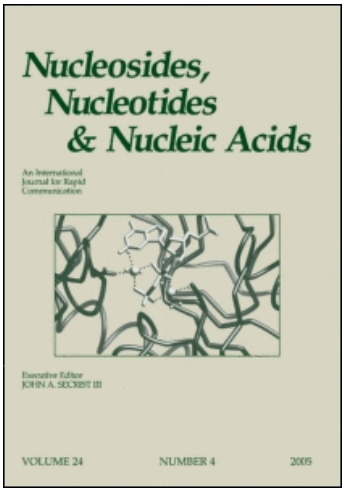

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Tauto Merism and Ionization of Xanthosine

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TAUTOMERISM AND IONIZATION OF XANTHOSINE

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Abstract: Tautomerism and ionization of xanthosine have been studied by infrared spectroscopy. N-methyl and O-methyl model compounds which are isoelectronic with possible keto and enol tautomers have been synthesized. Comparisons of their spectra with neutral and with ionized xanthosine demonstrate that unionized xanthosine has the diketo form and that on acid dissociation (pK 5.7), the first proton is lost from N3 (rather than N1) to give the 6-keto-2-enolate anion. Specific labelling at the 2- and 6-positions with ^{18}O confirms these conclusions and supports assignment of the highest frequency bands in the double bond region to carbonyl stretching vibrations. These conclusions are relevant to two different proposed structures for the ordered form of polyxanthylic acid (pH 7), one based upon keto and one upon enol tautomeric forms.

INTRODUCTION

One of the most important chemical characteristics of the nucleic acid bases is the site of attachment of dissociable hydrogen atoms: to nitrogen or oxygen, the ring or exocyclic atom, to N1 or N3. Profoundly different structural consequences, may follow from placing a proton on O or N in the constitutent bases of a macromolecule, and the energetic differences may also be large. Similarly, knowlege of the site of ionization is essential for understanding the chemistry of the bases and of polymers containing them (see, for example, references 1 and 2). In considering candidate macromolecular structures evidence of the tautomeric and ionic forms provides constraints which permit elimination of structures based upon incorrect forms. The present study examines the tautomerism and ionisation of xanthosine. We have previously published evidence that the ordered form of polyxanthylic acid in

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neutral solution has a four stranded structure with the 6-keto form and is ionised at N3 rather than N1[3]. The conclusions on tautomeric form were based on assignment of bands to predominantly carbonyl stretching vibrations and on analogy to the chemically similar 1-methyluracil molecule, for which extensive isotopic labeling data were available. A recent paper[4] has interpreted fibre diffraction data of poly (X) as supporting a two-stranded helix, a structure requiring enolic forms of xanthine residues. In view of the basic chemical difference of keto and enol forms between the proposed structures, it has become necessary to examine the tautomerism and ionisation of xanthosine in greater detail. We have used infrared spectroscopy of isoelectronic model compounds and specific isotopic substitution with ^{18}O. These methods have been discussed in the references cited above. We report our results below and show that uncharged xanthosine, indeed, does exist in diketo form in aqueous solution, and that the singly charged species is ionized at N3 and has a 6-keto structure.

MATERIALS AND METHODS

Model compounds fixed in 6-keto (1-methylxanthosine), 6-enol (6-O-methylxanthosine) and 2,6-dienol (2,6-di-O-methylxanthosine) form were prepared as described below. Numerous attempts to prepare 2-O-methylxanthosine by deamination of spongosine with adenosine deaminase (from calf intestinal mucosa, Sigma cat. No. A-9626) indicated this nucleoside is not a substrate for the enzyme, though Bergman et al. reported[5] that it was deaminated with adenosine deaminase. A more recent study has found that a series of 2-substituted adenosines are not substrates for adenosine deaminase.[6] Spongosine, like isoguanosine,[7] was found resistant to deamination by nitrous acid. Partial hydrolysis of 2,6-di-O-methylxanthosine also did not yield the desired product since the glycosidic bond cleaved more readily than the enol ether linkage in the 2-position.

