Acid–Base Properties of Xanthosine 5′-Monophosphate (XMP) and of Some Related Nucleobase Derivatives in Aqueous Solution: Micro Acidity Constant Evaluations of the (N1)H versus the (N3)H Deprotonation Ambiguity**

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Abstract: The first acidity constant of fully protonated xanthosine 5′-monophosphate, that is, of $H_3(XMP)^+$, was estimated by means of a micro acidity constant scheme and the following three deprotonations of the $H_2(XMP)^{\pm}$ ($pK_a = 0.97$), $H(XMP)^-$ (5.30), and XMP^{2-} (6.45) species were determined by potentiometric pH titrations; further deprotonation of $(XMP-H)^{3-}$ is possible only with $pK_a > 12$. The most important results are that the xanthine residue is deprotonated before the $P(O)_2(OH)^-$ group loses its final proton; that is, twofold negatively charged XMP carries one negative charge in the pyrimidine ring and one at the phosphate group. Micro acidity constant evaluations reveal that this latter mentioned species occurs with a formation degree of 88%, whereas its tautomer with a neutral xanthine moiety and a PO_3^{2-} group is formed only to 12%; this distinguishes XMP from its related nucleoside 5′-monophosphates, like guanosine 5′-monophosphate. At the physiological pH of about 7.5 mainly $(XMP-H)^{3-}$ exists. The question, which of the purine sites, (N1)H or (N3)H, is deprotonated in this species cannot be answered unequivocally, though it appears that the (N3)H site is more acidic. By application of several methylated xanthine species intrinsic micro acidity constants are calculated and it is shown that, for example, for 7-methylxanthine the N1-deprotonated tautomer occurs with a formation degree of about 5%; a small but significant amount that, as is discussed, may possibly be enhanced by metal ion coordination to N7, which is known to occur preferably to this site.

Keywords: acidity · micro acidity constants · nucleotides · tautomerism · xanthosine monophosphate

Introduction

Xanthosine 5′-monophosphate (XMP^{2-}) and its nucleobase xanthine are both important intermediates in the metabolism of purines and their nucleotides,[1] and only recently the production of XMP in dependence on the cellular energetic status was studied for a microorganism.[2] The structure of XMP^{2-}, together with its related guanosine 5′-monophosphate (GMP^{2-}) is shown in Figure 1.[3–6] The xanthosine residue is unique among the common purine-type nucleosides due to the presence of two ionizable protons in the pyrimidine ring, that is, the (N1)H and the (N3)H sites (see Figure 1). Indeed, different conclusions exist concerning the relative acidities of the (N1)H and (N3)H units: In some studies the (N1)H site is considered as being the more acidic,[7–9] in analogy to the situation in guanosine and its de-

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[**] Abbreviations and definitions used throughout this paper (see also Figures 1 and 4): AMP^{2-}, adenosine 5′-monophosphate; 7,9DiMeUrA, 7,9-dimethyluric acid; 1,7DiMeXan, 1,7-dimethylxanthine; 1,9DiMeXan, 1,9-dimethylxanthine; 3,7DiMeXan, 3,7-dimethylxanthine; 3,9DiMeXan, 3,9-dimethylxanthine; Gua, guanine; Guo, guanosine; GMP^{2-}, guanosine 5′-monophosphate; *I*, ionic strength; IMP^{2-}, inosine 5′-monophosphate; K_a, general acidity constant; 7MeXan, 7-methylxanthine; 9MeXan, 9-methylxanthine; NMP^{2-}, nucleoside 5′-monophosphate; $RibMP^{2-}$, D-ribose 5-monophosphate; 1,7,9TriMeUrA, 1,7,9-trimethyluric acid; 3,7,9TriMeUrA, 3,7,9-trimethyluric acid; UMP^{2-}, uridine 5′-monophosphate; UrA, uric acid; Xan, xanthine; Xao, xanthosine; XMP^{2-}, xanthosine 5′-monophosphate (see legend of Figure 1). Species written without a charge (e.g., XMP) either do not carry one or represent the species in general (i.e., independent of their protonation degree); which of the two possibilities applies is always clear from the context. A formula like $(XMP-H)^{3-}$ means that the compound has lost a further proton and is to be read as XMP *minus* H^+.

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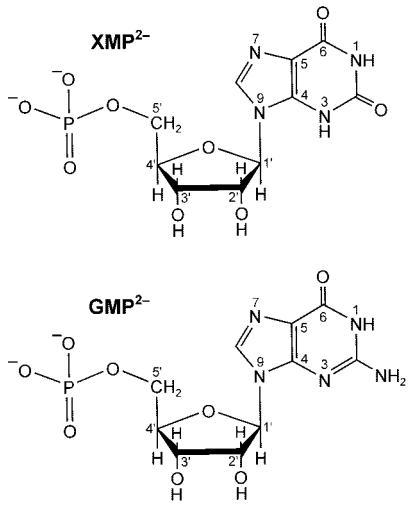


Figure 1. Chemical structure of xanthosine 5′-monophosphate (XMP^{2-}) together with that of its relative guanosine 5′-monophosphate (GMP^{2-}). Both purine nucleotides are shown in their dominating *anti*-conformation.[3–6] It is important to note that the structure shown above for *twofold* negatively charged XMP is the one commonly found in the literature including textbooks. Unfortunately this structure is incorrect, because it presents a wrong distribution of the protons: The fact is that the (N3)H/(N1)H sites of the pyrimidine ring have lost *one* proton, whereas the phosphate group still carries one; this means that one of the two negative charges is located in the pyrimidine ring and the other one at the phosphate group and this species is symbolized as $(X-H{\cdot}MP{\cdot}H)^{2-}$ and discussed in Section 3 (see also Figure 3).

rivatives,[10–13] whereas in others (N3)H is favored;[14–17] it appears that now some agreement exists regarding the acidity order (N3)H > (N1)H.[18,19] In fact, a crystal structure analysis[20] of the sodium salt of xanthine shows that deprotonation takes place at N3.

