Seelig, A., & Seelig, J. (1978) *Hoppe-Seyler's Z. Physiol. Chem. 359*, 1747.
Seelig, J., Tamm, L., Hymel, L., & Fleischer, S. (1981) *Biochemistry 20*, 3922.
Sefcik, M. D., Schaefer, J., Stejskal, E. O., McKay, R. A., Ellena, J. F., Dodd, S. W., & Brown, M. F. (1983) *Biochem. Biophys. Res. Commun. 114*, 1048.
Smith, R. L., & Oldfield, E. (1984) *Science (Washington, D.C.) 225*, 280.
Valic, M. I., Enga, E., Burnell, E. E., & Bloom, M. (1974) *Proc. Colloq. AMPERE (18th)*, 573.
Wennerstrom, H. (1973) *Chem. Phys. Lett. 18*, 41.

Articles

Proton Nuclear Magnetic Resonance Studies of Mast Cell Histamine†

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ABSTRACT: The state of histamine in mast cells was studied by ^{1}H NMR spectroscopy. Spectra were measured for histamine in situ in intact mast cells, for histamine in suspensions of mast cell granule matrices that had been stripped of their membranes, and for histamine in solutions of heparin. The ^{1}H NMR spectrum of intact mast cells is relatively simple, consisting predominantly of resonances for intracellular histamine superimposed on a weaker background of resonances from heparin and proteins of the cells. All of the intracellular histamine contributes to the NMR signals, indicating it must be relatively mobile and not rigidly associated with the negatively charged granule matrix. Spectra for intracellular histamine and for histamine in granule matrices are similar, indicating the latter to be a reasonable model for the in situ situation. The dynamics of binding of histamine by granule matrices and by heparin are considerably different; exchange of histamine between the bulk water and the granule matrices is slow on the ^{1}H NMR time scale, whereas exchange between the free and bound forms in heparin solution is fast. The chemical shifts of resonances for histamine in mast cells are pH dependent, decreasing as the intragranule pH increases without splitting or broadening. The results are interpreted to indicate that histamine in mast cells is relatively labile, with rapid exchange between bound histamine and pools of free histamine in water compartments confined in the granule matrix.

Mast cells are a major tissue storage site of histamine. In the adult rat (5–7 months old) 25 ± 5 μg of histamine is present per 10^6 mast cells (Kruger & Lagunoff, 1981). To the limits of available methods of measurement, all of the histamine in a normal mast cell is contained in specialized storage granules. The mean volume of a peritoneal mast cell from a 5–7 month old rat is 1080 ± 80 μm^3, of which the aggregate granule volume represents 50% (Hamel et al., 1983). If the 25 pg of histamine in a mast cell were in solution in the total granule volume, the concentration of histamine would accordingly be 0.42 M.

It is reasonable to believe on the basis of equilibrium sedimentation in Percoll that the aqueous compartment comprises of the order of 10% of the granule volume (Kruger et al., 1980) and that much of the histamine is bound to the granule matrix rather than in solution. However, the binding site of histamine remains controversial, and the actual distribution of histamine between bound and free states in situ is unknown. In consideration of the large amount of intracellular histamine and the location of the chemical shifts of the imidazole protons in a biologically uncluttered portion of the ^{1}H NMR[1] spectrum, it seemed likely that ^{1}H NMR measurements performed on intact mast cells in suspension could add to an understanding of the status of granule histamine.

Materials and Methods

Mast cells were washed from the peritoneal cavities of rats decapitated under CO_2 anesthesia and separated from the other cells on Percoll (Bauza & Lagunoff, 1983). Immediately before the ^{1}H NMR measurements were made, the mast cells were washed twice in balanced salt solution (Bauza & Lagunoff, 1983) prepared in D_2O (BSS-D_2O). Several million mast cells were suspended in 0.4 mL of BSS-D_2O; 0.25% 2-methyl-2-propanol was usually added as an internal chemical shift reference. Histamine released from the mast cells was assayed (Kruger et al., 1980) on supernatants after collecting the cells by centrifugation at the completion of the NMR experiments. Granule matrices stripped of their histamine

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[1] Abbreviations: NMR, nuclear magnetic resonance; BSS-D_2O, balanced salt solution in D_2O; Gly-His-Gly, glycylhistidylglycine; DSS, sodium 2,2-dimethyl-2-silapentane-5-sulfonate; FID, free induction decay.

