

Protonation of hypoxanthine, guanine, xanthine, and caffeine

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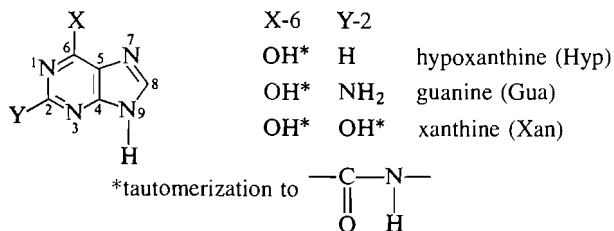
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The protonation equilibria of three hydroxypurines, hypoxanthine, guanine, and xanthine, and of related caffeine have been studied by ^1H and ^{13}C nmr and uv spectroscopies in aqueous sulfuric or perchloric acids. The results have been interpreted on the basis of the excess acidity method. The $\text{p}K_{\text{BH}_n^+}$ values and the protonation sites are discussed and comparisons are made with results of recent theoretical calculations.

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Les équilibres de protonation de trois hydroxypurines, l'hypoxanthine, la guanine, la xanthine, et du composé voisin la caféine ont été étudiés par les spectroscopies rmn du ^1H et du ^{13}C et uv dans les acides sulfurique et perchlorique aqueux. On a interprété les résultats sur la base de la méthode dite "d'acidité en excès". Les valeurs des $\text{p}K_{\text{BH}_n^+}$ et les sites de protonation sont examinés et des comparaisons sont faites avec les résultats de calculs théoriques récents.

In addition to their importance in biological systems, purines offer a challenging thermodynamic and structural problem specifically in the determination of the reactivity of their many basic sites. Establishing correlations between the reactivity of the purines basic sites with the proton and other electrophiles such as metal ions also presents an interesting challenge. We recently reported results of a study of the first protonation step of some purines and of the second and third protonation steps of purine and adenine in more acidic media (1). We have now extended our study to the protonation of 3 hydroxypurines,



hypoxanthine (6-hydroxypurine), guanine (2-amino-6-hydroxypurine), and xanthine (2,6-dihydroxypurine), and of related caffeine (1,3,7-trimethylxanthine). For this work we abandoned the calorimetric method because a strong medium effect at high acidity interfered with the interpretation of the results, and we used, instead, uv spectroscopy. Adding uv spectroscopy to ^{13}C and ^1H nmr spectroscopies makes it also possible to compare data at widely different concentrations (10^{-4} M vs. 0.2 M).

The known $\text{p}K_{\text{BH}^+}$ values for the first protonation step of hypoxanthine and guanine, which occur in dilute acid media, are respectively 1.9 (2) and 3.3 (2), while for xanthine the values quoted are 0.8 (3) and 1.2 (4) and for caffeine 0.5 (4). As for $\text{p}K_{\text{BH}_2^+}$, the only value reported is -1.05 for guanine (5); GuaH_2^{2+} has also been identified in both trifluoroacetic acid (TFA) and HSO_3F (6).

We present here the results of our study of the protonation reactions of the three hydroxypurines and caffeine between 0.05 and 18 M H_2SO_4 . Our $\text{p}K$ values as well as the protonation sites are discussed, and comparisons are made with results of recent theoretical calculations.

Experimental

Hypoxanthine (L. Light), guanine (L. Light), xanthine (L. Light and Aldrich), and caffeine (Baker) were used as received. Our uv

spectra of HypH^+ and GuaH^+ were in agreement with those published (7). The spectra of Xan for both xanthine samples were similar to those reported (7, 8). Previous values of ϵ , the molar absorptivity, are somewhat lower than ours. We suspect that this was so because some decomposition of Xan takes place on standing. Sulfuric acid, H_2SO_4 fuming (15% SO_3) (Fisher) and 70% HClO_4 (Biopharm) were reagent grade. Triple-distilled HSO_3F (Aldrich) was used.