However, in a study evaluating the binding modes in metal ion complexes formed with xanthosine, deprotonation at (N3)H is favored, yet application of $\log K_{\text{complex stability}}$ versus pK_a straight-line plots for N1-type nitrogen ligands yields reasonable results.[19] Similarly irritating are the results of a study[21] that presents NMR evidence that Cd^{2+} coordinates in solution to xanthosinate through N3, whereas X-ray diffraction analysis of $[Zn(xanthosinate)_2(H_2O)_4]{\cdot}2\text{-}H_2O$ reveals N7 binding of the metal ion in the solid state. These and related observations[18,22,23] indicate to us that the acidities of the (N1)H and (N3)H sites may possibly be different, but that there is still a tautomeric equilibrium in the anionic form, that is, the remaining proton may reside largely at N1, but to a certain extent at N3 as well. Such a tautomeric situation is known for the structurally related uracil nucleobase.[24]

In this study we report a comprehensive set of the acidity constants of threefold protonated XMP^{2-}, that is, of $H_3(XMP)^+$. In several instances the buffer regions of the deprotonation reactions overlap; in other words, the acidity constants (pK_a values) are relatively similar. In these cases micro acidity constant analyses are presented, which allow a quantification of the intrinsic acid–base properties of the various sites. By also employing known acidity constants of some methylated xanthine derivatives, the ambiguity indicated above concerning (N1)H/(N3)H could be resolved to some extent and formation degrees for the tautomeric species are given. This analysis of the acid–base properties of XMP will allow now an evaluation of the metal-ion-binding properties[25] of this interesting nucleotide.

Results and Discussion

Based on our previous experience with nucleotides[26–28] related to XMP, great care was taken to measure the acid–base properties of XMP under conditions in which its self-association is expected to be negligible. Most measurements were made in solutions that were 0.3 mM in XMP; this guarantees[5,29,30] that the properties of the monomeric species are studied.

1. Definition of the acidity constants of $H_3(XMP)^+$ and of related species: Approximate site attributions of the protons: The nucleoside 5′-monophosphates (NMP^{2-}) shown in Figure 1 are tribasic species; they may accept two protons at the phosphate group and one at the purine moiety giving $H_3(NMP)^+$. Considering further that the nucleobase residue can be deprotonated at least once, possibly even twice, or if not that an additional proton can be released in the strongly alkaline pH range from the sugar part, overall five deprotonation reactions need to be considered. These steps are expressed for $H_3(XMP)^+$ in the Equilibria (1)–(5):

$$H_3(XMP)^+ \rightleftharpoons H_2(XMP)^{\pm} + H^+ \quad (1a)$$

$$K^{H}_{H_3(XMP)} = [H_2(XMP)^{\pm}][H^+]/[H_3(XMP)^+] \quad (1b)$$

$$H_2(XMP)^{\pm} \rightleftharpoons H(XMP)^- + H^+ \quad (2a)$$

$$K^{H}_{H_2(XMP)} = [H(XMP)^-][H^+]/[H_2(XMP)^{\pm}] \quad (2b)$$

$$H(XMP)^- \rightleftharpoons XMP^{2-} + H^+ \quad (3a)$$

$$K^{H}_{H(XMP)} = [XMP^{2-}][H^+]/[H(XMP)^-] \quad (3b)$$

$$XMP^{2-} \rightleftharpoons (XMP{-}H)^{3-} + H^+ \quad (4a)$$

$$K^{H}_{XMP} = [(XMP{-}H)^{3-}][H^+]/[XMP^{2-}] \quad (4b)$$

$$(XMP{-}H)^{3-} \rightleftharpoons (XMP{-}2\,H)^{4-} + H^+ \quad (5a)$$

$$K^{H}_{(XMP-H)} = [(XMP{-}2\,H)^{4-}][H^+]/[(XMP{-}H)^{3-}] \quad (5b)$$

The results obtained for the deprotonation reactions of $H_2(XMP)^{\pm}$ by means of potentiometric pH titrations are listed in Table 1 together with the acidity constants of related species.[31–34] The data banks given in references [35–37] contain no information[19] about $H_3(XMP)^+$.

Comparison of entry 5 with 4 and 9 with 8 of Table 1 reveals that replacement of the hydrogen at (N9)H in the nucleobase by a ribose residue makes the resulting nucleosides more acidic; hence, it is appropriate to use the values of guanosine (Guo), xanthosine (Xao), and D-ribose 5-monophosphate ($RibMP^{2-}$) for the site attributions in the depro-

Table 1. Negative logarithms of the acidity constants of $H_3(XMP)^+$, mainly determined by potentiometric pH titrations in aqueous solution (25 °C; I = 0.1 M, $NaNO_3$), together with some related data that refer to the same conditions.[a]

	Acid	$pK^H_{H_3(XMP)}$ [Eq. (1)] and pK_a of $P(O)(OH)_2$	$pK^H_{H_2(XMP)}$ [Eq. (2)] and pK_a of $(N7)H^+$	$pK^H_{H(XMP)}$ [Eq. (3)] and pK_a of (N1/N3)H	pK^H_{XMP} [Eq. (4)] and pK_a of $P(O)_2(OH)^-$	$pK^H_{(XMP-H)}$ [Eq. (5)] and pK_a of (N1)H
1	$H_3(AMP)^+$	0.4 ± 0.2[b]	3.84 ± 0.02[c,d]		6.21 ± 0.01[d]	
2[e]	$H_3(IMP)^+$	0.45 ± 0.25	1.30 ± 0.10		6.22 ± 0.01	9.02 ± 0.02
3[e]	$H_3(GMP)^+$	0.3 ± 0.2	2.48 ± 0.04		6.25 ± 0.02	9.49 ± 0.02
4[e]	$H(Guo)^+$		2.11 ± 0.04			9.22 ± 0.02
5[f]	$H(Gua)^+$		3.29 ± 0.03			9.36 ± 0.01
6[g]	$H(RibMP)^-$				6.24 ± 0.01	
7	$H_3(XMP)^+$	0.44 ± 0.27[h]	0.97 ± 0.15	5.30 ± 0.02	6.45 ± 0.02	> 12.0
8[i]	$H(Xao)^+$		0.74 ± 0.06[j]	5.47 ± 0.03		> 12.0
9	$H(Xan)^+$		1.2[k]	7.41 ± 0.02[l]		

