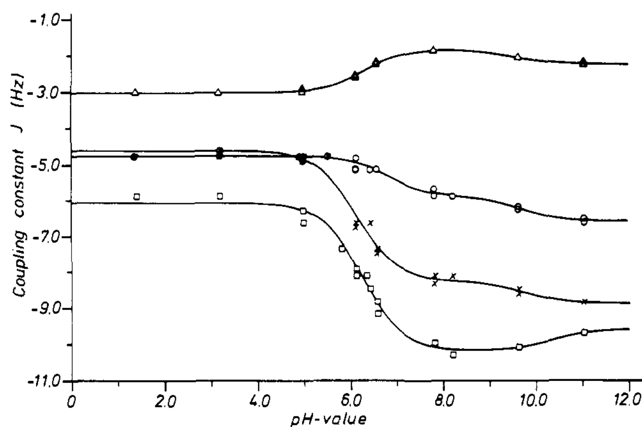



**Figure 3.** pH dependence of the $^{15}N-^{1}H$ coupling constants of the histidine imidazole nitrogens ($^{2}J_{^{15}N\pi-^{1}H2}$, □; $^{2}J_{^{15}N\tau-^{1}H2}$, ×; $^{2}J_{^{15}N\tau-^{1}H5}$, ○; $^{3}J_{^{15}N\pi-^{1}H5}$, △). Solid lines are calculated assuming rapid exchange between the different ionized species.

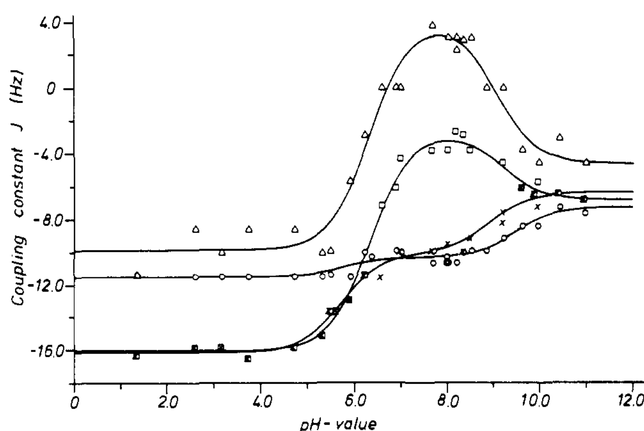



**Figure 4.** pH dependence of the $^{13}C-^{15}N$ coupling constants of the histidine imidazole nitrogens ($^{1}J_{^{13}C2-^{15}N\pi}$, □; $^{1}J_{^{13}C2-^{15}N\tau}$, ×; $^{1}J_{^{13}C5-^{15}N\tau}$, ○; $^{1}J_{^{13}C4-^{15}N\pi}$, △). The solid lines are calculated using the Henderson-Hasselbalch equation.

## Discussion

The imidazole ring of histidine in its neutral form may exist in two tautomeric structures (Figure 6): the proton is attached either to the $\tau$ or the $\pi$ position.[26-29] It is of interest to discuss the NMR data in view of this tautomeric equilibrium and to try to derive structural features from the various experimental parameters for the ring system itself and the transitions which occur in the ring upon the pH-dependent protonation processes, i.e., the protonation of the amino group at high pH, the protonation of the imidazole ring at neutral pH, and the protonation of the carboxyl group at low pH.

**Chemical Shifts.** The two $^{15}N$ resonances which are observed in the spectrum of the isotopically enriched histidine could be clearly assigned to the $\pi$ and the $\tau$ nitrogens of the imidazole ring due to the coupling of the $^{15}N$ nuclei to the neighboring protons. The chemical shift values of both resonances are very similar at low pH values (−152.7 ppm for N-$\tau$ and −155.1 for N-$\pi$). Apparently there is no change in the resonance position upon deprotonation of the carboxyl group. With further increase of the pH value both resonances shift to lower field or higher frequencies. However, the shift of the N-$\pi$ signal is about 55.9 whereas the shift of the N-$\tau$ signal is only 5.0 ppm. This shift in signal position of both resonances is clearly due to the deprotonation of the imidazole ring, and the titration curve with its p$K$ value of 6.15 for N-$\pi$ (6.19 for N-$\tau$) which has been fitted to the chemical shift data is in excellent agreement with literature data.[30] Above pH ~8 both resonances shift in opposite direction owing to the deprotonation of the $\alpha$-amino group of histidine.

The chemical shift values agree very well with those reported by Kawano and Kyogoku[31] for low pH values. Also the

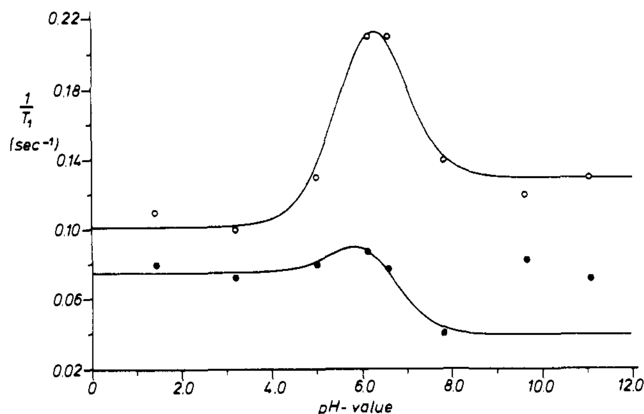



**Figure 5.** pH dependence of the relaxation rates of both imidazole nitrogens (N-$\pi$, ●; N-$\tau$, ○). The standard deviation of the $T_1$ values each of which is calculated from about 50 data points (50 $\tau$ values) is 2-4%. The solid lines represent the calculated pH dependence of the relaxation rates of both nuclei as discussed in the text. The $1/T_1$ values above pH 9 are probably erroneous, especially for the N-$\pi$ nucleus, because of paramagnetic impurities.

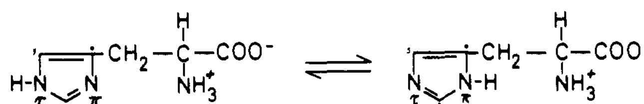


**Figure 6.** Tautomeric equilibrium of the imidazole ring of histidine above pH 6.2.

**Table II.** Coupling Constants[a] of the Imidazole Nitrogens for the Different Ionic Species of Histidine

| | Coupling constants, Hz | | |
|---|---|---|---|
| | Cation[b] | Amphion | Anion |
| $^{2}J_{^{15}N\pi-^{1}H2}$ | −6.1 | −10.2 | −9.6 |
| $^{3}J_{^{15}N\pi-^{1}H5}$ | −3.0 | −1.8 | −2.2 |
| $^{2}J_{^{15}N\tau-^{1}H2}$ | −4.6 | −8.2 | −8.8 |
| $^{2}J_{^{15}N\tau-^{1}H5}$ | −4.8 | −5.9 | −6.6 |
| $^{1}J_{^{13}C2-^{15}N\pi}$ | −16.0 | −2.7 | −6.9 |
| $^{1}J_{^{13}C4-^{15}N\pi}$ | −9.9 | +4.1 | −4.7 |
| $^{2}J_{^{13}C\beta-^{15}N\pi}$[c] | nd[d] | −4.6 | −3.8 |
| $^{1}J_{^{13}C2-^{15}N\tau}$ | −16.1 | −10.1 | −6.4 |
| $^{1}J_{^{13}C5-^{15}N\tau}$ | −11.6 | −10.4 | −7.3 |
| $^{2}J_{^{15}N\pi-^{15}N\tau}$[c] | ±0.9 | ±0.6 | ±0.9 |

[a] The values given are those calculated by the least-squares program for the various ionized species. The digital resolution provided by the BNC 12 computer is 0.7 Hz for the $^{13}C$ data (32 K Fourier transformation, 10.5 kHz spectral width, quad-detection) and 0.15 Hz for the $^{15}N$ data (8 K Fourier transformation, 600 Hz spectral width). [b] Cation represents the dicationic as well as the cationic species of histidine. [c] Data are not fitted by the least-squares program. The values given are measured at pH 1.3, cation; pH 7.6, amphion; and pH 10.9, anion. [d] Not detected.