The uv spectra were recorded within 10 min of preparing the solutions on a 545 Perkin-Elmer spectrophotometer and absorbancies at a fixed wavelength were measured on a PMQ II Zeiss spectrophotometer. The ^{13}C nmr spectra were acquired at 28°C on a Bruker WP-80 spectrometer operating at 20.2 MHz. Data acquisition and Fourier transformation were performed with 8K data points. Proton decoupled spectra were recorded when needed to assist signal assignments. Carbon-13 chemical shifts were measured relative to external dioxane in D_2O (coaxial tube) and converted to the Me_4Si scale using the relationship $\delta_{\text{Me}_4\text{Si}} = \delta_{\text{dioxane}} + 67.40$ ppm. The ^1H nmr spectra were obtained on a Bruker WH-90 spectrometer operating at 90.0 MHz. The ^1H chemical shifts were determined by using the CH_3 protons of external DSS ($(\text{CH}_3)_3\text{Si}(\text{CH}_2)_3\text{SO}_3^-\text{Na}^+$) in D_2O and taking $\delta_{\text{Me}_4\text{Si}} = \delta_{\text{DSS}}$. The nmr samples were prepared by adding weighed portions of the solid bases to known volumes of titrated H_2SO_4 or HClO_4 solutions. The uv samples were obtained by diluting aliquots of the bases in acid solutions to 5 mL with titrated acid solutions.

Results and discussion

Chemical shifts

The nmr spectra were recorded for Hyp, Xan, and Caf with 0.05–18 M H_2SO_4 solutions and for Gua with 4–16 M H_2SO_4 . For guanine, 0.5–6 M HClO_4 solutions were also used because of the low solubility of the base in H_2SO_4 below 4 M. In order to interpret our ^{13}C and ^1H shifts, H_0 values were computed from the corrected acid concentrations and literature data (9). These corrected concentrations were obtained by subtracting from the initial acid concentrations n times the concentrations of the protonated BH_n^+ species formed. In turn, these latter concentrations were obtained from the total base concentrations and eq. [2] and [3] given later in this discussion. The chemical shifts of the 5 carbons of Gua, Hyp, and Xan, those of C-8 and $-\text{CH}_3$ of Caf, and those of the H-8 protons of Gua and Xan were plotted against H_0 . Carbons were assigned by using off-resonance spectra and data in Me_2SO .

The plots of ^{13}C and ^1H shifts are for the most part sigmoid curves indicative of protonation reactions. According to their $\text{p}K_{\text{BH}^+}$, Gua, Hyp, and Xan are all present as BH^+ at the lowest HClO_4 and H_2SO_4 concentrations used so that the ^{13}C curves show the formation of GuaH_2^{2+} and HypH_2^{2+} . The sigmoid ^1H

TABLE 1. ^{13}C and ^1H shift values (ppm) of protonated species

Hydroxypurine	C-2	C-4	C-5	C-6	C-8	H-8
GuaH ⁺	155.9	150.7	108.7	155.9	138.2	8.67
GuaH ₂ ²⁺	152.3	137.9	110.1	152.3	138.8	8.89
HypH ⁺	150.4	148.1	116.1	155.1	139.9	—
HypH ₂ ²⁺	152.2	135.5	117.3	149.9	141.9	—
XanH ⁺	152.4	140.5	108.8	156.1	137.2	8.88
CafH ⁺	152.3	141.0	108.8	155.8	137.8	—

plot confirms GuaH₂²⁺. In the case of Xan (and Caf), there is no clear evidence for BH₂²⁺: C-8 is the only carbon noticeably shifted, but no plateau is reached, just as an early plateau, found when raw H-8 data were plotted, disappeared after bulk susceptibility corrections (10) were made.

The first protonation of Gua, Hyp, Xan, and Caf on the imidazole ring is well established (2) so that the choice of the second protonation site is restricted to N-3 or O-6 for Gua and Hyp. The shifts to high field of $\delta\text{C-4}$ and C-2 when passing from GuaH⁺ to GuaH₂²⁺ (Table 1) favour protonation at N-3 according to the α -protonation effect found by Pugmire and Grant for five- and six-membered nitrogen heterocycles (11). This assignment is in agreement with that of Wagner and von Philipsborn (6) deduced from ^1H nmr spectra in TFA at low temperature. However, these authors assumed that GuaH₂²⁺ was the protonated species in TFA, whereas by comparing our ^{13}C data in TFA and H₂SO₄ we calculated that for TFA solutions GuaH₂²⁺/GuaH⁺ = 1:1. In the case of Hyp, C-4 shows a large upfield shift on passing from HypH⁺ to HypH₂²⁺, as expected from protonation at N-3, and C-2 is moved to lower field as was observed for the third protonation of adenine at N-3 (1).