[a] So-called practical, mixed, or Brønsted constants are listed; see Experimental Section. The error limits given are *three times* the standard error of the mean value or the sum of the probable systematic errors, whichever is larger. Those values for which no source is given have been determined in this study. For the sites at which the protons are listed, see also the text in the Results and Discussion section. [b] Determined by ^{1}H NMR shift experiments.[4] [c] This value refers to the deprotonation of the $(N1)H^+$ site of the adenine residue. [d] From reference [31]; see also reference [32]. [e] From reference [29]; see also reference [32]. [f] From reference [33]. [g] From reference [34]. [h] See Figure 2 in Section 2. [i] From reference [8]. [j] Determined by UV spectrophotometry at 25 °C and I = 0.5 M ($NaClO_4$).[8] [k] Determined by UV spectrophotometric measurements; conditions undefined, but most likely in the presence of some buffer at room temperature.[16] [l] From. reference [9]; potentiometric pH titrations at 25 °C and I = 0.1 M, $NaNO_3$.

tonation reactions occurring with $H_3(GMP)^+$ and $H_3(XMP)^+$. In a first approximation one may conclude (see also Section 2) that all $H_3(NMP)^+$ species of Table 1 release their first proton [Eq. (1)] from the $P(O)(OH)_2$ group and the second one [Eq. (2)] from the $(N7)H^+$ site of the purine system, with the exception of $H_2(AMP)^\pm$, for which this proton is from the $(N1)H^+$ unit.[11,38]

At this point the pathways of AMP, IMP, and GMP separate from that of XMP: With the former nucleotides the species $H(NMP)^-$ release their third proton [analogous to Eq. (3)] from the $P(O)_2(OH)^-$ group (Table 1, column 6) and, as far as IMP^{2-} and GMP^{2-} are concerned, this is followed by deprotonation of the (N1)H site of the nucleobase residue (Table 1, column 7);[29] a further proton can only be released [analogous to Eq. (5)] from the sugar moiety, but for this reaction it holds $pK_a > 12$.[7] The situation with $H(XMP)^-$ is quite different; here the third proton [Eq. (3)] is released from the (N1/N3)H sites giving the XMP^{2-} species, which carries one negative charge at the pyrimidine ring and one at the $P(O)_2(OH)^-$ group, and which is therefore better written as $(X{-}H{\cdot}MP{\cdot}H)^{2-}$ (see also legend to Figure 1 and Section 3 below). Only in the next step is the still monoprotonated phosphate group deprotonated [Eq. (4)] giving $(XMP{-}H)^{3-}$ (see also columns 5 and 6 in Table 1). It is thus not surprising to find that the final phosphate proton from $(X{-}H{\cdot}MP{\cdot}H)^{2-}$ is released with a pK_a value that is approximately 0.2 units larger than those for the $H(NMP)^-$ species of AMP, IMP, and GMP (Table 1, column 6); clearly, the negative charge at the pyrimidine ring, which may be partly delocalized to the carbonyl oxygen atoms, inhibits the phosphate deprotonation somewhat.

As far as the deprotonation reaction of $H(XMP)^-$ is concerned, and this holds for Xao as well, an ambiguity exists: Which site, (N1)H or (N3)H, is deprotonated first? This question will further be addressed in Section 4 and evidence will be provided that a tautomeric equilibrium for the monodeprotonated pyrimidine ring exists. However, after monodeprotonation of either (N1)H or (N3)H, the remaining (N)H can be deprotonated as well,[17] as can the ribose residue,[7] and thus it is not certain which deprotonation reaction in Equilibrium (5a) actually takes place; most likely first the ribose ring loses a proton,[7] followed by that from the remaining (N)H site ($pK_a \approx 13$).[14] In any case, under our experimental conditions a deprotonation reaction according to Equilibrium (5a) occurs only with $pK_a > 12.0$; in other words, under strongly alkaline conditions and it is thus of no biological relevance.

2. Micro acidity constant scheme for $H_3(XMP)^+$ and estimation of its macro acidity constant: From the results assembled in Table 1 it is evident that the buffer regions of the individual deprotonation steps of $H_3(AMP)^+$ and $H_3(GMP)^+$ (entries 1,3) are separated by about two or more pK units; hence, there is no significant overlap of the buffer regions and the macro acidity constants are identical with the micro acidity constants of the individual sites in these instances. This is different with $H_3(IMP)^+$ because $pK^H_{H_3(IMP)} = 0.45$ and $pK^H_{H_2(IMP)} = 1.30$ (entry 2) are relatively close to each other and in fact, a micro constant scheme was derived for this case[29] and it was shown that both tautomeric species, $(IMP{\cdot}H_2)^0$ and $(H{\cdot}IMP{\cdot}H)^\pm$, occur in appreciable amounts.

Considering that $pK^H_{H_2(XMP)} = 0.97$ (Table 1, entry 7, column 4) is also a rather low value, a similar situation for $H_2(XMP)^\pm$ is expected as found previously[29] for $H_2(IMP)^\pm$. Therefore, a micro acidity constant,[39] $k^{XMP{\cdot}H}_{H{\cdot}XMP{\cdot}H}$, for Equilibrium (6),

$$(H \cdot XMP \cdot H)^\pm \rightleftharpoons (XMP \cdot H)^- + H^+ \qquad (6)$$

in which $(H{\cdot}XMP{\cdot}H)^\pm$ represents a species that carries one proton each at N7 and the phosphate group, is estimated based on the following reasoning: The *difference* between the pK_a values for the deprotonation of $(N7)H^+$ in the guanine and xanthine residues is expected to be independent of the presence of a phosphate group and should be the same if calculated by means of the pK_a values of the two nucleosides, Guo and Xao, or by those of the two NMPs, though the acidity constants themselves are different of course. Hence, application of the values in Table 1 gives $\Delta pK_a =$

$pK^{H}_{H(Guo)} - pK^{H}_{H(Xao)} = (2.11 \pm 0.04) - (0.74 \pm 0.06) = 1.37 \pm 0.07$, and therefore $pk^{XMP\cdot H}_{H\cdot XMP\cdot H} = pK^{H}_{H_2(GMP)} - \Delta pK_a = (2.48 \pm 0.04) - (1.37 \pm 0.07) = 1.11 \pm 0.08$; this value describes now the position of Equilibrium (6). Furthermore, this micro acidity constant for the deprotonation of the $(N7)H^+$ site, with one standard deviation $pk^{XMP\cdot H}_{H\cdot XMP\cdot H} = 1.11 \pm 0.03 (1\sigma)$, is close but still different from the macroconstant, $pK^{H}_{H_2(XMP)} = 0.97 \pm 0.05 (1\sigma)$ (Table 1), indicating a contribution from the deprotonation of the $P(O)(OH)_2$ group to this macroconstant; hence, a more rigorous evaluation is required.