**Table III.** NOE Factors of Both Imidazole Nitrogens for the Different Ionic Species of Histidine

| | Cation[a] | Amphion[a] | Anion[a] |
|---|---|---|---|
| N-$\pi$ | −4.8 | −2.3 | −1.8 |
| N-$\tau$ | −4.9 | −2.7 | −2.0 |

[a] The values given represent the NOE factors at pH 1.4, 7.8, and 11.0 for the cationic, amphionic, and anionic species of histidine, respectively.

chemical shift values of the $^{14}N$ resonances of histidine described by Richards and Thomas are very similar to our data if one considers the different choice of the standard.[32] A deviation of about 8-9 ppm to the $^{15}N$ chemical shift data of Pregosin, Randall, and White is found. These data are, however, not comparable because these authors measured the $^{15}N$ chemical shifts of the imidazole nitrogens of histidine methyl ester.[33]

According to Witanowski the nitrogen in five-membered heterocyclic ring systems can essentially be found in two different states.[34] The $\alpha$-type nitrogen provides the two electrons of its lone pair to the aromatic $\pi$ system, whereas the $\beta$-type nitrogen provides only one electron to the $\pi$ system. Its lone pair is not involved in the $\pi$ system and therefore accessible for processes like protonation. Since both types of nitrogen are found in the imidazole ring, it would seem useful to prove whether the chemical shift values found for the $^{15}N$ resonances are in qualitative agreement with the predicted behavior for the two types of nitrogen in five-membered heterocyclic systems.

The chemical shift or the screening constant $\sigma$ is usually discussed with respect to contributions from a diamagnetic ($\sigma^d$) and a paramagnetic ($\sigma^p$) term as defined by Ramsey.[35] The diamagnetic term is assumed to be small only in the case of $^{15}N$ nuclei. Moreover, Ebraheem et al. were able to show that the diamagnetic term remains roughly constant upon smaller changes of the chemical environment of the nitrogen atom.[36] Such a small change would be a protonation process in the case of the imidazole nitrogens. Thus the shift differences—especially for $sp^2$- or sp-hybridized nitrogens—are essentially induced by the paramagnetic term. The paramagnetic term may be evaluated using some simplifying assumptions. Following the AEE (average excitation energy) approximation the chemical shifts of nitrogen nuclei in heterocyclic ring systems are proportional to three terms:[65] the so-called orbital expansion term, the $\Sigma Q_{ij}$ term which represents the distribution of the 2p electrons in the ring system, and the average excitation energy term $\Delta E_{av}$ (eq 3)

$$\sigma_j^p \propto -\frac{1}{\Delta E_{av}} \langle r^{-3} \rangle_{2p} \sum_i Q_{ij} \qquad (3)$$

where $\langle r^{-3} \rangle_{2p}$ is the so-called orbital expansion term, the mean value of the reciprocal cube of the 2p orbital radius.

The first two terms are influenced to a considerable content by variations in the $\pi$-electron density $q_N^\pi$ at the nitrogen atom. Both terms decrease with increasing $\pi$-electron density. Witanowski et al.[34] were able to show that a linear relationship exists between the nitrogen chemical shift and the $\pi$-electron density $q_N^\pi$ in various heterocyclic compounds. The $q_N^\pi$ values for $\alpha$-type nitrogens are higher than for $\beta$-type ones. The protonation of the lone pairs of the $\beta$-type nitrogens causes therefore an increase in $\pi$-electron density and consequently a shift of the resonance to higher field. This interpretation does not mean that the protonation induces an increase of the overall electronic charge at that nitrogen. Also the $\sigma$ part of the electronic system is affected by protonation of such a lone pair, and its electronic density is expected to decrease at that nitrogen with protonation.

From their calculations they concluded furthermore that there has to be a difference in the excitation energy $\Delta E$ between $\alpha$- and $\beta$-type nitrogens, the third term affecting the chemical shifts. This difference is quite reasonable since in the $\beta$-type nitrogen a low-lying n–$\pi^*$ transition is possible, whereas in the $\alpha$-type nitrogen such a transition is not possible. Therefore the protonation should have an influence on the $\Delta E_{av}$ value. Obviously all three terms of the AEE approximation influence the chemical shifts of the two imidazole nitrogens. Only a qualitative explanation of the chemical shift values following the protonation of the imidazole ring seems possible.

With an increase of the pH value above pH 5, a drastic decrease of the chemical shift of one of the two nitrogen resonances to lower field is observed, whereas the second resonance is only slightly affected. Both resonances at low pH values clearly represent $\alpha$-type nitrogens. The deprotonation of the $\pi$ nitrogen decreases the $q_N^\pi$ value and diminishes the polarization of the $\sigma$ framework, and the occurrence of the lone electron pair seems to lower the $\Delta E_{av}$ value. All these effects are expected to produce such a remarkable shift of the N-$\pi$ resonance to lower field.

The much smaller shift of the $\tau$ nitrogen resonance to lower field cannot be explained in terms of decreasing $q_N^\pi$ values and decreasing $\sigma$ polarization. The release of $\pi$-electron density at the deprotonating nitrogen and the decreasing polarization of the $\sigma$ core should increase the shielding because the electronic charge at the $\alpha$-type nitrogen is increased even if only the paramagnetic term of the shielding is considered. However, the small shift of the $\tau$ nitrogen resonance may be explained in terms of the tautomeric equilibrium described above for the deprotonated imidazole ring. Since we have to take into account that we are observing weighted averages of two states of either of the two nitrogens the slight shift of the $\tau$ nitrogen resonance to lower field upon deprotonation of the imidazole indicates that the $\tau$ nitrogen is also transformed—to a much lesser extent than the N-$\pi$ to a $\beta$-type nitrogen being deprotonated in the equilibrium. Yet, the downfield shift of the $\tau$ nitrogen produced by the tautomeric equilibrium is nearly cancelled by the upfield shift owing to the electronic changes which occur as a consequence of the deprotonation process at the $\pi$ nitrogen. Hence only a small shift of about 5 ppm is observed.

With deprotonation of the $\alpha$-amino group around pH 9.3, again remarkable changes of the chemical shift of both nitrogen resonances are observed (Figure 2 and Table I). The N-$\pi$ signal is shifting upfield whereas the N-$\tau$ signal is shifting downfield. The magnitude of both shifts in signal position is about 15 ppm. Obviously there must be an interaction between the imidazole ring and the $\alpha$-$NH_3$ group, whereas an interaction between the carboxyl group and the imidazole ring seems unlikely because no change in resonance position is observed when the carboxylate is protonated around pH 1.8.

An interaction between the imidazole ring and the $\alpha$-amino group is only possible via the $\pi$ nitrogen because of sterical reasons. This interaction between the lone electron pair of the $\beta$-type nitrogen and the protonated amino group does not induce as strong effects on the chemical shift as a protonation process. Presumably the interaction consists of an asymmetric hydrogen bond with the proton attached to the amino nitrogen. However, it seems that because of this interaction the tautomeric equilibrium described by Figure 6 is shifted to the species with the $\pi$ nitrogen in the deprotonated state.

With deprotonation of the $\alpha$-amino group which causes the breakdown of that interaction, a considerable amount of species with $\tau$ nitrogen being a $\beta$-type nitrogen is formed, which produces the downfield shift of the $\tau$ nitrogen resonance position. The $\pi$ nitrogen resonance is shifted upfield owing to the partial conversion of this nucleus to an $\alpha$-type nitrogen. Because of the tautomeric equilibrium the same amount of $\pi$ and $\tau$ nitrogen is converted to an $\alpha$- or $\beta$-type nucleus, respectively, during the deprotonation process, so the shift in signal position of $\pi$ and $\tau$ nitrogen of the same magnitude but inverted sign is quite reasonable. It should be emphasized that above pH 9.3 the species with the $\pi$ nitrogen being deprotonated is still the predominant one.