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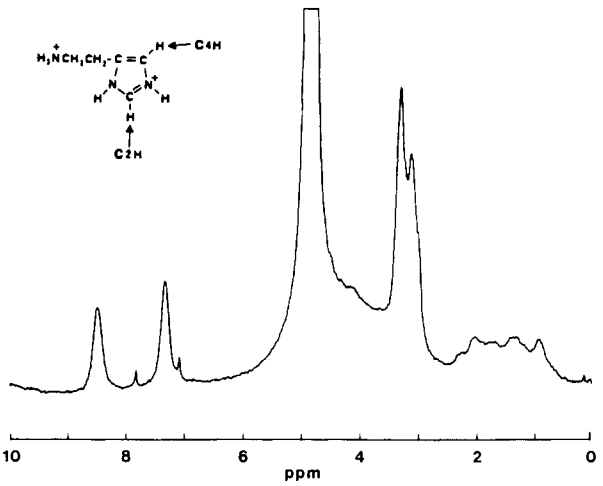

FIGURE 1: ^1H NMR spectrum of 10×10^6 mast cells in BSS-D_2O. The spectrum was obtained from 320 FIDs (90° pulse and 2.1-s repetition time). The truncated signal is from HDO and had a peak height approximately 256 times those of the aromatic resonances. Assignments of other resonances are given in the text.

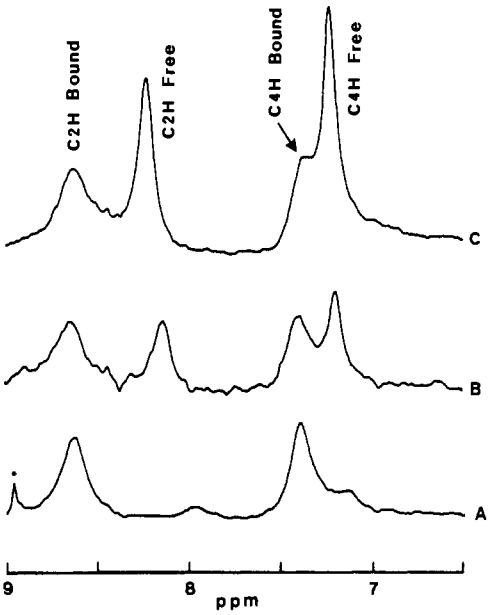

FIGURE 2: Portions of the ^1H NMR spectra of D_2O suspensions of mast cell granule matrices to which (A) 0.0, (B) 1.0, and (C) 2.0 mM histamine was added. The pH of the suspensions was 6.1, 5.9, and 5.8, respectively. Spectra were measured by the pulse sequence 45°–τ–45°–acquisition (Clore et al., 1983). The peak identified by the asterisk is an artifact from the carrier.

were prepared by sonicating mast cells (Kruger et al., 1980) suspended in D_2O, collecting the granules by centrifugation, washing them twice in BSS-D_2O, and resuspending them in D_2O.

Histamine and heparin solution were prepared in 0.5 mM phosphate buffers in D_2O; the pH of the solutions was adjusted as necessary with DCl or NaOD, and the pH was determined with a glass electrode uncorrected for the deuterium isotope effect (Glascoe & Long, 1960).