Figure 2 summarizes the complete equilibrium scheme for $H_3(XMP)^+$ to $H(XMP)^-$ defining the microconstants (k) and giving their interrelation with the macro acidity con-

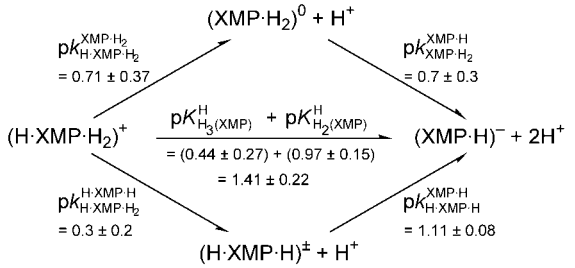


Figure 2. Equilibrium scheme for $(H\cdot XMP\cdot H_2)^+$ to $(XMP\cdot H)^-$ defining the micro acidity constants (k) and showing their interrelation with the macro acidity constants (K) and also the interrelation between $(XMP\cdot H_2)^0$ and $(H\cdot XMP\cdot H)^{\pm}$ and the other species present. In $(XMP\cdot H_2)^0$ both protons are at the phosphate group while in $(H\cdot XMP\cdot H)^{\pm}$ one proton is at N7 and the other at the phosphate group (Figure 1). $(H\cdot XMP\cdot H_2)^+$ is also often written as $H_3(XMP)^+$; it carries one proton at N7 and the two others at the phosphate group. The arrows indicate the direction for which the acidity constants are defined. For the origin of the various constants see the text in Section 2.

stants (K). The microconstant derived in the preceding paragraph, $pk^{XMP\cdot H}_{H\cdot XMP\cdot H}$, appears in the lower part of the scheme at the right. Since the macroconstant $K^{H}_{H_3(XMP)}$ could not be measured by potentiometric pH titrations, because it is so small, one more constant needs to be estimated. From entry 3 of Table 1 it follows that the acidity constants for the deprotonation of $H_3(GMP)^+$ and $H_2(GMP)^{\pm}$ are well separated from each other; hence, $pK^{H}_{H_3(GMP)} = 0.3 \pm 0.2$ certainly represents well the micro acidity constant $pk^{H\cdot XMP\cdot H}_{H\cdot XMP\cdot H_2}$, which describes the release of one of the protons from the $P(O)(OH)_2$ group in a purine nucleotide with a $(N7)H^+$ unit, that is, from $(H\cdot XMP\cdot H_2)^+$. With this value, given at the left in the lower part of the scheme in Figure 2, due to the properties of a cyclic system, the sum of $pK^{H}_{H_3(XMP)} + pK^{H}_{H_2(XMP)}$ can now be calculated; namely, $pk^{H\cdot XMP\cdot H}_{H\cdot XMP\cdot H_2} + pk^{XMP\cdot H}_{H\cdot XMP\cdot H} = (0.3 \pm 0.2) + (1.11 \pm 0.08) = 1.41 \pm 0.22$. This value in turn furnishes now together with $pK^{H}_{H_2(XMP)} = 0.97 \pm 0.15$ (Table 1) a value for the macro acidity constant $pK^{H}_{H_3(XMP)} = (1.41 \pm 0.22) - (0.97 \pm 0.15) = 0.44 \pm 0.27$, which is also listed in entry 7 of Table 1 (column 3).

With the lower circle in Figure 2 complete, it would now be possible by following known routes[13,31,39,40] to calculate values for the micro acidity constants that appear in the upper pathway of the scheme. However, as the error limits of some of the acidity constants employed are rather large, we prefer in the present case to make a further sophisticated estimate: In the upper pathway of the scheme at the right a micro acidity constant is needed for the deprotonation of $(XMP\cdot H_2)^0$, that is, for the release of the first proton from $P(O)(OH)_2$ of an NMP which carries a neutral nucleobase residue. Such a value has previously been measured[34] for $H_2(UMP)$, that is, $pK^{H}_{H_2(UMP)} = 0.7 \pm 0.3$, and this value is considered as a good estimate for $pk^{XMP\cdot H}_{XMP\cdot H_2}$. Due to the properties of a cyclic system now also the upper pathway of the scheme in Figure 2 can be completed and a value for $pk^{XMP\cdot H_2}_{H\cdot XMP\cdot H_2}$ can be calculated. It is most satisfying to note that $pk^{XMP\cdot H_2}_{H\cdot XMP\cdot H_2} = 0.71 \pm 0.37$ agrees excellently with $pK^{H}_{H(Xao)} = 0.74 \pm 0.06$ (Table 1, entry 8); clearly, in $(H\cdot XMP\cdot H_2)^+$ and $H(Xao)^+$ deprotonation of the $(N7)H^+$ site of the xanthine moiety occurs in both cases from an otherwise neutral species and, therefore, the corresponding acidity constants should be identical. This result indicates that the micro acidity constants summarized in Figure 2 are more reliable than their error limits (3σ!!) might suggest.

Finally, one may apply the micro acidity constants of Figure 2 to estimate the ratio R [Eq. (7)] of the twofold protonated and isocharged tautomeric species $(H\cdot XMP\cdot H)^{\pm}$ and $(XMP\cdot H_2)^0$, which carry one proton at N7 and one at the phosphate or both protons at the phosphate, respectively:

$$R_{H_2/XMP} = \frac{[(H \cdot XMP \cdot H)^{\pm}]}{[(XMP \cdot H_2)^0]} = \frac{k^{H\cdot XMP\cdot H}_{H\cdot XMP\cdot H_2}}{k^{XMP\cdot H_2}_{H\cdot XMP\cdot H_2}} \quad (7a)$$

$$= \frac{10^{-(0.3\pm0.2)}}{10^{-(0.71\pm0.37)}} = 10^{0.41\pm0.42} \quad (7b)$$

$$= \frac{2.57}{1} \quad (7c)$$

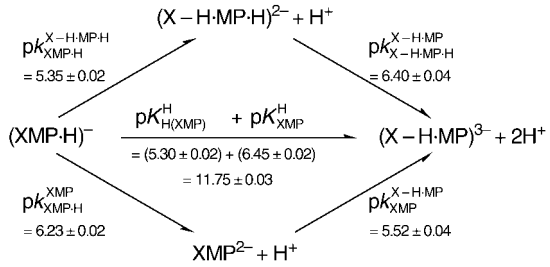
$$= \frac{72}{28}\left(\frac{49}{51}; \frac{87}{13}\right) \quad (7d)$$