Since the chemical shift values of nuclei are strongly affected by chemical substitution in the environment, it is not possible

to evaluate the tautomeric equilibrium by comparison of the observed shifts with the shifts of compounds which resemble the pure tautomers like pyrrole ($\alpha$-type nitrogen) or *N*-methylimidazole ($\alpha$- and $\beta$-type nitrogen). However, the shift of the nitrogen resonance positions in the alkaline region which should be exclusively produced by changes in the tautomeric equilibrium can in principle be used to estimate the magnitude of the change of that equilibrium. Since the shift difference between the pyrrole nitrogen ($\alpha$-type nitrogen) and the $\beta$-type nitrogen of *N*-methylimidazole is about 120 ppm in $CCl_4$, we are able to estimate the shift of the mole fractions of each tautomer to be 0.12 unit. It should be stressed that this value is only a rough *estimate* especially since $^{15}N$ chemical shifts are known to be strongly solvent dependent. A better method to derive absolute values of mole fractions of the tautomeric equilibrium will be discussed later.

This interpretation of the pH dependence of the imidazole $^{15}N$ resonances is in agreement with $^{13}C$ NMR investigations of histidine derivatives by Reynolds et al.[27] In some of these derivatives the $\alpha$-amino group is blocked such that an interaction of the imidazole ring with the amino group is impossible. The observed difference of the investigated $^{13}C$ resonances of histidine derivatives with histidine led to the conclusion that a smaller amount of tautomer with the $\pi$ nitrogen deprotonated exists in these compounds.[27] The difference of the tautomeric equilibrium of those compounds in comparison with histidine between pH 6.2 and pH 9.3 can now be understood, if the interaction of the deprotonated $\pi$ nitrogen and the protonated $\alpha$-amino group in histidine itself is considered, since the tautomeric equilibrium of the deprotonated imidazole ring is dependent on this interaction.

## Coupling Constants

**$^1J_{^{13}C-^{15}N}$.** Usually coupling constants are interpreted as being governed by three terms: $J_{AB}^{FC}$, the Fermi contact term, $J_{AB}^{o}$, the term of interaction between the angular momentum of the electron orbitals and the nuclear spin, and $J_{AB}^{sd}$, the term representing the interaction between the dipoles of the electrons and the nucleus.[37] In most cases the Fermi contact term is the predominant one, and there has been good success in the interpretation of coupling constants using only the Fermi contact term.[38] Based on this assumption a relationship between the s character of hybrid orbitals of the bound nuclei and the $^1J$ coupling constant can be derived. Following Binsch and coworkers[39] there is a simple empirical relation between $^1J_{^{13}C-^{15}N}$ and the product of percent s characters ($s_{^{15}N}$, $s_{^{13}C}$) in the corresponding bonding hybrids:

$$80\,^1J = s_N s_C \quad (4)$$

This equation is valid for many compounds. However, remarkable deviations are observed in the case of those nitrogen compounds, the $^{15}N$ resonances of which are shifted downfield to a large extent. Schulman and Venanzi[40] found out from theoretical considerations that the contribution of the Fermi contact term is large and negative for some aromatic nitrogen-containing compounds; for other aromatic heterocyclic compounds it is zero or small and negative. From their data based on a Hartree-Fock-type calculation, these authors concluded that the investigated aromatic compounds can be divided into two groups: compounds which contain a lone electron pair ($\sigma$ lone pair)[40] at the nitrogen and those which do not. This classification corresponds to that of $\beta$- and $\alpha$-type nitrogens in histidine.

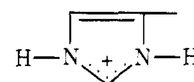
In the case of such lone electron pairs at the nitrogen the $J_{AB}^{FC}$ term is small. Therefore the $^1J_{^{13}C-^{15}N}$ coupling constant is also determined by the terms $J_{AB}^{o}$ and $J_{AB}^{sd}$. Since the Binsch relationship is based upon the predominant contribution of the Fermi contact term, it is only valid for those aromatic compounds which do not contain a $\sigma$ lone electron pair at the nitrogen.

The imidazole ring of histidine contains two nitrogens of both types, the $\pi$ and the $\tau$ nitrogen. Two electrons of the $\tau$ nitrogen are involved in the aromatic system having almost pure p character, whereas the $\sigma$ lone pair at the $\pi$ nitrogen presumably cancels the Fermi contact contribution of the $^1J_{^{13}C-^{15}N}$ coupling. The determination of the $^1J_{^{13}C-^{15}N}$ coupling constants of the imidazole ring of histidine (Figure 4) has been carried out according to the assignment of the carbon resonances to the corresponding carbon atoms.[27,41] The pH dependence of the $^1J_{^{13}C-^{15}N}$ values reflects the various protonation states of histidine. The corresponding titration curves are calculated using the Henderson-Hasselbalch equation. The p*K* values obtained by this fitting procedure are in good agreement with those obtained from the pH dependence of the chemical shift values of the $^{15}N$ resonances (Figure 1 and Table I) and the corresponding $^{13}C$ resonances.

At pH values below 5 the imidazole ring is protonated. As already discussed explicitly above, the $\beta$-type nitrogen is converted to an $\alpha$-type nitrogen; the $J_{AB}^{FC}$ term dominates the coupling of the $\pi$ nitrogen nucleus with the neighboring carbon nuclei. In Table II the $^1J_{^{13}C4-^{15}N\pi}$ and the $^1J_{^{13}C2-^{15}N\pi}$ values at low pH values are negative (−9.9 and −16.0 Hz). Although we were not able to determine the sign of the $^1J_{^{13}C-^{15}N}$ coupling constants with our experimental device, it should be clear from the theoretical outline of Schulman and Venanzi[40] that these couplings between an $\alpha$-type nitrogen and a carbon are negative. Experimental determinations and calculations of the sign of $^1J_{^{13}C-^{15}N}$ couplings for substances, the coupling constants of which are governed predominantly by the FC term, support this assumption.[42-46] Also from the pH dependence the sign of these $^1J$ values can be derived as will be discussed below.

The Binsch relation[39] predicts a $^1J_{^{13}C-^{15}N}$ value of about −14 Hz for the coupling between both the $sp^2$-hybridized nitrogen and the $sp^2$-hybridized carbon atom. The value of −16.0 Hz for $^1J_{^{13}C2-^{15}N\pi}$ is in fairly good agreement with this value, whereas the lower value of −9.9 Hz for $^1J_{^{13}C4-^{15}N\pi}$ indicates a reduced s character of the C-4 hybrid.

For the coupling of the $\tau$ nitrogen with the neighboring carbon atoms, the values of −16.1 Hz ($^1J_{^{13}C2-^{15}N\tau}$) and −11.6 Hz ($^1J_{^{13}C5-^{15}N\tau}$) were obtained at low pH values (Table II). It seems that the coupling constants $^1J_{^{13}C2-^{15}N\tau}$ and $^1J_{^{13}C2-^{15}N\pi}$ correspond to each other as well as the $^1J_{^{13}C5-^{15}N\tau}$ and the $^1J_{^{13}C4-^{15}N\pi}$ values. It seems plausible to divide the imidazole ring into two parts which can be characterized by their electronic structure: the bonds of the part N-$\tau$-C-2-N-$\pi$ seem to be mainly $sp^2$-hybridized whereas the hybrids of the part N-$\tau$-C-5-C-4-N-$\pi$ obviously lost some of their s character and tend to be more $sp^3$-hybridized. A difference of the two parts can also be derived from the coupling constants $^1J_{^{13}C2-^1H2}$ and $^1J_{^{13}C5-^1H5}$.[47] The protonated imidazole ring is usually depicted as[48]




This structural formula indicates that the positive charge is delocalized over the N-$\tau$-C-2-N-$\pi$ part which leads to the above-described electronic structure. Although this formula seems to be brought about merely by intuition, it has led in this case to a correct description.