^1H NMR spectra were obtained at 25 °C and 360 MHz by the pulse/Fourier transform mode on a Bruker WM-360 spectrometer. Even though the samples were in D_2O solution, the residual HDO resonance was generally much more intense than the histamine resonances. When the histamine resonances were weak, the strong HDO resonance was suppressed by use of the pulse sequence $(45°–\tau–45°–\text{acquisition})_n$ with the carrier set 50 Hz to the high-frequency side of the aromatic histamine resonance (Clore et al., 1983). τ was chosen so that the HDO magnetization had precessed through 180° relative to the carrier when the second 45° pulse was applied. Attempts to suppress the HDO resonance with a selective saturation pulse prior to the observation pulse resulted in loss of histamine resonances because of negative nuclear Overhauser effects on the histamine resonances. Typically 100–400 transients were collected. Chemical shifts were measured relative to the methyl resonance of 2-methyl-2-propanol but are reported relative to the methyl resonance of sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS).

Results

A typical ^1H NMR spectrum of mast cells in suspension in BSS-D_2O is presented in Figure 1. The spectrum is surprisingly simple. The dominant features are (1) a resonance at 4.80 ppm from residual HDO, (2) four resonances to the high-frequency side of the HDO resonance from the carbon-bonded protons of the histamine imidazole ring, and (3) two intense, broad resonances (2.9–3.5 ppm) to the low-frequency side of the HDO resonance from the two methylene groups of histamine. These resonances are superimposed on a broad, rather featureless background presumably from heparin and proteins of the cell. The resonances at 8.504 and 7.327 ppm were assigned to C2H and C4H, respectively, of the imidazole ring of intracellular histamine and the less intense, sharper resonances at 7.853 and 7.091 ppm to the same protons of extracellular histamine. A solution of histamine in BSS-D_2O yielded only the 7.853 and 7.091 ppm resonances. With time, the two resonances assigned to extracellular histamine increased in intensity at the expense of those assigned to intracellular histamine. When the cells were centrifuged and the NMR spectrum of the supernatant was examined, the chemical shifts and the intensity of the resonances matched those assigned to extracellular histamine.

In order to determine if the NMR spectrum observed for intact mast cells arose from the total cell histamine or only a portion of it, we added the tripeptide Gly-His-Gly as an extracellular intensity standard. The areas under the resonances for histamine C2H and the histidine C2H of Gly-His-Gly were measured, the cells were lysed by freezing and thawing 3 times, and the areas under the resonances were remeasured. The lack of any significant difference between the ratio of areas (histamine/histidine) before and after lysis provided evidence that the total intracellular histamine was observed in the ^1H NMR spectra of intact mast cells.[2]

Since the histamine in mast cells is associated with the cell's specific granules, studies of the binding of histamine to isolated mast cell granules were undertaken with ^1H NMR. Spectra were measured for histamine added to suspensions of granule matrices stripped of their membrane. In this model system, a mixture of bound and free histamine is expected. Four resonances were observed for the imidazole protons of histamine (Figure 2A). The two most intense resonances, at 8.64 and 7.40 ppm, are assignable to C2H and C4H of bound histamine. The weaker resonances at 7.98 and 7.13 ppm are attributable to the corresponding protons of free histamine. As histamine is added to the suspension (Figure 2B,C), the

[2] The release of mast cell histamine by cell lysis was characterized independently. Mast cells were extracted with trichloroacetic acid to quantitatively release the histamine, which was then determined by fluorometric assay (Kruger et al., 1980). A second portion of mast cells was suspended in BSS and then lysed by taking the suspension through three freeze–thaw cycles, and the histamine released was also determined by fluorometric assay. Lysis by the freeze–thaw method released 92.3 ± 0.4% of the histamine released by extraction.

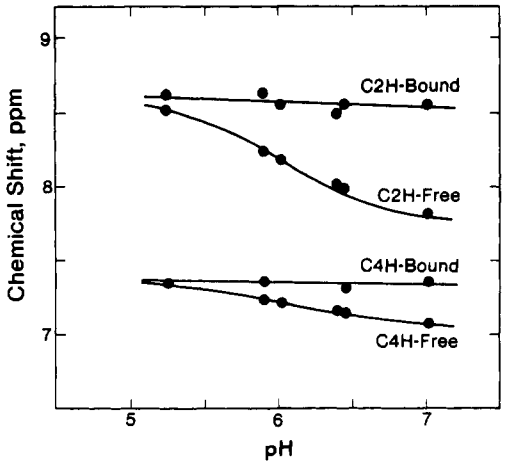

FIGURE 3: pH dependence of the chemical shifts of the aromatic resonance from histamine in suspensions of mast cell granule matrices. The solid curves through the points labeled C2H-free and C4H-free are the chemical shift vs pH curves obtained for free histamine in D_2O solution.