The ratio in Equation (7d) corresponds to the approximate percentages of the $(H\cdot XMP\cdot H)^{\pm}$ and $(XMP\cdot H_2)^0$ species. The first ratio given in parentheses represents the lower limit (3σ) following from $0.41 - 0.42 = -0.01$ [Eq. (7b)], and the second ratio the upper limit, which follows from $0.41 + 0.42 = 0.83$; with one standard error (1σ) only, Equation (7d) reads: 72/28 (65/35; 78/22). Hence, we may conclude that the zwitterionic species $(H\cdot XMP\cdot H)^{\pm}$ dominates with about 70%, while $(XMP\cdot H_2)^0$ forms to about 30%. Certainly, this result is only an estimation, but it proves that both tautomeric forms of $H_2(XMP)$ occur simultaneously in appreciable amounts.

3. Is there a tautomeric equilibrium between $(X-H\cdot MP\cdot H)^{2-}$ and XMP^{2-}? In Section 1 we have concluded, based on a comparison of the acidity constants (Table 1) of $H(XMP)^-$ and XMP^{2-} with those of Xao and $H(RibMP)^-$, that in $H(XMP)^-$ first one proton is released from the (N1/N3)H sites before the monoprotonated phosphate group is further ionized. Therefore, the species XMP^{2-} was also written as $(X-H\cdot MP\cdot H)^{2-}$. Naturally, this leads to the question: Does an XMP^{2-} species with a neutral

xanthine residue and a twofold negatively charged phosphate group not exist at all?

We attempt to answer the above question by applying micro acidity constants to the various species. Figure 3 summarizes the equilibrium scheme for the deprotonation reactions of $H(XMP)^-$ to $(XMP-H)^{3-}$; these species are written in Figure 3 as $(XMP{\cdot}H)^-$ and $(X-H{\cdot}MP)^{3-}$ to indicate that in the first species the proton is at the phosphate group and that in the second one the xanthine residue has lost a proton. More important, the tautomers XMP^{2-} and $(X-H{\cdot}MP{\cdot}H)^{2-}$ are on the pathway from $(XMP{\cdot}H)^-$ to $(X-H{\cdot}MP)^{3-}$. The macro acidity constants connected with the two deprotonation reactions [Eqs. (3) and (4)] have been measured (Table 1) and are given on the horizontal arrow in the scheme of Figure 3. This figure defines further four micro acidity constants (k) and gives their interrelation with the macro acidity constants (K) according to the definitions provided in the lower part of Figure 3 by following known routes.[13,31,39,40]



$$K^{H}_{H(XMP)} = k^{X-H\cdot MP\cdot H}_{XMP\cdot H} + k^{XMP}_{XMP\cdot H} \quad (a)$$

$$\frac{1}{K^{H}_{XMP}} = \frac{1}{k^{X-H\cdot MP}_{X-H\cdot MP\cdot H}} + \frac{1}{k^{X-H\cdot MP}_{XMP}} \quad (b)$$

$$K^{H}_{H(XMP)} \cdot K^{H}_{XMP} = k^{X-H\cdot MP\cdot H}_{XMP\cdot H} \cdot k^{X-H\cdot MP}_{X-H\cdot MP\cdot H} = k^{XMP}_{XMP\cdot H} \cdot k^{X-H\cdot MP}_{XMP} \quad (c)$$

Figure 3. Equilibrium scheme for $(XMP{\cdot}H)^-$ to $(X-H{\cdot}MP)^{3-}$ defining the micro acidity constants (k) and showing their interrelation with the measured macro acidity constants (K) and the connection between $(X-H{\cdot}MP{\cdot}H)^{2-}$ and XMP^{2-} and the other species present. In $(X-H{\cdot}MP{\cdot}H)^{2-}$ the xanthine residue is deprotonated and the proton located at the phosphate group; in its XMP^{2-} tautomer the nucleobase is uncharged and the phosphate group deprotonated. $(XMP{\cdot}H)^-$ and $(X-H{\cdot}MP)^{3-}$ are also often written as $H(XMP)^-$ [Eq. (3)] and $(XMP-H)^{3-}$ [Eq. (4)], respectively. The arrows indicate the direction for which the acidity constants are defined. Use of the average of the acidity constants measured for the deprotonation of $H(AMP)^-$, $H(IMP)^-$, $H(GMP)^-$ and $H(RibMP)^-$ (Table 1, column 6), that is, $pK^{H}_{H(NMP)} = 6.23 \pm 0.02$, for the microconstant $pk^{XMP}_{XMP\cdot H}$ (lower pathway at the left) permits calculation of the other microconstants with Equations (a), (b) and (c). The error limits of the various constants were calculated according to the error propagation after Gauss; they correspond to three times the standard error (see Table 1; footnote [a]).

Figure 3 shows that there are four unknown micro acidity constants, but only three independent equations interrelating them with the macroconstants; this means, one of the microconstants needs to be obtained independently: Equilibrium (8) describes the deprotonation of a $P(O)_2(OH)^-$ group of a nucleotide with an uncharged nucleobase residue.

$$(XMP\cdot H)^- \rightleftharpoons XMP^{2-} + H^+ \quad (8)$$

Clearly, the corresponding acidity constant, $k^{XMP}_{XMP\cdot H}$, is well represented by the constants of the deprotonations of $H(AMP)^-$, $H(IMP)^-$, $H(GMP)^-$ and $H(RibMP)^-$; therefore, the corresponding four values (Table 1, column 6) are averaged to give $pK^{H}_{H(NMP)} = pk^{XMP}_{XMP\cdot H} = 6.23 \pm 0.02$. Use of this value in the lower pathway at the left in Figure 3 allows us to calculate according to the properties of a cyclic system the microconstant for the release of one proton from the (N1/N3)H sites of an XMP^{2-} with a free phosphate group, that is, $pk^{X-H\cdot MP}_{XMP} = 5.52 \pm 0.04$. Furthermore, application of Equation (a) in Figure 3 allows the calculation of a value for $pk^{X-H\cdot MP\cdot H}_{XMP\cdot H}$ and thus also completion of the microconstant values for the upper pathway.