1-N-Methylxanthosine: To a solution of 1-methylguanosine[8] (500 mg) and sodium nitrite (1 gm) in water (17 ml) was added dropwise 0.8 ml of 85% phosphoric acid with constant stirring at room temperature. After 20 hours the reaction mixture was diluted to 40 ml and titrated to pH 6.0 and left at room temperature for 3 days. The solution was then

applied to a charcoal column (2.5 cm X 25 cm) and eluted with 1.2 liter of methanol: concentrated ammonium hydroxide (3:1). Appropriate fractions were combined and evaporated to dryness. The colored residue was then purified by chromatography on DEAE cellulose preparative plates with isopropanol:NH_3:H_2O (7:1:2). Purified material (24 mg), m.p. 250°-264°C (d) UV λ_{max}, (H_2O) 242 nm, 265 nm; (0.1 N HCl) 237 nm, 263 nm; (0.1 N NaOH) 252 nm, 279 nm; Bartlett[9] found m.p. 250°-280°C (d) UV λ_{max} (pH 6.0) 243 nm, 265 nm; (pH 1) 239 nm, 264 nm; (pH 13) 253 nm, 280 nm.

6-O-methylxanthosine: 6-O-methylguanosine[10] (10 mg) and sodium nitrite (18 mg) were dissolved in water (0.5 ml) by warming. The solution was cooled to 25°, and glacial acetic acid (17 µl) was added. Vigorous nitrogen evolution ceased within 5 minutes and in a half hour crystals of 6-O-methylxanthosine appeared. After an hour crystals were filtered and washed with cold water, then redissolved in water and lyophilised. Pure material was chromatographically homogeneous by tlc (DMF:n-BuOH:H_2O, 1:1:1) and had a sharp m.p. 188°-190°C. UV (0.1 N HCl) λ_{max} 240 nm, 270 nm, ratio 0.75; (H_2O) 245 nm, 284 nm; ratio 0.84; (0.1 N NaOH) λ_{max} 246 nm, 283 nm; ratio 0.89. The material on hydrolysis (0.1 N HCl, 5 mins, 50°C) yielded xanthosine as shown by UV and IR spectra of hydrolysate.

2,6-di-O-methylxanthosine: 1.7 gm of 9(tri-O-acetyl-β-ribofuranosyl)-2,6-dichloropurine riboside[11] was refluxed with 8.69 m moles of freshly prepared sodium methoxide in 50 ml of absolute methanol for four hours. The solution was neutralised with HCl and evaporated. The residue was dissolved in a minimum volume of ethanol and filtered to remove salts. Ethanol extracts were evaporated and the residue was twice crystallised from ethanol/ethyl acetate. Yield 600 mg., m.p. 155-159° (softening at 120°). UV λ_{max} (pH 1.5) 235 nm, 267 nm; (H_2O) 243 nm, 263 nm; (pH 12.0) 243 nm, 263 nm. Schaeffer and Thomas reported[12] m.p. 163°C (softening 120°), UV λ_{max} (pH 1.0) 235 nm, 267 nm; (pH 7.0) 243 nm, 263 nm; (pH 13.0) 244 nm, 264 nm.

Xanthosine-6-^{18}O: Guanosine-6-^{18}O was prepared as described.[13] A solution of labeled guanosine (15 mg) in 0.5 ml water was then deaminated with $NaNO_2$ (20 mg) and 85% phosphoric acid (20 µl) at room tempera-

ture. After a half hour, the solution was diluted to 1 ml and left at room temperature for two hours. Subsequent tlc and UV spectra showed essentially complete conversion to xanthosine. The reaction mixture was neutralised, lyophilised, and used directly for IR spectra.