resonances assigned to the bound histamine remained relatively unchanged, indicating saturation of the granule binding sites, while those assigned to the free histamine increased in intensity. In other experiments, granules that had been stripped of histamine were suspended in D_2O and then titrated with histamine. Resonances were observed for both free and bound histamine before saturation of the histamine binding sites. After saturation, the resonances for free histamine continued to increase in intensity as more was added, with no change in the intensity of the bound resonance. The pH dependence of the chemical shifts of the resonances of bound and free histamine is presented in Figure 3, where the chemical shifts of the four resonances are plotted versus pH. The chemical shifts of the two resonances of free histamine exhibit a pH dependence identical with that of free histamine in the absence of granules. Only a slight shift to lower frequency occurred in the resonances of bound histamine.

The assignment of the broad resonances in Figure 2 to bound histamine was examined in experiments in which the HDO resonance was suppressed with a selective saturation pulse prior to the nonselective observation pulse. In experiments with both intact mast cells and granule suspensions, the bound histamine resonances disappeared from the spectrum, indicating a negative internuclear Overhauser effect as a result of association of the histamine with a macromolecule with resonances at the frequency of the saturation pulse. However, since the ^1H NMR spectra of both heparin and protein contain resonances in this frequency range, the experiment does not distinguish between binding to these two principal constituents of the granule matrix.

Considering that the bulk of the evidence favors heparin as the molecule to which histamine binds in the granule (Lagunoff, 1974, 1985), we also examined the effect of heparin on the ^1H NMR spectrum of histamine. Spectra were obtained for solutions containing histamine and heparin at various pH values and varying ratios of histamine to heparin. In all cases, sharp, exchange-averaged resonances (i.e., intermediate in chemical shift between free and bound histamine) were observed for the imidazole ring protons as illustrated by the spectra in Figure 4. The pH dependence of the chemical shifts of the C2H and C4H resonances for histamine in the presence of heparin at a histamine to heparin dimer molar ratio of 1:100 is shown in Figure 5.

The difference between the spectra of histamine bound to heparin or to granules prompted us to investigate the effect

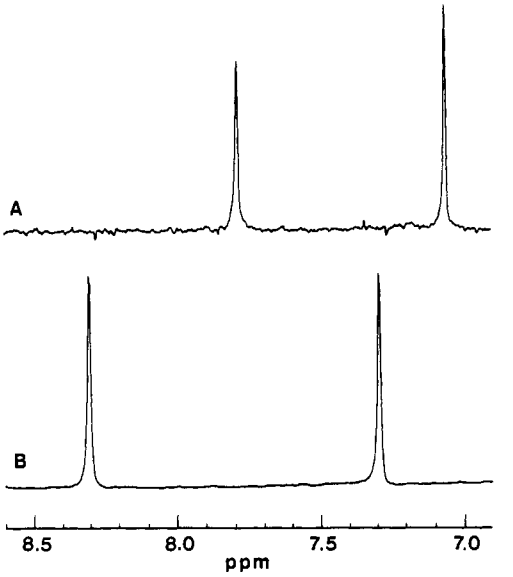

FIGURE 4: Portions of the ^1H NMR spectra of D_2O solutions containing (A) 5.0 mM histamine at pH 7.00 and (B) 5.0 mM histamine with 20 mM heparin dimer at pH 6.95.