Comparison of the data in Figure 3 reveals that the micro acidity constants in the upper pathway are close to the measured acidity constants; this means that this pathway is the dominating one. However, application of these micro acidity constants also allows now to answer the above question and to calculate the ratio R [Eq. (9)] of the isocharged species $(X-H{\cdot}MP{\cdot}H)^{2-}$ and XMP^{2-}:

$$R_{XMP} = \frac{[(X-H\cdot MP\cdot H)^{2-}]}{[XMP^{2-}]} = \frac{k^{X-H\cdot MP\cdot H}_{XMP\cdot H}}{k^{XMP}_{XMP\cdot H}} \quad (9a)$$

$$= \frac{10^{-(5.35\pm0.02)}}{10^{-(6.23\pm0.02)}} = 10^{0.88\pm0.03} \quad (9b)$$

$$= \frac{7.59}{1} \quad (9c)$$

$$= \frac{88.4}{11.6}\left(\frac{89.0}{11.0};\frac{87.6}{12.4}\right) \quad (9d)$$

The ratio in Equation (9d) corresponds to the percentages of the $(X-H{\cdot}MP{\cdot}H)^{2-}$ and XMP^{2-} species formed in solution in a tautomeric equilibrium. For the limiting values given in parentheses [Eq. (9d)] the analogous explanations hold as given in the final paragraph of Section 2. From the results in Equation (9d) it is evident that, as expected, the species $(X-H{\cdot}MP{\cdot}H)^{2-}$ with a deprotonated xanthine residue and a monoprotonated phosphate group dominates with a formation degree of about 88%. However, with a formation degree of about 12% the simple XMP^{2-} species, with a neutral nucleobase moiety and a twofold negatively charged phosphate group, is not negligible; it still occurs in a remarkable concentration and thus, a minority tautomer of XMP exists in solution that corresponds structure-wise to AMP^{2-}, IMP^{2-} and GMP^{2-} (see Figure 1).

4. Evaluation of the tautomeric equilibrium between (N1)H and (N3)H in monoprotonated xanthine derivatives: In Section 3 we have seen that the twofold negatively charged XMP species, which has lost one proton from the xanthine

residue and which carries another one at the phosphate group, that is, $(X-H\cdot MP\cdot H)^{2-}$, dominates with a formation degree of about 88% [Eq. (9c)]. However, the question that remains to be answered is: Which site is deprotonated, (N1)H or (N3)H? This question cannot be answered for XMP and also not for xanthosine with the information presently at hand.

However, in recent research with other purine derivatives[12,13,41] we have shown that the positions of tautomeric equilibria can be quantified by employing *N*-methylated derivatives; this then allows us to obtain micro acidity constants. This approach is possible because the electronegativity of an H atom and a methyl group are quite alike. In other words, such a substitution does not significantly alter the acid–base properties of another nearby site,[12] for example, N1-protonated adenosine and 1-methyladenosine have the same pK_a value for the $(N7)H^+$ site, meaning that $H^+(N1)$ and $CH_3{}^+(N1)$ have the same effect on the $(N7)H^+$ site.

For the present evaluation, methylated xanthine derivatives[14,15,42] and their published acidity constants were used. 9-Methylxanthine (9MeXan) (Figure 4, top) has the same

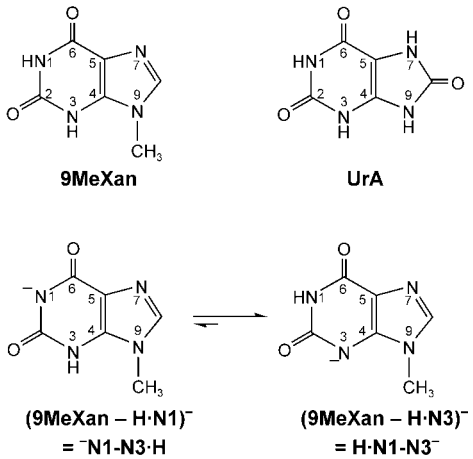


Figure 4. Chemical structures of 9-methylxanthine (9MeXan) and uric acid (UrA). In the lower part the tautomeric equilibrium for 9-methylxanthinate is indicated (of course, the negative charge at the N atoms may partly be delocalized to the carbonyl oxygen atoms).

ambiguity as xanthosine and XMP with regard to (N1)H and (N3)H; however, in its derivatives 1,9-dimethylxanthine (1,9DiMeXan) and 3,9-dimethylxanthine (3,9DiMeXan) the situation is unambiguous, because a proton can only be released either from the (N3)H *or* the (N1)H site, respectively. Hence, the pK_a value of 1,9DiMeXan may be considered as being representative for the (N3)H deprotonation of 9MeXan; that is, its anion represents the tautomer shown in the lower part of Figure 4 at the right, whereas the properties of the tautomer at the left are described by the anion of 3,9DiMeXan.

The above reasonings are summarized in the micro acidity constant scheme of Figure 5, in which the various deprotonation reactions of 9MeXan are given, together with the acidity constants from reference [16] and in which the

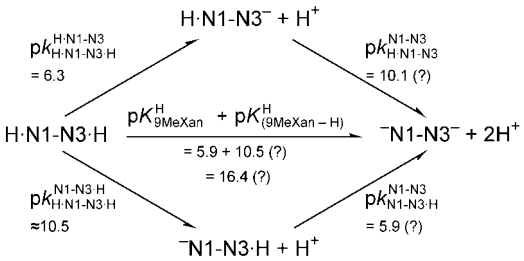


Figure 5. Micro acidity constant scheme for 9-methylxanthine (9MeXan), in which H·N1-N3·H represents 9MeXan. Shown is the interrelation between the micro acidity constants (k) and the macro acidity constants (K) as well as the connection between H·N1-N3⁻ and ⁻N1-N3·H and the other species present (see also Figure 4). The arrows indicate the direction for which the acidity constants are defined. The inserted constants at the left of the scheme and at the horizontal arrow are from entries 1–3 of Table 2; the value of 10.5 for $pK^{H}_{(9MeXan-H)}$ appears to us as uncertain and it is therefore given with a question mark (see also the comment to this value in Table 2). However, this uncertainty has no effect on the calculations which are based on the values given at the left.