Xanthosine-5'-phosphate-2-^{18}O: GMP (5 mg) was deaminated with $NaNO_2$ (10 mg) and 85% phosphoric acid (10 µl) in 300 µl of $D_2{}^{18}O$ (97.7% ^{18}O). After 15 min the reaction mixture was diluted with a further 300 µl of $D_2{}^{18}O$ and left for an hour at 25° in a stoppered vial. Tlc and UV analysis showed complete conversion to xanthosine.

1-Methylxanthosine-2-^{18}O: 1-methylguanosine (6 mg) was deaminated with $NaNO_2$ (10 mg) and 85% phosphoric acid (10 µl) in 300 µl of $D_2{}^{18}O$ (97.7% ^{18}O). The reaction mixture was left at room temperature for 40 hours before it was titrated with a minimum volume of alkali (5 µl) to pH 5.5 and left at room temperature for three days. Products were then separated on DEAE cellulose plates with isopropanol:NH_3:H_2O (7:1:2). A minor band having the same UV spectrum as that of 1-N-methylxanthosine was cut out and extracted with water. An infrared spectrum of the material revealed the presence of a non-UV-absorbing contaminant, which was removed by further chromatography on pre-washed paper using the same solvent system. The appropriate band was eluted, lyophilised and used for IR spectra.

Ultraviolet spectra were measured on a Cary 118 spectrophotometer. Infrared spectra were recorded with a Perkin Elmer 580B spectrophotometer in D_2O solutions using CaF_2 cells.[2] UV and IR spectral data were acquired by a dedicated DEC 11/03 computer using the LDACS system developed by the Division of Computer Research and Technology of NIH.[14] pH measurements were made with a Radiometer pH meter-26 at room temperature.

RESULTS AND DISCUSSION

Spectrophotometric titration of 1-methylxanthosine and 6-O-methylxanthosine are shown in Fig. 1. Both compounds have only one ionisable proton at N3 and show a pK of 5.85 for 1-methylxanthosine and 6.15 for 6-O-methylxanthosine. The close similarity of these values to

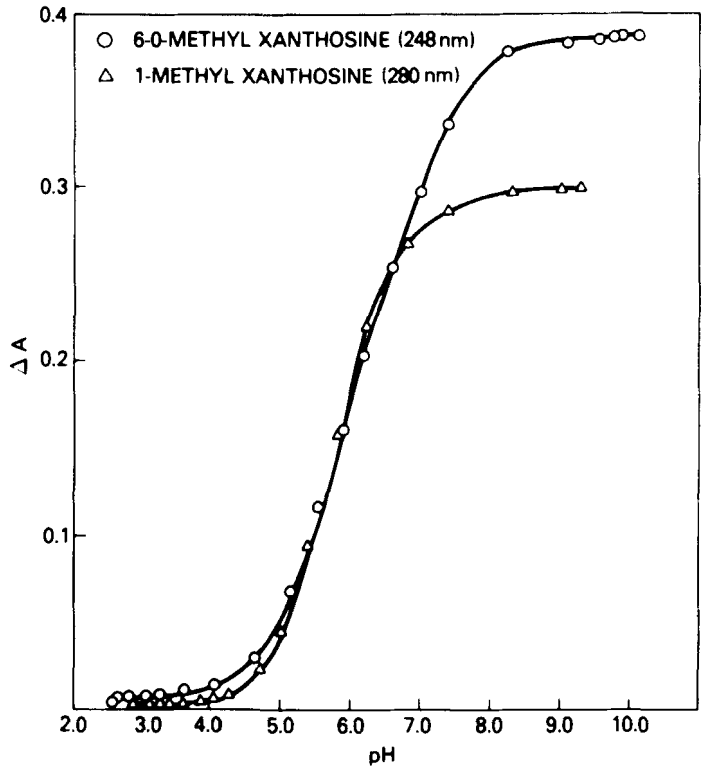


FIG. 1: Spectrophotometric titration of 6-O-methylxanthosine (O) and 1-N-methylxanthosine (Δ). The N3 proton is dissociated in both molecules.