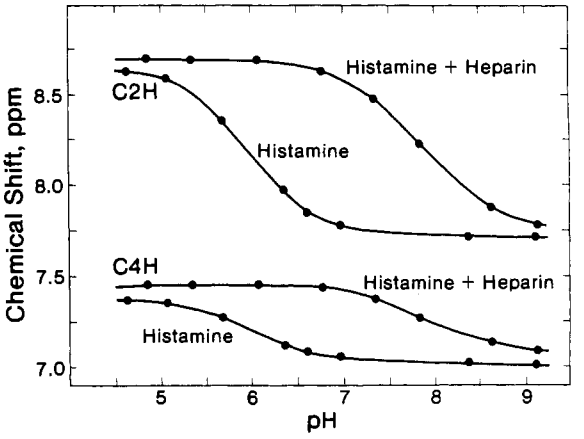

FIGURE 5: pH dependence of the chemical shifts of the aromatic resonances from histamine in D_2O solutions containing 1.0 mM histamine and 1.0 mM histamine with 100 mM heparin dimer.

of changing pH on histamine in situ. The sequential addition of NH_4Cl to the media in which the mast cells were suspended has previously been shown to increase the granule pH (Lagunoff & Rickard, 1983). When this procedure was carried out while monitoring the chemical shift of the imidazole ring protons, the resonances were observed to shift progressively upfield without any perceptible signal broadening or the appearance of additional lines (Figure 6).

Discussion

The spectrum of intracellular histamine presents single, broadened peaks with chemical shifts of 8.504 and 7.327 ppm for C2H and C4H, respectively. The fact that all the intracellular histamine contributes to the NMR signal indicates it must be relatively mobile and not rigidly associated with the negatively charged granule matrix. The apparent similarity in chemical shift and in peak width for spectra of intracellular histamine and histamine bound to isolated granule matrixes would appear to indicate that the latter is a reasonable model for the in situ situation. A significant difference is the presence of separate resonances for bound histamine and the histamine free in the extragranular solution in the spectrum of isolated granules and only a single peak for intracellular histamine. However, the spectrum of histamine in the presence of heparin,

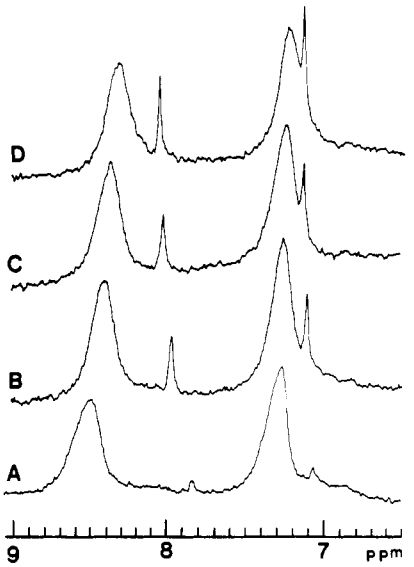

FIGURE 6: ^1H NMR spectra of mast cells in BSS-D_2O to which (A) 0.0, (B) 20, (C) 40, and (D) 80 mM ammonium chloride was added to raise the intracellular pH. Resonances at 7.84 and 7.07 ppm in (A) are from extracellular histamine. The extracellular histamine concentration in (B) was increased by adding histamine to the suspension medium. Note the progressive shift of resonances for mast cell histamine to lower frequency as the granule pH increases. The progressive shift of resonances for extracellular histamine to higher frequency is in accord with the expected decrease in external pH when the NH_3 form is selectively taken up by the cells.

the proposed binding site in the granule, exhibits sharp, exchange-averaged resonances.

The separate resonances observed for free and granule-bound histamine in equilibrium are indicative of average lifetimes greater than 0.01 s for the two species before exchange, whereas the exchange-averaged resonances for free and heparin-bound histamine indicate preexchange lifetimes less than 0.0001 s for free and heparin-bound histamine.

We have considered two explanations for the marked difference. Tighter binding of the histamine to the granule matrix than to heparin in solution (i.e., $K_{a,granule} \gg K_{a,heparin}$) would result in longer mean lifetimes for the histamine bound to the granule and separate resonances for free and bound histamine. Direct measurements of affinity constants[3] indicate that the affinity of histamine binding to the granule is essentially the same as that of histamine binding to heparin, and this cannot account for the apparent difference in exchange rates. A second possibility is that in the case of isolated granules there are three classes of histamine: that bound to the matrix, that free in water confined within the granule matrix, and that free in the bulk medium. The presence of separate resonances is dependent on rapid exchange between bound histamine and the small pool of free histamine in the water confined in the granule matrix but slow exchange between free intragranule histamine and the large pool of free, extragranule histamine. The slow exchange is reasonable considering that the positively charged, intragranule histamine must migrate through the highly negatively charged granule matrix to the surface before exchange with that free in the extragranular solution can occur.