values of 1,9DiMeXan and 3,9DiMeXan are applied at the left in the upper and lower pathways, respectively. A comparison of the macro constants with the micro constants shows that the upper pathway dominates. The two micro acidity constants at the left allow us to calculate, according to Equation (10), the ratio and, thus, the formation degree of the two tautomers seen in the bottom part of Figure 4.

$$R_{N1/N3} = \frac{[H\cdot N1\text{-}N3^-]}{[^-N1\text{-}N3\cdot H]} = \frac{k^{H\cdot N1\text{-}N3}_{H\cdot N1\text{-}N3\cdot H}}{k^{N1\text{-}N3\cdot H}_{H\cdot N1\text{-}N3\cdot H}} \quad (10)$$

The corresponding results for 9MeXan are given in entry 3 of Table 2,[43] and they indicate that the N1-deprotonated isomer occurs only in traces. It needs to be noted here that the acidity constants of ref. [16] in the high pK_a range do not seem to be very exact (see also below the discussion regarding the systems of entries 4–12 of Table 2), but if one assumes that the K_a of 3,9DiMeXan is off by a factor of 10 and that $pK^{H}_{3,9DiMeXan} \approx 9.5$, then the formation degree of the N1-deprotonated species, ⁻N1-N3·H, increases only to about 0.1%. In any case, traces of this species are certainly present.

Another xanthine derivative with (N1)H and (N3)H sites is 7-methylxanthine (7MeXan), for which also the necessary methylated species exist, that is, 1,7- and 3,7-dimethylxanthine (1,7DiMeXan and 3,7DiMeXan). In fact, the necessary acidity constants are available for this evaluation from three different sources.[14,16,42] The corresponding data are given in entries 4–12 of Table 2 and the results regarding the formation degrees of the tautomers in entries 6, 9, and 12. It is evident that the results of entries 9 and 12 agree well with each other, whereas that of entry 6 is off. A closer look at the macro acidity constants reveals that this is due to the high pK_a value (11.0) given in reference [16] for 3,7DiMeXan. To conclude, 7-methylxanthinate exists to about 5% as the N1-deprotonated and to about 95% as the N3-deprotonated tautomer, that is, in this case clearly both isomers are formed in remarkable quantities.

Table 2. Evaluation of the tautomeric equilibrium between the (N1)H and (N3)H sites in monodeprotonated derivatives of xanthine and uric acid (Figure 4) by means of micro acidity constants. These microconstants are derived from analogous species in which one of the (N)H units is alternatively replaced by a (N)CH_3 group. For the definition of the micro acidity constants see the equilibrium scheme in Figure 5. The tautomers are quantified by their ratios R [Eq. (10)] and their formation degrees given in percentages.[a,b]

	Acid	pK_a for the site (N1/N3)H	(N3)H	(N1)H	$pk^{H\cdot N1\text{-}N3}_{H\cdot N1\text{-}N3\cdot H}$	$pk^{N1\text{-}N3\cdot H}_{H\cdot N1\text{-}N3\cdot H}$	$R=\frac{[H\cdot N1\text{-}N3^-]}{[^-N1\text{-}N3\cdot H]}$	% H•N1-N3$^-$	% $^-$N1-N3•H
1	1,9DiMeXan		6.3						
2	3,9DiMeXan			~10.5					
3	9MeXan	5.9		~10.5[c]	6.3	~10.5	$10^{4.2}$	99.99	0.01
4	1,7DiMeXan		8.6						
5	3,7DiMeXan			11.0					
6	7MeXan	8.4		~10.5[d]	8.6	11.0	$10^{2.4}$	99.6	0.4
7	1,7DiMeXan		8.71						
8	3,7DiMeXan			9.97					
9	7MeXan	8.33		~13	8.71	9.97	$10^{1.26}$	94.8	5.2
10	1,7DiMeXan		8.5						
11	3,7DiMeXan			9.9					
12	7MeXan	8.5			8.5	9.9	$10^{1.4}$	96	4
13	1,7,9TriMeUrA		5.28						
14	3,7,9TriMeUrA			9.42					
15	7,9DiMeUrA	5.05		>13	5.28	9.42	$10^{4.14}$	99.99	0.01

[a] The values refer to aqueous solutions close to 25 °C; the ionic strength is not defined. The constants were measured by spectrophotometry,[14,16,43] except for entries 10–12 which are the results from potentiometric measurements.[42] [b] The values in columns 3–5 are for entries 1–6 from reference [16], for entries 7–9 from reference [14],for entries 10–12 from reference [42] and for entries 13–15 from reference [43]. [c] Considering that this value refers to the release of a proton from an already negatively charged xanthinate residue, it appears as low if compared with the value due to the deprotonation of 3,9DiMeXan (entry 2). However, this shortcoming has no effect [see also the paragraph following Eq. (10) in the text], since this value does not enter into the calculations (see also Figure 5). [d] For this value comments analogous to those given in [c] hold.

That the N1/N3 tautomeric ratio depends significantly on the substituents of the purine moiety is also evident from entries 13–15 of Table 2, in which the situation for 7,9-dimethyluric acid (7,9DiMeUrA; see also Figure 4 at the top, right) is considered. In this case only traces of the N1-deprotonated tautomer are formed, meaning that the N3-deprotonated species dominates very strongly.

To conclude, from the above results it follows that N1- and N3-methylxanthosine need to be studied to see which effect the sugar residue has on the distribution of the tautomers. That it can have a significant effect is known from other purine derivatives in which a methyl group at N9 was replaced by ribose.[12,13] It is further interesting that a methyl group at N7 evidently favors the formation of the N1-deprotonated tautomer. Has metal ion coordination at N7 the same effect? If so, this would immediately explain why N7 coordination to the xanthosine residue in aqueous solution is well known.[19]

Conclusions

The acid–base properties of XMP are now well described in the pH range 0 to 12 and it is shown that micro acidity constant schemes are useful "tools" to quantify the intrinsic acid–base properties of certain sites. Thus, about 70% of the H_2(XMP) species exist in the zwitterionic form, that is, N7 is protonated and P(O)$(OH)_2$ monodeprotonated, whereas the remaining 30% refer to a neutral molecule with a P(O)$(OH)_2$ residue.