With deprotonation of the imidazole ring a $\beta$-type nitrogen is produced. The $\sigma$ lone pair reduces the contribution from the Fermi contact term which results in a less negative value of the coupling constant if the $J_{AB}^{FC}$ term is predominant and negative in the protonated imidazole ring. It should be mentioned that the terms $J_{AB}^{o}$ and $J_{AB}^{sd}$ of the corresponding couplings of pyr-

idine are nearly constant upon deprotonation of the nitrogen.[40] Hence we would like to assume a similar behavior for the coupling of C-2 and C-4 to the $\pi$ nitrogen. Since the coupling constants $^1J_{^{13}C2-^{15}N\pi}$ and $^1J_{^{13}C4-^{15}N\pi}$ are becoming less negative or even slightly positive, it can be concluded that the $\pi$ nitrogen is the predominant site of deprotonation as was already derived from the pH dependence of the chemical shifts. The $^1J_{^{13}C4-^{15}N\pi}$ value obviously inverts its sign at around pH 7 and increases to a positive value of about 4 Hz (Figure 4). A similar algebraic increase of $^1J_{^{13}C-^{15}N}$ upon deprotonation is also observed in the case of pyridine,[7,40] quinoline,[49] and 3,4-dihydroisoquinoline.[50] The coupling constant $^1J_{^{13}C5-^{15}N\tau}$ is roughly independent on deprotonation of the imidazole ring, whereas the value of $^1J_{^{13}C2-^{15}N\tau}$ is reduced from $-16$ to $-10$ Hz. The influence of deprotonation of $^1J_{^{13}C2-^{15}N\tau}$ is difficult to estimate. The conversion of N-$\pi$ to a $\beta$-type nitrogen which induces a change of the electronic structure of neighboring bonds certainly has an effect on $^1J_{^{13}C2-^{15}N\tau}$. However, an additional effect has to be considered. According to the abovementioned tautomeric equilibrium of the deprotonated imidazole ring (Figure 6), we have to assume that part of the N-$\tau$ is also converted to a $\beta$-type nitrogen. Both these effects contribute to the change of $^1J_{^{13}C2-^{15}N\tau}$ at pH 6.2. The small change of $^1J_{^{13}C5-^{15}N\tau}$ ($\sim$1 Hz) is obviously induced by the tautomeric equilibrium which reduces the amount of $\alpha$-type nitrogen at the N-$\tau$ position of the imidazole ring. It seems that other electronic changes of the bonding system between C-5 and N-$\tau$ upon deprotonation of the imidazole ring are small.

At pH values above 8.0 the $^1J_{^{13}C-^{15}N}$ values are again subject of a change due to the deprotonation of the $\alpha$-amino group (Figure 4). From the pH dependence of the $^{15}N$ chemical shifts it has been concluded that at pH 9.3 the tautomeric equilibrium of the deprotonated imidazole ring is shifted toward a larger amount of species with the $\tau$ nitrogen being deprotonated. As a consequence of this behavior the $^1J_{^{13}C4-^{15}N\pi}$ value becomes less positive, inverts its sign, and finally gets negative. In a similar manner $^1J_{^{13}C2-^{15}N\pi}$ becomes more negative. The other carbon-nitrogen coupling constants ($^1J_{^{13}C2-^{15}N\tau}$ and $^1J_{^{13}C5-^{15}N\tau}$) become less negative. Again from the shift of the tautomeric equilibrium which is reflected in the changes of the $^1J_{^{13}C-^{15}N}$ values, our assumption of an interaction between the imidazole ring and the $\alpha$-amino group is confirmed.

**$^2J_{^{13}C-^{15}N}$.** This coupling constant is dependent on the orientation of the lone pair at the nitrogen relative to the coupled carbon.[44,51,52] The proximity of a lone pair to the coupled carbon nucleus should make a positive contribution to the reduced nitrogen-carbon coupling constant.[53] The trans configuration of the lone electron pair with respect to the corresponding carbon atoms produces a relatively small coupling constant, and hence the values of $^2J_{^{13}C5-^{15}N\pi}$ and $^2J_{^{13}C4-^{15}N\tau}$ could not be determined, also because protonation of the lone pair has only a small influence on the coupling constant in this conformation.[7] The $^2J_{^{13}C\beta-^{15}N\pi}$ value can only be determined at pH values above 6.2; the lone pair at the N-$\pi$ being predominantly a $\beta$-type nitrogen at that pH is in a cis configuration to the $\beta$-carbon atom. Because of the probable positive contribution of the cisoid lone pair to the reduced coupling constant, we suppose the $^2J_{^{13}C\beta-^{15}N\pi}$ value to be negative.

**$^2J_{^{15}N\pi-^{15}N\tau}$.** This coupling also is dependent on the orientation of the lone pair electrons at either nitrogen relative to the considered nitrogen nucleus. At lower pH values both nitrogens are $\alpha$-type nitrogens. With deprotonation of the imidazole ring the coupling is becoming smaller and goes through zero at pH 6.2 (Table II). However, the effect is only small as observed for the correspondent $^{13}C-^{15}N$ couplings in pyridine[7,44] and quinoline.[49,51] It is not possible to estimate the sign of this coupling by comparison of its pH dependence with the pH dependence of $^2J_{^{15}N-^1H}$ and $^2J_{^{15}N-^{13}C}$ values in compounds with similar geometry because the changes of these couplings in pyridine[44] and acetaldoxime[54] on protonation are just opposite.

**$^1J_{^{15}N-^1H}$.** For the whole investigated pH range the exchange of the protons attached to the nitrogens of the imidazole ring is so fast that no direct coupling is observed. The line width of the $^{15}N$ resonances with and without proton decoupling is identical and should represent the natural line width ($1/\pi T_2^*$). If a value of about 80-90 Hz for this coupling constant is assumed, the averaging out of this coupling indicates that the exchange rate of the protons must be larger than $10^3$ $s^{-1}$.

**$^2J_{^{15}N-^1H}$.** This coupling also strongly depends on the orientation of the lone electron pair at the nitrogen, if there is any, relative to the considered hydrogen atom.[54-56] In the case of the cisoid configuration usually relatively large coupling constants (10-15 Hz) are observed whereas in the transoid configuration small values of $^2J_{^{15}N-^1H}$ (only a few hertz) are found.

In histidine the $^2J_{^{15}N-^1H}$ coupling constants are supposed to be negative from theoretical considerations of some other heterocyclic compounds.[7,57] For the protonated imidazole ring all these coupling constants resemble those which are usually found for a transoid arrangement. Apparently the lone pair only influences $^2J_{^{15}N-^1H}$ when arranged in a cis configuration, whereas a trans configuration does not have a remarkable influence. Upon deprotonation of the imidazole ring the $^2J_{^{15}N-^1H}$ values become more negative.

$^2J_{^{15}N\pi-^1H2}$ gets down to a value of about $-10.2$ Hz reflecting the fact that N-$\pi$ is converted to a $\beta$-type nitrogen with a lone electron pair cisoid to the C-2 hydrogen atom. However, the coupling constant $^2J_{^{15}N\tau-^1H2}$ also becomes a considerable amount more negative. The rearrangement of the electronic system due to the conversion of the $\pi$ nitrogen and the part conversion of the $\tau$ nitrogen itself in the tautomeric equilibrium of the deprotonated imidazole is certainly responsible for this effect. Even the $^2J_{^{15}N\tau-^1H5}$ value is becoming slightly more negative at pH 6.2. This effect should be entirely due to the occurrence of a lone pair in a cisoid configuration to H-5 at N-$\tau$. From the pH dependence of the carbon-nitrogen couplings it was concluded that the electronic structure of the C-5-N-$\tau$ bond does not change upon deprotonation of the imidazole, because no remarkable change of $^1J_{^{13}C5-^{15}N\tau}$ is observed at pH 6.2, whereas the $^1J_{^{13}C2-^{15}N\tau}$ constant does change. Hence, it seems justified to assume that the change in $^2J_{^{15}N\tau-^1H5}$ at pH 6.2 is almost completely reflecting the partial conversion of the N-$\tau$ into a $\beta$-type nitrogen according to the tautomeric equilibrium (Figure 6).