the first pK of 5.7 for xanthosine and XMP as well as for poly X provides strong evidence that first ionisation of xanthosine is due to loss of proton from N3. A similar conclusion was drawn in an earlier UV spectroscopic study on model methylated xanthines by Cavilleri et al.[15] though these workers did not observe 1,9-dimethylxanthine and could not provide direct evidence for the diketo tautomeric form of xanthosine. UV spectra of 1-methylxanthosine (a 6-keto model) and XMP at different pH's are shown in Fig. 2. Identity of two sets of spectra demonstrates that these compounds are isoelectronic and that the same ionization process is occurring in both. It follows that in xanthosine the 6-oxygen atom must be in the keto form and that ionisation is due to the N3H proton. The spectrum of isocaffeine[16] (1,3,9-trimethylxanthine), shown in inset, resembles closely spectra of 1-methylxanthosine and XMP at acid pH, further supporting the 2,6-diketo form for these unionised molecules.

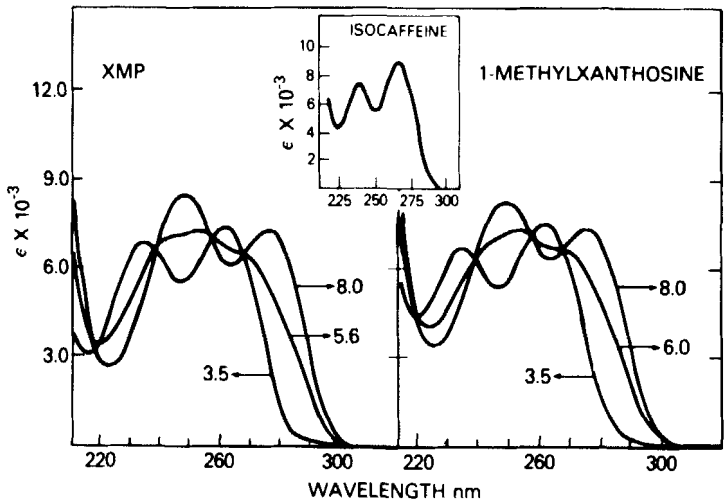
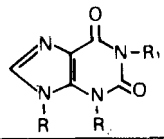
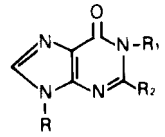
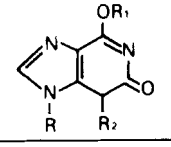
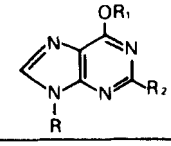


FIG. 2: UV spectra of XMP and 1-N-methylxanthosine. pH values are chosen to keep the compounds in the unionised (pH 3.5), half-ionised (pH 5.6 and 6.0), and monoionised (pH 8.0) state. Inset is a typical spectrum of 1,3,9-trimethylxanthine (isocaffeine) adapted from Gulland et al., (1934).

Infrared spectra: It is advantageous to be able to make reliable vibrational assignments of the infrared bands in the region of interest, and iostopic ^{18}O substitution is employed in a later section for this purpose. It is not essential, however, to have such information for tautomeric assignments, provided models are available for both tautomers in question and provided the spectrum of the nucleoside in the double bond region closely resmebles that of one model and is clearly different from the other. In such cases one can reach a reliable conclusion on an empirical basis even in the absence of vibrational band assignments.[1,2] We have used this approach to study xanthosine, which has two dissociable protons (at N1 and N3) and consequently possess several tautomeric possibilities, such as, 2,6-dienol, 2-keto-6-enol, 6-keto-2-enol, and 2,6-diketo forms. We prepared suitable model compounds for these forms and report their infrared spectra in Table 1 and Fig. 3. A close correspondence within each group of isoelectronic structures in the table is obvious as are the sharp contrasts between groups. We discuss below our relevant conclusions.