However, more important is the fact that XMP^{2-} is actually present to about 88% as $(X-H\cdot MP\cdot H)^{2-}$, a species with a negative charge at the pyrimidine ring and another one at the P$(O)_2(OH)^-$ group. In the physiological pH range of 7.5 $(XMP-H)^{3-}$ is the dominating species, that is, the xanthine residue has lost a proton and thus the nucleobase is negatively charged. There is no other purine nucleotide with such a property. It is expected that this affects the metal-ion-binding properties of XMP and the structures of its complexes in solution.[25]

Application of micro acidity constant schemes to methylated xanthine derivatives demonstrate that deprotonation at the nucleobase occurs mainly at N3, but that the N1-deprotonated tautomer may also be formed in appreciable amounts under certain conditions. Clearly, here more research is needed, especially of xanthosine and its methylated derivatives. However, it is revealing that remarkable amounts (ca. 5%) of the N1-deprotonated tautomer of 7-methylxanthine form and it appears as rather likely that N7 coordination of a metal ion, which is known to occur in the solid state,[21,44] also favors this tautomer in aqueous solution,[19] in which in addition an outersphere chelate, involving (C6)O with a metal-ion-coordinated water molecule, may easily form.

Of interest in the above context is also the solid-state structure of a titanocene–xanthine 3:1 complex,[23] in which xanthine is twofold deprotonated, namely at the N1 and N9 sites (Figure 4, top left). In this molecule $(\eta^5\text{-}C_5H_5)_2Ti^+$ forms a four-membered chelate involving N1 and (C2)O as well as a five-membered chelate with N7 and (C6)O; the third $(\eta^5\text{-}C_5H_5)_2Ti^+$ interacts in a unidentate manner with N9 and a Cl^- ion. Most remarkable is that the (N3)H unit in this complex is still intact. All this indicates that a shift from an (N3)H to a (N1)H deprotonation is easily achieved in the xanthine residue.

Experimental Section

Materials: The disodium salt of xanthosine 5′-monophosphate was purchased from Sigma, St. Louis, MO (USA). Potassium hydrogen phthalate, HNO_3, NaOH (Titrisol), and sodium nitrate (all pro analysi) were from Merck, Darmstadt (Germany). All solutions were prepared with deionized ultrapure (MILLI-Q 185 PLUS, from Millipore S.A., 67120 Molsheim, France) and CO_2-free water.

The aqueous stock solution of XMP was freshly prepared daily, and its exact concentration was newly determined each time by titrations with NaOH (see below). The titer of the NaOH used for the titrations was established with potassium hydrogen phthalate.

Potentiometric pH titrations: The pH titrations were carried out with a Metrohm E536 potentiograph equipped with a E665 dosimat and a 6.0202 100(NB) combined glass electrode. The buffer solutions (pH 4.64, 7.00, and 9.00 based on the NBS scale, now U.S. National Institute of Standards and Technology (NIST)) used for calibration were also from Metrohm, Herisau (Switzerland).

The direct pH-meter readings were used to calculate the acidity constants, that is, these constants are so-called practical, mixed, or Brønsted constants.[45] Their negative logarithms given for aqueous solutions at $I=0.1$ M ($NaNO_3$) and 25 °C may be converted into the corresponding concentration constants by subtracting 0.02 from the listed pK_a values; this conversion term contains both the junction potential of the glass electrode and the hydrogen ion activity.[45,46] The ionic product of water (K_W) and the mentioned conversion term do not enter into our calculation procedures, because we evaluate the *differences* in NaOH consumption between a pair of solutions; that is, a solution with and one without ligand are always titrated (see also below; for further details references [45] and [47] may be consulted).

All acidity constants were calculated by curve-fitting procedures using a Newton–Gauss nonlinear least-squares program in the way and with the computer equipment described recently.[12,48]

Determination of the acidity constants of $H_2(XMP)^{\pm}$: The acidity constants $K^{H}_{H(XMP)}$ [Eq. (3)] and K^{H}_{XMP} [Eq. (4)] were determined by titrating aqueous HNO_3 (0.54 mM, 50 mL, 25 °C; $I=0.1$ M, $NaNO_3$) in the presence and absence of XMP (0.3 mM, adjusted in its stock solutions to pH 5.9) under N_2 with NaOH (0.03 M, 1.5 mL). In a second series some experiments were also carried out by titrating aqueous HNO_3 (31.7 mM, 15 mL, 25 °C; $I=0.1$ M, $NaNO_3$) in the presence and absence of XMP (4.8 mM, adjusted in its stock solution to pH 7.9) under N_2 with NaOH (0.2 M, 2.5–3.5 mL). In this second series of experiments the conditions were such that also the acidity constant $K^{H}_{H_2(XMP)}$ [Eq. (2)] could be determined and for $K^{H}_{(XMP-H)}$ [Eq. (5)] a lower limit could be defined.

In the first series of experiments the data were collected every 0.1 pH unit in the pH range 3.7 to 8.2 and used for the calculations; this range corresponds initially to about 2% neutralization for the equilibrium $H(XMP)^{-}/XMP^{2-}$ [Eq. (3)] and finally, to about 98% neutralization for the equilibrium $XMP^{2-}/(XMP-H)^{3-}$ [Eq. (4)]. In the second series the calculation was carried out in the pH range 1.8 to 10.8, which corresponds initially already to a neutralization degree of about 85% for the equilibrium $H_2(XMP)^{\pm}/H(XMP)^{-}$ [Eq. (2)] and, therefore, the error in the corresponding acidity constant is relatively large. For the constant regarding the equilibrium $(XMP-H)^{3-}/(XMP-2H)^{4-}$ [Eq. (5)] only a lower limit could be obtained.

The results are the averages of four independent pairs of titrations for the acidity constant $K^{H}_{H_2(XMP)}$ [Eq. (2)], of 25 independent pairs of titrations for the acidity constants $K^{H}_{H(XMP)}$ [Eq. (3)] and K^{H}_{XMP} [Eq. (4)], and of two independent pairs of titrations for the determination of the lower limit of the acidity constant K^{H}_{XMP-H} [Eq. (5)].

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