According to the second model, the single broadened resonance observed for each of the imidazole ring protons of histamine in intact mast cells is the exchange-averaged signal expected for rapid exchange between bound and intragranule histamine free in the pools of water confined in the granules.[4] This interpretation is consistent with the shift of the resonance lines without additional broadening when the pH of the granule compartment was increased by the addition of NH_4Cl to the medium. The chemical shifts of the resonances for the small pool of free histamine in the water confined in the granule matrix, and thus the chemical shifts of the exchange-averaged resonances, will decrease with increasing pH. An increase in free histamine due to a decrease in the binding affinity with the increase in pH may also contribute to the shift. If on the contrary the resonances observed for in situ histamine had represented separate, slowly exchanging, overlapping signals of bound and free histamine at the low pH in situ, estimated to be 5.2, then alkalinization should have resulted in the progressive separation of the two signals or at least a broadening of the resonances.

The somewhat smaller chemical shifts of the resonances for histamine in situ compared to those for histamine in isolated granule matrices suggests that the fraction of free histamine is larger in situ than the fraction of free, rapidly exchanging histamine in the isolated matrices. An accurate knowledge of the pH of the intragranule milieu under the conditions of the NMR experiments is necessary to allow determination of the distribution of histamine between the free and bound forms for in situ granules from the exchange-averaged chemical shift.

Registry No. Histamine, 51-45-6.

REFERENCES

Bauza, M. T., & Lagunoff, D. (1983) *Biochem. Pharmacol. 32*, 59.

Clore, G. M., Kimber, B. J., & Gronenborn, A. M. (1983) *J. Magn. Reson. 54*, 170.

Glascoe, P. D., & Long, F. A. (1960) *J. Phys. Chem. 64*, 188.

Hammel, I., Lagunoff, D., Bauza, M., & Chi, E. Y. (1983) *Cell Tissue Res. 228*, 51.

Kruger, P. G., & Lagunoff, D. (1981) *Int. Arch. Allergy Appl. Immunol. 65*, 291.

Kruger, P. G., Lagunoff, D., & Wan, H. (1980) *Exp. Cell Res. 129*, 83.

Lagunoff, D. (1974) *Biochemistry 13*, 3982.

Lagunoff, D. (1985) *Bronchial Asthma: Mechanism and Therapeutics* (Weiss, E. B., Segal, M. S., & Stein, M., Eds.) p 236, Little, Brown and Co., Boston, MA.

Lagunoff, D., & Rickard, A. (1983) *Exp. Cell Res. 144*, 353.

[3] The affinity of heparin for histamine was determined by equilibrium dialysis with tritiated histamine. Association constants (K_a) were measured at 2×10^{-3} M Na^+, a concentration sufficient to eliminate any contribution of the Donnan effect to the distribution of histamine. The constants were estimated from the slopes of Scatchard plots. The K_a's for histamine binding to mast cell granules were evaluated by measuring tritiated histamine bound to the granules after centrifugation at varying initial concentrations of free histamine. Again, the slope of a Scatchard plot of the data was used to estimate the K_a. These methods yielded values for K_a at pH 6.0 of 1.18×10^4 M^{-1} and 2.86×10^4 M^{-1} for heparin and mast cell granules, respectively.

[4] The chemical shift, δ_o, of an exchange-averaged resonance is the weighted average of the chemical shifts of bound and free histamine, $f_f\delta_f + (1 - f_f)\delta_b$, where f_f is the fraction of the total histamine in the free form and δ_f and δ_b are the chemical shifts of resonances for free and bound histamine.