We have used the pH dependence of the $^2J_{^{15}N\tau-^1H5}$ to determine the amount of $\beta$-type nitrogen at N-$\tau$ in the equilibrium at several pH values. Above pH 6.2 the observed coupling constant can be expressed by

$$^2J_{^{15}N\tau-^1H5} = \gamma_{\beta N\tau}{}^2J_{\beta N} + \gamma_{\alpha N\tau}{}^2J_{\alpha N} \quad (5)$$

where $\gamma_{\beta N\tau}$ and $\gamma_{\alpha N\tau}$ are the mole fractions of N-$\tau$ being a $\beta$-type nitrogen in the deprotonated imidazole and an $\alpha$-type nitrogen, respectively. $^2J_{\beta N}$ is the coupling constant $^2J_{^{15}N-^1H}$ for a five membered heterocyclic system with an electron lone pair at the nitrogen. Its value is found to be $-14.4$ Hz for various compounds.[7,56,58] $^2J_{\alpha N}$ is the corresponding coupling constant $^2J_{^{15}N-^1H}$ of a heterocyclic system with its nitrogen fully protonated. From our measurements this value is determined to be $-4.8$ Hz. Very similar values are obtained for other fully protonated five-membered heterocyclic compounds.[7,8,56,58,59] From eq 5 we obtain:

$$\gamma_{\beta N\tau} = ({}^2J_{^{15}N\tau-^1H5} - {}^2J_{\alpha N})/({}^2J_{\beta N} - {}^2J_{\alpha N}) \quad (6)$$

Using eq 6 we get a value for $\gamma_{\beta N\tau}$ of 0.12 at pH 8.2. It should be mentioned that Reynolds and co-workers[27] have estimated

$\gamma_{\beta N_\tau}$ using chemical shift data. Since these authors have to compare derivatives of histidine modified at either ring nitrogen to get the limiting values of the chemical shift for the corresponding tautomers, they have to consider the effect of substituents on the chemical shift values, which may produce errors in the determination of $\gamma_{\beta N_\tau}$. Nevertheless, their estimate of $\gamma_{\beta N_\tau} = 0.2$ corresponds to our value of $\gamma_{\beta N_\tau} = 0.2$ for pH values above pH 9.3. The assumed interaction between the protonated $\alpha$-amino group and the deprotonated imidazole ring between pH 6.2 and 9.3 shifts the tautomeric equilibrium as already outlined discussing the chemical shift data. A larger portion of the $\pi$ nitrogen is converted to a $\beta$-type nitrogen. Since the $^2J_{^{15}N_\tau-^1H5}$ coupling constant is a probe for this conversion, this constant should be more negative above pH 9.3, and indeed this is observed (Figure 3). In our quantitative treatment according to eq 6 the $\gamma_{\beta N_\tau}$ value is shifting from 0.12 to 0.2. This is in reasonable agreement with the value of 0.12 *estimated* for the change of $\gamma_{\beta N_\tau}$ from $^{15}N$ chemical shift data. We can estimate the free enthalpy of the interaction between the $\alpha$-amino group and the imidazole ring between pH 6.2 and 9.3 from this shift of the tautomeric equilibrium ($\Delta G = -2.6$ kJ/mol). Also the values of $^2J_{^{15}N_\pi-^1H2}$ and $^2J_{^{15}N_\tau-^1H2}$ are changed upon deprotonation of the $\alpha$-amino group. As outlined above both couplings are sensitive to the conversion of an $\alpha$-type nitrogen into a $\beta$-type nitrogen or vice versa. Hence the $^2J_{^{15}N_\pi-^1H2}$ coupling constant becomes less negative in going from pH 8 to above pH 9.3 because the amount of $\alpha$-type nitrogen character at N-$\pi$ is increased, and the $^2J_{^{15}N_\tau-^1H2}$ value is getting more negative due to the larger amount of $\beta$-type nitrogen character at N-$\tau$. The magnitude of these changes in $^2J_{^{15}N-^1H}$ is nearly the same, namely about 0.6 Hz (Figure 3, Table II).

**$^3J_{^{15}N-^1H}$.** Two couplings of this type can be observed: $^3J_{^{15}N_\pi-^1H5}$ and $^3J_{^{15}N_\pi-^1H\beta}$. (The analysis of the $^{15}N$ spectra is shown in Figure 1.) The pH dependence of the $^3J_{^{15}N_\pi-^1H5}$ coupling constant again reflects the conversion of an $\alpha$-type nitrogen into a $\beta$-type nitrogen at pH 6.2. The value of that coupling constant increases at the p$K$ value of the imidazole ring whereas at the p$K$ of the $\alpha$-amino group a decrease is observed (Figure 3).

From investigations on $^3J_{^{15}N-^1H}$ couplings in pyridine[7] and quinoline[54] and from comparison of the $^3J_{^{15}N-^1H}$ couplings in imines and enamines,[60] it is known that such couplings are relatively small if there exists a lone pair at the nitrogen whereas these $^3J$ values are larger if the influence of that lone pair is reduced by protonation. Again the sign of this constant is assumed to be negative analogous to the corresponding values in pyridine[7] and some oximes.[54] Further evidence for this assumption follows from the similar behavior of the $^3J_{^{15}N-^1H}$ coupling constants of these compounds on protonation.

As outlined above, a lone pair is produced at pH 6.2 to a certain amount at the $\pi$ nitrogen, which increases the $^3J_{^{15}N_\pi-^1H5}$ coupling constant algebraically. The shift of the tautomeric equilibrium above pH 9.3 causes a larger amount of $\alpha$-type character at position N-$\pi$, which results in a slightly more negative coupling constant above this pH value.

The other coupling constant $^3J_{^{15}N_\pi-^1H\beta}$ should allow certain insights into the conformation of the bond between C-$\beta$ and C-4, since $^3J$ couplings are known to be strongly dependent on the dihedral angle between the coupled nuclei.[61-63]

The coupling constants of the coupling between the $\pi$ nitrogen and the $\beta$ protons can be represented by the following equations:

$$^3J_{^{15}N_\pi-^1H\beta A} = g_1{}^3J_{I} + g_2{}^3J_{II} + g_3{}^3J_{I} \quad (7)$$

$$^3J_{^{15}N_\pi-^1H\beta B} = g_1{}^3J_{II} + g_2{}^3J_{I} + g_3{}^3J_{I} \quad (8)$$

if rapid exchange between the possible rotamers in solution is assumed. $g_1$, $g_2$, and $g_3$ are the mole fractions of these rotamers

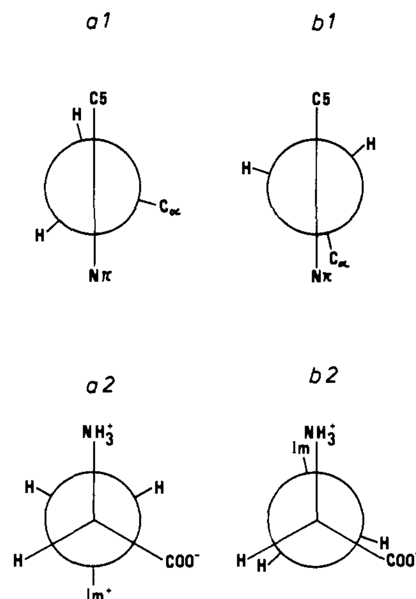



**Figure 7.** Conformations of histidine in aqueous solution as proposed by $^{15}N$ and $^{13}C$ NMR studies: (a) conformation of histidine in the acid pH region below pH 6.2; (b) conformation of histidine between pH 6.2 and 9.3: (upper part) conformation of the C4–C$\beta$ bond; (lower part) conformation of the C$\alpha$–C$\beta$ bond.

in solution as depicted in Figure 7. $g_1$ and $g_2$ correspond to the rotamers with either $H_A$ or $H_B$ in gauche position to N-$\pi$, whereas $g_3$ is related to the rotamer with both protons gauche to N-$\pi$ (Figure 7,a1). $^3J_I$ and $^3J_{II}$ are the coupling constants for the pure gauche and trans conformations of the coupling between the $\beta$ protons and the $\pi$ nitrogen, respectively. Since we observe different values for the coupling $^3J_{^{15}N_\pi-^1H\beta A}$ and $^3J_{^{15}N_\pi-^1H\beta B}$ at low pH values we have to conclude that the rotamers $g_1$ and/or $g_2$ have to be considered in this pH region.