Ultraviolet spectra

On the basis of the nmr results, H₂SO₄ concentrations were selected to ensure the formation of BH⁺ and BH₂²⁺. The corresponding uv spectra were recorded and used to determine the most suitable wavelengths for measuring the absorbancies. Table 2 shows the H₂SO₄ concentrations used for the various protonated species together with the wavelengths of maximum absorption and the logarithms of the molar absorptivities ϵ . The plots of the absorbancies measured at several wavelengths (from 6 to 12 λ values) against H_0 were sigmoid curves and confirmed the formation of GuaH₂²⁺ and HypH₂²⁺. It is worth noting that the second protonation causes a hypsochromic shift. For Xan, there is also a hypsochromic shift of both absorption bands above 14.5 M H₂SO₄, but the absorbancies did not reach a plateau at 18 M H₂SO₄, and increasing the acidity further by adding oleum gave background absorbancies which were too high for meaningful measurements. Previous uv studies such as that for Gua (12) have already shown that it is difficult to assign the first protonation site on the basis of observed uv spectra, so that no attempt was made in this direction.

Calculation of pK_n

The values of pK_n for BH_nⁿ⁺ were first calculated using the equation

$$[1] \quad H_0 = pK_n - m_n \log I$$

with

$$[2] \quad I = \frac{|\text{BH}_n^{n+}|}{|\text{BH}_{n-1}^{(n-1)+}|}$$

TABLE 2. Ultraviolet spectral data of hydroxypurines

Hydroxypurine	[H ₂ SO ₄] (M)	λ_{max} (nm)	Log ϵ
Gua	0	(246, 275) ^a	(4.01, 3.89) ^a
GuaH ⁺	0.11	248, 271	4.03, 3.85
GuaH ₂ ²⁺	10.85	236, 252	4.01, 3.95
Hyp	0	249 ^a	(4.02) ^a
HypH ⁺	1.78	247	4.02
HypH ₂ ²⁺	18.09	241	4.00
Xan	0	267	3.99, (3.90) ^a
XanH ⁺	6.03–14.5	230, 259	3.87, 3.91
Caf	0	272.5	4.02
CafH ⁺	5.74	233, 265	3.79, 3.98

^aData from ref. 7.

The ratio I was obtained from the measured shifts δ and from the estimated limit chemical shifts δ_{n+} and $\delta_{(n-1)+}$ (Table 1) according to the standard equation

$$[3] \quad I = \frac{\delta - \delta_{(n-1)+}}{\delta_{n+} - \delta}$$

for the nmr shift data and the corresponding equation for the uv absorbancies data. However, the values of m_n were consistently well above 1.00, indicating that H_0 was not a satisfactory acidity function for following the protonation of these bases, just as it was not for adenine and purine (1). Two other acidity function, H_+ and H_A , were also used instead of H_0 . Although H_A proved to be more satisfactory than H_+ and H_0 , since our bases are very different from the amides used for H_A , we have finally chosen the Cox and Yates approach to calculated pK_n .

The excess acidity method (9) is based on the following equation:

$$[4] \quad \log I - \log C_{H^+} = m^*X + pK_n$$

Values for $\log C_{H^+}$ and X are independent of the bases and are taken from literature data (13) for each corrected H₂SO₄ or HClO₄ concentration. The program developed by Cox was used to solve simultaneously eqs. [3] and [4]. For each selected carbon and proton (or wavelength), values of C_{H^+} , X , δ , and the estimated values of δ_{n+} and $\delta_{(n-1)+}$ (or of the corresponding absorbancies data) were entered in the program. Thus, values of pK_n with their standard deviations (13) and m^* were obtained, as well as refined values of δ_{n+} and $\delta_{(n-1)+}$. These latter values differed from the estimated ones by less than 0.5 ppm (^{13}C), 0.01 ppm (^1H) (or 0.02 absorbancies unit). The pK_n values for the 4 bases, as well as those for adenine (Ade) and purine (Pur) (1) are summarized in Table 3. The differences found between the successive pK_n values are large enough to justify our treating the experimental data as if the protonation steps take place stepwise. An implicit, but reasonable, assumption in our treatment is that if tautomeric protonated species are present, their proportion is independent of the acid concentration. Since the agreement between pK_n values obtained by nmr and uv methods is generally satisfactory (although less so at higher acidities), considering the very different base concentrations used, 0.2 M vs. 10^{-4} M, large medium effects in one method can be ruled out. The presence of 2 isosbestic points in the uv spectra of Hyp (246 and 268 nm) between 1.8 and 18 M H₂SO₄ also suggests the absence of medium effect. Furthermore, no appreciable medium effect had previously been found for the protonation of Ade and Pur (1). For XanH₂²⁺, the uv pK_2 value is -10.0 ± 0.6 . Additional nmr data taken on a HSO₃F

TABLE 3. pK values and protonation sites for hydroxypurines, caffeine, purine, and adenine