2,6-Di-O-methylxanthosine, model for the 2,6 dienol form, shows four major bands at 1612,1603,1516 and 1487 cm^{-1} corresponding to the expected

TABLE 1: OBSERVED INFRARED FREQUENCIES IN WAVE NUMBERS

			
R_1, R_2 = H, H (2, 6-diketo) 1689, 1659, ~ 1605 1554, 1478	R_1, R_2 = H, O^- (6 keto-2 enolate) 1648, ~ 1568, 1557 1529, 1466	R_1, R_2 = Me, H (2 keto-6 enol) 1633, 1612 (sh), 1591, 1570, 1488, 1468	R_1, R_2 = Me, O^- (2 enolate-6 enol) 1614, 1580, 1520 1472
R_1, R_2 = Me, H (2,6-diketo) 1701, 1647, ~ 1556	R_1, R_2 = Me, O^- (6 keto-2 enolate) 1652, ~ 1584, 1566 1554, 1525		R_1, R_2 = Me, OMe. (2, 6-dienol) 1612, 1603, 1516 1487, 1472
	R_1, R_2 = H, NH_2 1665, 1580, ~ 1568 1540		R_1, R_2 = Me, NH_2 1612, 1597, 1522 1481

four fundamental modes. 6-O-Methylguanosine and the enolate anion of 6-O-methylxanthosine, having similar electronic structures, also show four major bands in this region at similar frequencies (Fig. 3, Table 1). None of these compounds of this group, however, has any band above 1614 cm^{-1}, while xanthosine, in the unionised state, has two strong bands above 1650 cm^{-1}, which we assign to carbonyl stretching vibrations (see below). This contrast is sufficient to exclude the 2,6-dienol form as a possible tautomeric form of xanthosine and strongly supports the diketo form.

The infrared spectrum of 6-O-methylxanthosine is quite different from the spectra of the 2,6-dienolic molecules discussed above and suggests its structure is rather 2-keto-6-enol. The highest frequency band at 1633 cm^{-1} presumably corresponds to the 2-carbonyl vibration, extensively mixed with other ring modes, since the band disappears on ionisation of N3H at pH 8.4 (Fig. 3). Absence of any band above 1633 cm^{-1} in 6-O-methyl-xanthosine is in sharp contrast to the presence of two strong bands above 1650 cm^{-1} in the spectrum of uncharged xanthosine. Hence the 2-keto-6-enol tautomer in the case of xanthosine can be ruled out.

Three of the models of the 6-keto-2-enol form are listed in Table 1. As shown in the Table, all the compounds in this group have only one band