Model building and the charges on the imidazole ring, carboxylate and $\alpha$-amino group prompted us to propose that the carboxylate group is turned over the ring plane where a positive charge is located. In this conformation the positive charges of the imidazole and the $\alpha$-amino group are furthest apart and because of the repulsive forces this arrangement becomes plausible. This structure implies also a restriction of the rotation around the C-$\alpha$-C-$\beta$ bond (Figure 7). This conformation resembles the crystal structure of histidine hydrochloride monohydrate to a large amount.[64] Only a rotation of a few degrees around the C-$\alpha$-C-$\beta$ bond is necessary to get our proposed solution conformation. The fact that we do not detect any change in the $^{15}N$ chemical shifts of both ring nitrogens during deprotonation of the carboxylate group supports this interpretation; such a change should be expected if there were any hydrogen bonding between the carboxylate group and the protonated imidazole ring (Figure 7).

With the deprotonation of the imidazole ring two effects are observed. The values of the $^3J$ coupling constants decrease and become undetectable above pH 7 which can be attributed to the electronic changes in the ring system itself and to changes of the conformation of the C-$\beta$-C-4 bond. The couplings of the $\pi$ nitrogen to the two C-$\beta$ protons become equivalent. The interaction between the $\alpha$-amino group and the imidazole ring in the pH range of 6.2–9.3 which is evident from the chemical shift data and nearly all discussed couplings is only possible if both C-$\beta$-H bonds are forming an angle of about 120° to the C-4-N-$\pi$ bond. In this position the C-$\beta$ protons become equivalent relative to the imidazole ring as depicted for the correspondent rotamer b1 in Figure 7. For the discussed interaction the axis C-5-N-$\pi$ is inverted in comparison with

Figure 7a. Hence $g_1$ and $g_2$ are now related to the rotamers with either $H_A$ or $H_B$ cis to N-$\pi$ whereas $g_3$ corresponds to the rotamer with both protons transoid to N-$\pi$ (Figure 7,b1). Correspondingly the definitions of $^3J_I$ and $^3J_{II}$ are changed. $^3J_I$ is the coupling constant for a transoid arrangement between N-$\pi$ and the $\beta$ protons, whereas $^3J_{II}$ is related to a cis conformation between these nuclei. However, this other view of the molecule does not influence the deductions which are derived from the $^3J$ coupling constants. We have to assume that the populations of the rotamers b (Figure 7) (mole fractions $g_1$ and $g_2$) are only small. Again the direct interaction between the $\alpha$-amino group and the imidazole ring determines the conformation of the C-$\alpha$-C-$\beta$ bond (Figure 7).

With deprotonation of the $\alpha$-amino group no change in $^3J_{^{15}N\pi-^1H\beta}$ is observed although the increasing amount of $\alpha$-type nitrogen character at N-$\pi$ should enlarge that $^3J$ value as it is found for $^3J_{^{15}N\pi-^1H5}$. However, the effect due to the conversion of N-$\pi$ is only small, and furthermore the averaged coupling constant $^3J_{^{15}N\pi-^1H\beta}$ due to three staggered conformations of the now freely rotating methylene group is supposed to be very small. From the work of Bystrov[62] and Gearhart[63] it is known that the $^3J_{^{15}N-^1H}$ couplings in amide systems which are to a certain amount similar to the electronic structure of the -$CH_2$-C-4-N-$\pi$ part of histidine are small and change sign when the dihedral angle is becoming 120°.

### Relaxation Rates $T_1$ and NOE Factors

The spin-lattice relaxation rates of nuclei ($I = \frac{1}{2}$) can be described[65] by

$$\frac{1}{T_1} = \frac{1}{T_1^{dd}} + \frac{1}{T_1^{SR}} + \frac{1}{T_1^{SA}} + \frac{1}{T_1^{EM}} + \frac{1}{T_1^{other}} \quad (9)$$

It is known for nitrogen atoms with directly attached protons from experiments on amino acids[11,12] and other compounds[66] that dipolar relaxation $1/T_1^{dd}$ caused by the protons plays the major role. Spin-rotational relaxation $1/T_1^{SR}$ is supposed not to be important for larger amino acids as proved from temperature-dependent relaxation time measurements on lysine and glutamine.[12] Relaxation due to the exchange modulation of the coupling interaction $1/T_1^{EM}$ does not contribute to the relaxation of nitrogen atoms as Leipert and Noggle[11] and Irving and Lapidot[67] deduced from their theoretical considerations. Relaxation due to the rotational modulation of the shielding anisotropy $1/T_1^{SA}$ is expected to contribute especially for nitrogens in unsaturated heterocyclic molecules, which are not bound directly to a proton, and for high magnetic fields.[68,69] The term $1/T_1^{other}$ describes all other relaxation mechanisms. Especially in the case of amino acids the contribution due to paramagnetic impurities has to be considered, because amino acids are known as excellent chelating compounds.[70]

In order to evaluate the importance of the term $1/T_1^{dd}$ we determined the NOE factor of the observed nitrogens. Broad-band irradiation of the proton resonance causes an NOE effect for those nitrogens, which are relaxed partly or predominantly by dipolar interactions with protons. If the relaxation is purely dipolar, the maximal value of the NOE is

$$NOE_{max} = +\frac{1}{2}(\gamma_{^1H}/\gamma_{^{15}N}) = -4.93 \quad (10)$$

Using the determined NOE values we are able to evaluate the contribution of the term $T_1^{dd}$ to the total relaxation rate $T_1^{obsd}$ by

$$T_1^{dd} = T_1^{obsd}\,(NOE_{max}/NOE_{obsd}) \quad (11)$$

In order to determine the influence of the deprotonation of the imidazole ring of histidine, we measured the pH dependence of relaxation times $T_1$ of both imidazole nitrogens and also the pH dependence of the NOE values (Figure 5, Table III).

In the acid region below pH 5 a $T_1$ value of about 13 s for the $\pi$ nitrogen and about 10 s for the $\tau$ nitrogen is found. These values are nearly constant between pH 1.4 and 5. The NOE values of both nitrogens are both near −4.9 which indicates that spin-lattice relaxation in that pH range is mainly produced by dipolar interaction with the attached protons. Increasing the pH to values near the p$K$ of the imidazole ring influences the relaxation behavior of the two nitrogens in a different manner.

The relaxation rate $1/T_1$ of the N-$\pi$ nucleus increases slightly at about pH 5.5; after this inflection point the relaxation rate is reduced due to the deprotonation of the imidazole ring. At pH 7.8 we determined a relaxation time of about 25 s for this nitrogen. The relaxation rate $1/T_1$ of N-$\tau$ increases in the pH range of 5.0 to 6.2; further increase of the pH causes a decrease of the relaxation rate of this nucleus to values, which are slightly larger than those measured in the acid pH region. Relaxation rates $1/T_1$ of $^1$H, $^{13}$C, or $^{15}$N resonances of amino acids reveal maxima at pH values around 8 which are in most cases attributed to paramagnetic impurities.[24,72,73] However, there are some reasons which prompted us to rule out this possibility for our measurements at least in the pH region around the imidazole p$K$ value.

If relaxation rates are affected by paramagnetic ions, a very broad maximum of the pH dependence of $1/T_1$ is produced. The proton relaxation rates of the imidazole proton resonances of histidine are changing gradually from about pH 1 up to pH 11 in samples contaminated with paramagnetic ions.[73] A sharp maximum in between only 2 pH units—like the maximum in Figure 5—is not observed.