Hydroxypurine	pK (nmr)	pK (uv)	pK (lit.)	Protonation sites
GuaH ⁺			3.3 ^a	N-7/N-9
GuaH ₂ ²⁺	-1.01 ± 0.03 (¹ H and ¹³ C)	-0.99 ± 0.01	-1.05^b	N-3
HypH ⁺			1.8, ^a 2.0 ^h	N-7/N-9
HypH ₂ ²⁺	-3.12 ± 0.05 (¹³ C)	-3.65 ± 0.10		N-3
XanH ⁺		0.91 ± 0.01	$0.8,c 1.2d$	N-7/N-9
XanH ₂ ²⁺		-10.0 ± 0.6		O-6 (?)
CafH ⁺	-0.13 ± 0.03 (¹³ C)	0.18 ± 0.01	0.5^d	N-9
PurH ⁺			2.39 ^a	N-1
PurH ₂ ²⁺	-1.66 ± 0.04^e		-1.5^e	N-7/N-9
AdeH ⁺			4.19 ^f	N-1
AdeH ₂ ²⁺	-0.43 ± 0.02^e	-0.47^g	-0.35^b	N-7/N-9
AdeH ₃ ³⁺	-4.23 ± 0.2^e			N-3

^aReference 2.^bReference 5.^cReference 3.^dReference 4.^eReference 1.^fData from ref. 17.^gR. L. Benoit and M. Fréchette. Unpublished data.^hReference 7.

solution ($-H_0 = 15$) of Xan and Caf to extend our results obtained with H₂SO₄ solutions ($-H_0 < 9.5$) and confirm XanH₂²⁺ were inconclusive: C-8 moved further downfield and C-6 upfield but the shifts were small and no OH signal corresponding to C=O protonation could be detected at -75°C . The substantial downfield shift for the carbonyl carbons on protonation, which might have been expected with the formation of XanH₂²⁺ and CafH₂²⁺, is absent. Although this absence might be caused by conjugation effects, it nevertheless makes us consider the uv pK_2 value as tentative.

The discussion of the pK_n results for the ionization of the BH_n⁺ species (Table 3) is a matter of some difficulty since the 3 tautomerized hydroxypurines first protonate on the imidazole ring while Ade and Pur first protonate on the pyrimidine ring. However, it is worth noting that for the 4 purines with successive protonations on N belonging on different rings, the differences between pK_2 and pK_1 correspond approximately to 6 kcal/mol.

Although it is difficult, we can try to relate our findings to the results of a recent molecular orbital study by Del Bene of the protonation of four DNA bases which include Gua and Ade as well as thymine and cytosine (14). The author computed the following protonation energies from optimized structures of the neutral and monoprotonated bases: for Gua, -245 kcal/mol (N-7), -233 (O-6 on the C-5 side), -228 (N-3), -220 (O-6 on the N-1 side), and for Ade, -241 (N-1), -240 (N-3), and -232 (N-7). Del Bene pointed out that these absolute values were overestimated because correlation and zero-point vibrational energy had to be neglected. In fact, Del Bene's calculated value for Ade, -241 kcal/mol, is at complete odds with the experimental gas phase proton affinity of -255 kcal/mol reported earlier by Meot-Ner (15). Furthermore, while the calculated value for thymine is right on Meot-Ner's -211 kcal/mol, for cytosine, Del Bene found a puzzling -249 kcal/mol to be contrasted with the experimental -225

kcal/mol. Thus, it is very difficult to say whether a discrepancy between data in Table 3 and Del Bene's values is due to the fact that the data in Table 3 were obtained in solution while the calculations refer to the gas phase, or to the unreliability of the reported MO calculations. For example, Gua is calculated by Del Bene to be 4 kcal/mol more basic than Ade, while the data in Table 3 indicate the reverse, with a 1.2 kcal/mol difference. This being said, her MO calculations should at least provide reasonable estimates of the relative protonation energies for the several basic sites of a given molecule. For Gua (but with the proton on N-9 although there is a N-7/N-9 tautomeric equilibrium (4, 16)), Del Bene found N-7 (-245 kcal/mol) to be the preferred protonation site and the solution data in Table 3 indicate N-7/N-9. Her next favored site is O-6 (on the C-5 side) (-233 kcal/mol) followed by N-3 (-288 kcal/mol). In contrast, our solution data show definitely that the second protonation takes place at N-3 rather than at O-6. It may be that the 5 kcal/mol difference between the calculated protonation energies at O-6 and N-3 is not high enough to counterbalance the repulsion which would exist between the second proton on O-6 (on the C-5 side) and the first proton on N-7. We must remember, however, that our results on successive protonations of the bases reflect factors additional to the relative intrinsic basicities of the various sites of the neutral bases.

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