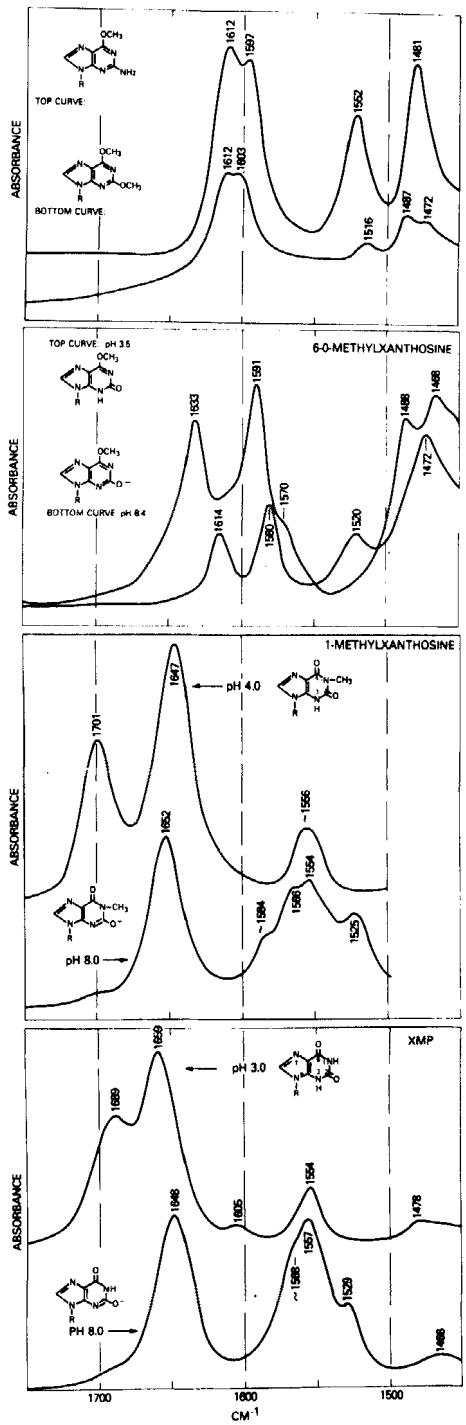


FIG. 3: Infrared spectra of tautomeric model compounds.

in the 1600-1700 cm^{-1} region whereas xanthosine or 1-methylxanthosine have two strong bands above 1630 cm^{-1} and clearly fall in a different group. One of these two bands,in both xanthosine and in 1-methyl-xanthosine, vanishes at alkaline pH, indicating that the 2-oxygen atom exists as carbonyl rather than hydroxyl function. Titration of the N3 proton converts the purine to enolate anion, whereby the 2-oxygen loses its carbonyl character and corresponding carbonyl stretching vibration.

1-Methylxanthosine could in principle have either the 2,6-diketo or the 6-keto-2-enol form. The latter, however, may be excluded by the presence of two strong, high frequency bands (Fig. 1, Table 1) shown in the following section to be carbonyl vibrations. The same conclusion, as noted above, applies to xanthosine itself in its uncharged form. The very close similarity of spectra XMP and 1-methylxanthosine is clear in Fig. 3. The site of ionization in 1-methylxanthosine and xanthosine is N3 (see above), and the spectroscopic consequence of ionization is loss of one of the two carbonyl bands.

Isotopic labeling: A significant frequency shift observed in a vibrational band when ^{18}O is substituted at a specific position in the molecule indicates that the band corresponds to a normal mode involving motion of oxygen in that position. In highly conjugated heteroaromatic molecules such as the nucleic acid bases the double bond vibrations are extensively mixed, and ^{18}O substitution may result in shifts of several bands, though usually one band has a larger shift than the others, indicating a larger contribution to this band of oxygen motion. Such isotopic labeling with ^{18}O has thus been used to assign carbonyl vibrations in other nucleic acid bases[1,13,17]. We have labeled the 2-position of XMP, the 6-position of xanthosine, and the 2-position of 1-methylxanthosine with ^{18}O. Infrared spectra of these labeled compounds are shown in Fig. 4. The spectrum of xanthosine-6-^{18}O, at pH 4.0, shows two strong bands at 1680 and at 1650 cm^{-1}. Corresponding bands in unlabeled material are at 1689 and at 1659 cm^{-1}, which we assigned earlier to predominantly C2=O and C6=O carbonyl vibrations respectively. We find that ^{18}O labeling at the 6-oxygen atom causes a frequency shift of 9 cm^{-1} in both these bands, thus providing an interesting example of strong coupling between two carbonyl vibrations. At pH 8.0, when C2 oxygen is converted to the enolate anion, we see a single band at 1638