Broad maxima in $1/T_1$ are reasonable in unpurified samples since it is well known that amino acids and in particular histidine are forming complexes with metal ions. With successive ionization of the three possible coordination sites (the carboxylate group, the imidazole $\pi$ nitrogen, and the $\alpha$-amino group), different distinguishable complexes are formed. In the acid pH region transition metal ions with octahedral coordination sphere are interacting with the carboxylate anion of the amino acid, whereas in the alkaline region strong complexation especially with the terdentate histidine is observed. Hence strongest complexation is occurring when both the imidazole ring and the $\alpha$-amino group are deprotonated.[74,77]

Copper ions prefer a planar coordination sphere. Complexes of histidine with $Cu^{2+}$ apparently involve only the $\alpha$-amino group and the carboxylate group of histidine.[78] In strong alkaline medium the copper ions tend to produce mixed histidine hydroxy complexes;[76] hence only one histidine molecule instead of two are complexed by $Cu^{2+}$ and the observed relaxation times should become again longer.

The purification procedures used in this work have certainly reduced the amount of paramagnetic impurities such that only the strong complexes with terdentate histidine are formed to a certain amount in the very alkaline pH region. Only after deprotonation of the $\alpha$-amino group should an influence on the relaxation rates be expected. Furthermore, even if we assume that the maximum of the relaxation rate of the $\tau$ nitrogen (Figure 5) is produced by paramagnetic ions, although this nitrogen is certainly not the site of complexation, it is difficult to explain the pH dependence of the relaxation rate of the N-$\pi$. Since this type of relaxation is mainly due to dipolar interaction of the $^{15}$N nuclei with the electron spins of the paramagnetic ions (the small amount of unpaired spin density possibly delocalized over the $\pi$ system of the imidazole ring should be neglected), the distance between the paramagnetic center and the observed nucleus should be considered.[37,79]

At pH 6.6 we observe a $T_1$ value of 4.7 s and a NOE value of −2.8 for the $\tau$ nitrogen. Using eq 10 the part of the relaxation time due to dipolar interaction of the N-$\tau$ nucleus with protons is 9.7 s; the remaining part of 9.2 s is produced by an-

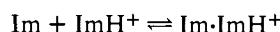
other mechanism. In the acid pH region the N-$\tau$ relaxation is almost entirely due to dipolar interaction with protons as can be derived from the maximal NOE value (Table III). Paramagnetic ions should not have any influence on its relaxation rate in this pH region. If we now attribute the increase in the relaxation rate of the N-$\tau$ nucleus at pH 6.6 only to paramagnetic ions bound at the $\pi$ nitrogen, we may estimate the influence of those ions on the $\pi$ nitrogen itself. Since the distance $\tau$ nitrogen-$\pi$ nitrogen is about 2.2 Å and the distance metal-$\pi$ nitrogen is about 2 Å, the ratio of the relaxation times produced by paramagnetic ions at both nitrogens should be:

$$T_1{}^{N\pi}{}_{para} = T_1{}^{N\tau}{}_{para}\,(2/4.2)^6 \tag{12}$$

Since the part of $T_1{}^{N\tau}$ which may be assigned to the influence of paramagnetic ions reaches at pH 6.6 a value of about 9.2 s, we should obtain for $T_1{}^{N\pi}$ values of <0.1 s. The removal of the proton at the N-$\pi$ should lead to an increase in $T_1$, but the influence of paramagnetic ions will certainly overcompensate for this effect. Furthermore, neither the NOE of about −2.5 for the $\pi$ nitrogen in the pH region of the imidazole p$K$ nor the maximum in the pH dependence of $T_1$ (24.8 s at pH 7.8) is consistent with the complexation of paramagnetic ions at this nucleus. At about this pH value a minimum in the pH dependence of the relaxation times is observed for all histidine samples contaminated with paramagnetic traces.

As a last argument for the success of the purification procedures it should be mentioned that in unpurified samples the NOE of the $\pi$ nitrogen signal is drastically reduced above pH 6.5 to values which are positive in some cases, whereas the $\tau$ nitrogen signal is only slightly affected.

Obviously two pH-dependent processes have to be considered to explain the observed maxima in the relaxation rates of both nuclei, since the influence of paramagnetic impurities can be neglected in this pH region. Beside the deprotonation of the imidazole ring we assume a pH-dependent association of a protonated [ImH+] and deprotonated [Im] imidazole ring system.



$$\mathrm{Im} + \mathrm{ImH^+} \rightleftharpoons \mathrm{Im{\cdot}ImH^+}$$

$$K_{ass} = [\mathrm{Im{\cdot}ImH^+}]/[\mathrm{Im}][\mathrm{ImH^+}] \tag{13}$$

From studies of the stacking phenomena in nucleic acid constituents it is known that in some cases maximal stacking[71] is observed at the p$K$ value of the nuclear base. It seems quite reasonable that in a similar manner stacking occurs in the histidine system. From the considerations concerning the conformation of histidine above and below pH 6.2 the predominant conformations of either species (Figure 7) would allow an interaction between the negatively charged carboxylate group of species b (Figure 7) and the positively charged $\alpha$-amino group of species a in addition to the stacking interaction.

The $^{15}$N chemical shift values are not affected by the stacking process. This seems quite reasonable, because a similar conformation of interacting groups (the negatively charged carboxylate group and the positively charged imidazole ring) between pH 1.8 and 6.2 does not influence the $^{15}$N chemical shifts also.

The total relaxation rate of the observed nitrogen nuclei can be expressed by weighting the relaxation rates of each of the species a,b of Figure 7 and the dimer formed by this interaction. Since we observe only one signal for each nitrogen nucleus in the ring, we have to assume rapid exchange between the possible species of histidine in solution:

$$\frac{1}{T_1{}^{tot}} = \gamma_{prot}\frac{1}{T_1{}^{prot}} + \gamma_{depr}\frac{1}{T_1{}^{depr}} + 2\gamma_{ass}\frac{1}{T_1{}^{ass}} \tag{14}$$

$$\gamma_{prot} = \frac{C_{prot}}{C_{prot} + C_{depr} + 2C_{ass}} \tag{15}$$

**Table IV.** Relaxation Rates of the Imidazole Nitrogens for the Different Species of Histidine in Solution and the Calculated Association Constants for the Dimeric Structure

| | $1/T_1^{prot}$, $s^{-1}$ | $1/T_1^{depr}$, $s^{-1}$ | $1/T_1^{ass}$, $s^{-1}$ | $K_{ass}$, $M^{-1}$ |
|---|---|---|---|---|
| N-$\pi$ | 0.075 | 0.039 | 0.26 | 2.0 |
| N-$\tau$ | 0.103 | 0.130 | 0.74 | 2.2 |

$$\gamma_{depr} = \frac{C_{depr}}{C_{prot} + C_{depr} + 2C_{ass}} \tag{16}$$

$$\gamma_{ass} = \frac{C_{ass}}{C_{prot} + C_{depr} + 2C_{ass}} \tag{17}$$

where $\gamma_{prot}$, $\gamma_{depr}$, and $\gamma_{ass}$ are the mole fractions of the protonated (a of Figure 7), the deprotonated (b of Figure 7), and the dimer species and $C_{prot}$, $C_{depr}$, and $C_{ass}$ and $T_1{}^{prot}$, $T_1{}^{depr}$, and $T_1{}^{ass}$ are the corresponding concentrations and relaxation times. It is not necessary to introduce further species due to the tautomeric or conformational equilibria, because a constant ratio of concentrations exists between them and the pH dependence of these ratios can be calculated by the overall deprotonation process outlined above.

From our determination of the pH dependence of the chemical shifts, we know the equilibrium constant of the deprotonation process. Introducing this value we get:

$$\gamma_{prot} = \frac{1}{1 + \dfrac{K_{prot}}{C_H} + 2K_{ass}C_{depr}} \tag{18}$$

$$\gamma_{depr} = \frac{1}{1 + \dfrac{C_H}{K_{prot}} + 2K_{ass}\dfrac{C_H}{K_{prot}}C_{depr}} \tag{19}$$

$$\gamma_{ass} = \frac{1}{\dfrac{1}{K_{ass}C_{depr}} + \dfrac{K_{prot}}{K_{ass}C_HC_{depr}} + 2} \tag{20}$$

where $C_H$ is the concentration of the hydrogen ion.

To remove the concentration parameter $C_{depr}$, we express this term by the total known concentration of histidine in the solution $C_{tot}$

$$C_{depr} = \sqrt{\frac{C_{tot}K_{prot}}{2K_{ass}C_H} + \left[\frac{1}{4}\frac{1 + (K_{prot}/C_H)}{K_{ass}}\right]^2} - \frac{1}{4}\left[\frac{1 + (K_{prot}/C_H)}{K_{ass}}\right] \tag{21}$$

We arrive at an equation which only depends on four unknown parameters: $T_1{}^{prot}$, $T_1{}^{depr}$, $T_1{}^{ass}$, and $K_{ass}$. Using a nonlinear least-squares program we were able to fit the observed relaxation time data with this equation. The calculated values of the relaxation rates of the three species and the equilibrium constants are given in Table IV. The solid line of Figure 5 shows the calculated pH dependence of the relaxation rates of both nitrogens.

With the assumption of this interaction the behavior of the N-$\pi$ nucleus can now be explained. The removal of the proton certainly causes the drastic decrease of the relaxation rate. However, even at pH values above the p$K$ of the imidazole ring relaxation is produced partly by dipolar interactions with protons as is also indicated the the NOE values. This dipolar relaxation can be explained with the tautomeric equilibrium, since to some extent the N-$\pi$-H tautomer is formed.

The large relaxation rate of N-$\pi$ in the associated species (Table IV) is induced by two effects. The larger molecular weight of such a species would cause an increase in the corre-

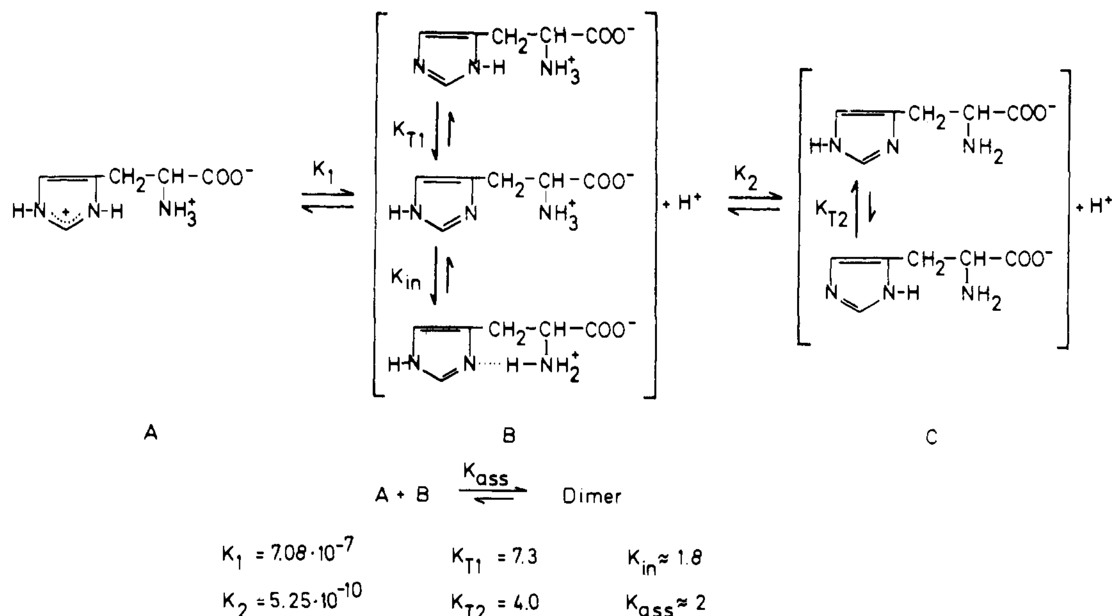



**Figure 8.** Equilibria of intramolecular and intermolecular interactions of histidine in aqueous solution as determined by nitrogen-15 and carbon-13 NMR. $K_1$ and $K_2$ refer to the ionization of the imidazole ring and the $\alpha$-amino group, respectively, $K_{T1}$ and $K_{T2}$ describe the tautomeric equilibria in the different pH regions, $K_{in}$ is related to the interaction between the N-$\pi$ lone pair and the $\alpha$-amino group between pH 6.2 and 9.3, and $K_{ass}$ reflects the pH-dependent association around pH 6.2.

lation time $\tau_c$. A larger $\tau_c$ implies a larger relaxation rate if we assume predominant dipolar relaxation. This assumption is quite reasonable if we consider the measured NOE values in the pH region at the p$K$ value. A further increase of the relaxation rate may be possible because of the increased number of protons, which are neighboring the N-$\pi$ nitrogen in the complex. In a similar way the relaxation behavior of N-$\tau$ can be explained. The pH-dependent association increases the relaxation rate of this nucleus even to a larger extent than that of the N-$\pi$ nucleus.

If we assume the interactions between the rings and the two charged groups in the dimer, two protons of each of the rings are very near to the two N-$\tau$ nuclei, whereas the N-$\pi$ nucleus is only neighbored by one proton. If we further consider the amount of tautomerization (88:12), it seems reasonable to assume, as supported by the NOE values, that this nucleus is relaxed to a larger amount by dipolar interaction than the N-$\pi$ nucleus in this pH region. Furthermore the influence of the larger correlation time $\tau_c$ should increase the relaxation rate to some extent.

We suppose that above the p$K$ value of the imidazole ring rotational modulation of the shielding anisotropy may contribute to the relaxation rate of both imidazole nitrogens to a certain amount. The smaller value of the relaxation rate of N-$\tau$ at low pH values compared to that at pH values above 7 may be explained by a larger bond length of the N-$\tau$ to the attached proton. Because of the electronic changes of the imidazole ring with deprotonation, this bond length is probably decreased. Owing to the $r^{-6}$ dependence of this parameter on the relaxation rate, it seems reasonable that such an effect would even overcompensate for the influence of the tautomeric equilibrium above pH 6.2 which should decrease the relaxation rate of N-$\tau$.

The difference in $1/T_1$ for N-$\pi$ and N-$\tau$ at low pH values may also be explained by the different bond length of the two N-H bonds. Since the NOE values indicate that the relaxation mechanism is predominantly dipolar, we may even estimate for the ratio of these bond lengths $r_{N\pi\text{-}H}/r_{N\tau\text{-}H}$ a value of 1.05. Because of the $r^{-6}$ dependence only a difference of about 0.05 Å in the bond length is detectable with the determination of the relaxation rates.[80] At pH values above the p$K$ value of the amino group, the relaxation rate of N-$\pi$ should be increased whereas that of N-$\tau$ should be decreased due to the breakdown of the interaction between the $\alpha$-amino group and the lone pair at N-$\pi$. There is some indication for this change in $1/T_1$. However, it seems that the values of $1/T_1$ are erroneous above pH 9, especially for the N-$\pi$ nucleus, since unavoidable traces of paramagnetic impurities are present in the solution. The NOE values are also small and do not change correspondingly with the expected change of the $T_1$ values above pH 9 which is additional proof for the presence of paramagnetic impurities.

## Conclusions

From our NMR investigations a complete description of the various equilibria of intramolecular and intermolecular interactions of histidine in aqueous solution can be presented (Figure 8). Changes in the electronic structure of the imidazole ring upon deprotonation are detected; the tautomeric equilibrium of the deprotonated imidazole as well as conformational changes induced by interactions between the various charged groups are determined partly in a quantitative manner. Even the existence of associated structures of histidine may be derived from pH-dependent $T_1$ measurements. It seems that in particular the $^{15}$N NMR spectroscopy of isotopically enriched compounds is a powerful tool for investigating the structure and conformation of these molecules and their interaction with the solvent water. The incorporation of $^{15}$N-labeled imidazoles or amino groups into the active sites of enzymes should give an insight into the various interactions using these methods.

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