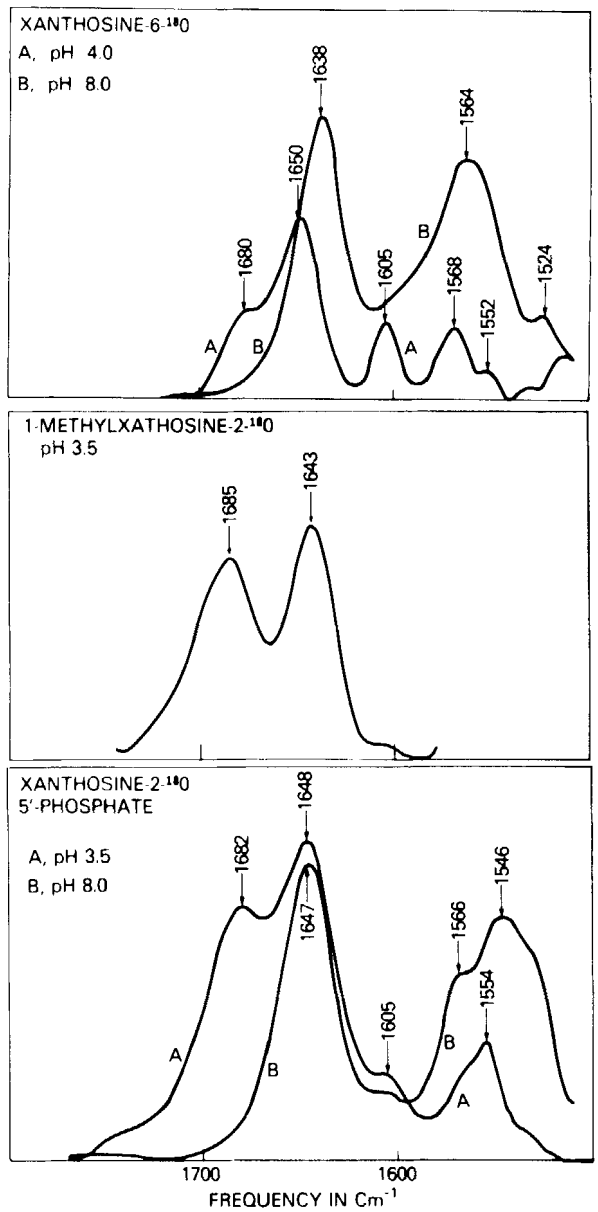


FIG. 4: Infrared spectra of ^{18}O derivatives.

cm^{-1} ($\Delta\nu$ 10 cm^{-1}), which is assigned to a mode with major C6 carbonyl stretching character. A similar observation is made with XMP-2-^{18}O. At pH 3.5, in unionised form, two carbonyl bands are shifted to 1682 ($\Delta\nu$ 7 cm^{-1}) and 1648 ($\Delta\nu$ 11 cm^{-1}) cm^{-1}, showing again very extensive mixing of the two carbonyl vibrations. All other bands in this region

remain essentially unaltered. The total shift in the two bands, as with xanthosine-6-^{18}O, is again 18 cm^{-1}. In the monoionized molecule of xanthosine-2-^{18}O a single band is observed at 1647 cm^{-1}. This is assigned to the C6 carbonyl stretch since it is shifted by 6-^{18}O substitution but remains essentially unaltered by ^{18}O labeling at the 2-position.

The situation is simpler in case of 1-methylxanthosine. This compound with ^{18}O at the 2-position, at pH 4.0, shows two carbonyl bands at 1685 and at 1643 cm^{-1}. The former band is greatly shifted ($\Delta\nu$ 16 cm^{-1}) from its usual value of 1701 cm^{-1} while the other band is shifted only slightly ($\Delta\nu$ 4 cm^{-1}). Here, therefore, the high frequency band is predominantly C2 carbonyl and lower frequency band is predominantly C6 carbonyl. Apparently extensive mixing between two carbonyl vibrations observed in xanthosine itself is removed by placing a methyl group at N1 between the oxygens. At pH 8.0 only the C6 carbonyl remains and the corresponding absorption observed at 1652 cm^{-1} (not shown) has the same frequency as that in the spectrum of the anion of unlabeled material. It is thus clear that the two high frequency bands are carbonyl bands with strong coupling of the C=O motions in xanthosine. This mixing is largely uncoupled by the Me group in 1-methylxanthosine, but both high frequency bands remain carbonyl stretching vibrations in this model compound.

Conclusion: In view of the results discussed above we conclude that XMP, or xanthosine, must exist very predominantly in the diketo tautomeric form in aqueous solution. The close similarity of the infrared spectra of poly $(X)^3$ to those of monomers and model compounds presented here shows that poly(X) has the diketo structure below pH ~5 and the 6-keto-2-enolate anion structure at neutral and slightly basic pH.

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