

Review

Xanthine, xanthosine and its nucleotides: solution structures of neutral and ionic forms, and relevance to substrate properties in various enzyme systems and metabolic pathways[⊛]

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The 6-oxopurine xanthine (Xan, neutral form 2,6-diketopurine) differs from the corresponding 6-oxopurines guanine (Gua) and hypoxanthine (Hyp) in that, at physiological pH, it consists of a \approx 1:1 equilibrium mixture of the neutral and monoanionic forms, the latter due to ionization of N(3)-H, in striking contrast to dissociation of the N(1)-H in both Gua and Hyp at higher pH. In xanthosine (Xao) and its nucleotides the xanthine ring is predominantly, or exclusively, a similar monoanion at physiological pH. The foregoing has, somewhat surprisingly, been widely overlooked in studies on the properties of these compounds in various enzyme systems and metabolic pathways, including, amongst others, xanthine oxidase, purine phosphoribosyltransferases, IMP dehydrogenases, purine nucleoside phosphorylases, nucleoside hydrolases, the enzymes involved in the biosynthesis of caffeine, the development of xanthine nucleotide-directed G proteins, the pharmacological properties of alkyl-xanthines. We here review the acid/base properties of xanthine, its nucleosides and

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Abbreviations: APRTase, adenine phosphoribosyltransferase; DHLP, dihydrolipoic acid; dOxo, 2'-deoxyoxanosine; Et, ethyl; HGPRase, hypoxanthine-guanine phosphoribosyltransferase; Hyp, hypoxanthine; Me, methyl; Me₂SO, dimethylsulphoxide; NTP, nucleoside-5'-triphosphate; Oxa, oxanine; Oxo, oxanosine; PNP, purine nucleoside phosphorylase; PRPP, α -D-5-phosphoribosyl-1-pyrophosphate; PRTase, purine phosphoribosyltransferase; SAR, structure-activity relationship; Xan, xanthine; Xao, xanthosine; XMP, xanthosine-5'-phosphate; XTP, xanthosine-5'-triphosphate.

nucleotides, their N-alkyl derivatives and other analogues, and their relevance to studies on the foregoing. Included also is a survey of the pH-dependent helical forms of polyxanthylic acid, poly(X), its ability to form helical complexes with a broad range of other synthetic homopolynucleotides, the base pairing properties of xanthine in synthetic oligonucleotides, and in damaged DNA, as well as enzymes involved in circumventing the existence of xanthine in natural DNA.

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1. INTRODUCTION

The 6-oxopurine, xanthine (Xan, 2,6-dioxopurine), and its nucleosides and nucleotides are involved in a variety of intracellular metabolic pathways as substrates and/or intermediates of numerous enzymes or enzyme systems, e.g. Xan, a substrate of both xanthine oxidase (XO) and xanthine dehydrogenase, is an intermediate in the formation, from Hyp, of urate, the end product of purine nucleotide catabolism. The initial methyl acceptor for caffeine biosynthesis in tea and coffee plants, a subject most recently reviewed, amongst others, by Ashihara and Suzuki (2004), is xanthosine (Xao) and/or xanthosine-5-phosphate (XMP). XMP is an intermediate in the formation of GMP from IMP by IMP dehydrogenase. XTP is as efficient a phosphate donor as ATP for human deoxycytidine kinase (Datta *et al.*, 1989). In purine salvage pathways, some purine phosphoribosyltransferases (PRTase) will accept xanthine, in several instances with high selectivity relative to other purines (Craig & Eakin, 2000; el Kouni, 2003). Although XTP and dXTP are moderate to good *in vitro* substrates for some polymerases, Nature has excluded Xan as a constituent of RNA and DNA for reasons which will become clear below.

The foregoing examples are far from exhaustive. What is puzzling, and even somewhat disconcerting, is that, with a few rare exceptions, the structures of the monoanionic species of Xan, its nucleosides and nucleotides, and some of their biologically important N-methyl counterparts, all highly relevant to their properties *in vivo*, have been widely overlooked or simply ignored. We herein survey these properties, relative to those of the corresponding 6-oxopurines Gua

and Hyp, and their 9-substituted analogues, which include Guo and Ino, followed by an examination of their behaviour in synthetic polynucleotides and natural nucleic acids, their substrate properties in various enzyme systems, and their involvement in some metabolic pathways.

2. NEUTRAL AND IONIC FORMS OF GUANINE, HYPOXANTHINE AND THEIR NUCLEOSIDES

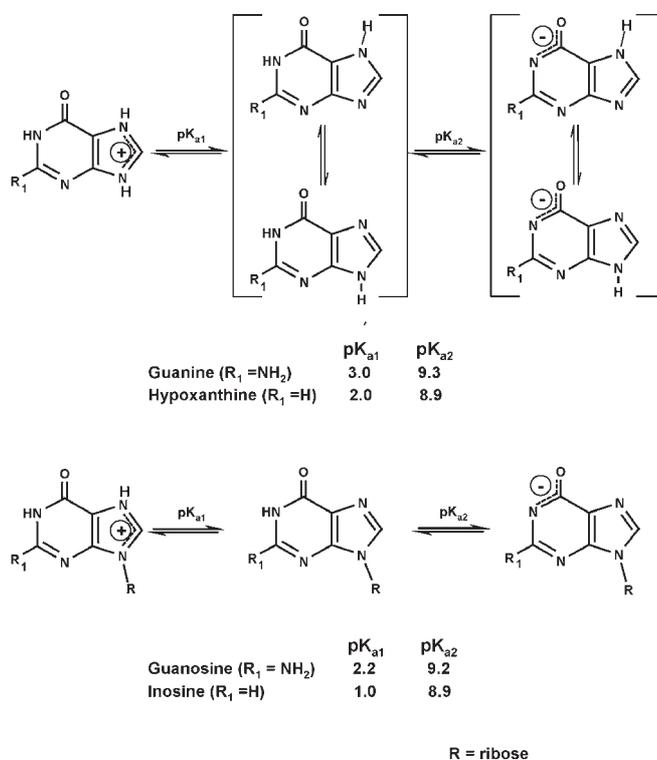
As will be seen from what follows, it is pertinent to first recall the structures of the cationic, neutral and ionic species of the 6-oxopurines Gua and Hyp, and of their corresponding nucleosides Guo and Ino, depicted in Scheme 1.

The structures of all the foregoing, as shown, have been well documented (Shugar & Psoda, 1990). In particular, it should be

noted, from the pK_a values for protonation and dissociation, that all of these exist predominantly as the neutral species at physiological pH. The same applies to the corresponding nucleotides. Furthermore, both the neutral and monoanionic species of the purine bases Gua and Hyp additionally exhibit N(7)-H N(9)-H prototropic tautomerism (see Shugar & Psoda, 1990).

3. NEUTRAL AND IONIC FORMS OF XANTHINE AND XANTHOSINE

The situation is quite different for Xan and its nucleosides and nucleotides. There is general agreement that the neutral forms of Xan and Xao in solution are 2,6-diketo, as shown in Scheme 2 (Shugar & Psoda, 1990), supported by the infrared spectrum of ^{18}O -labeled Xao and 1-methyl-Xao in solution earlier reported by Roy & Miles (1983), and by



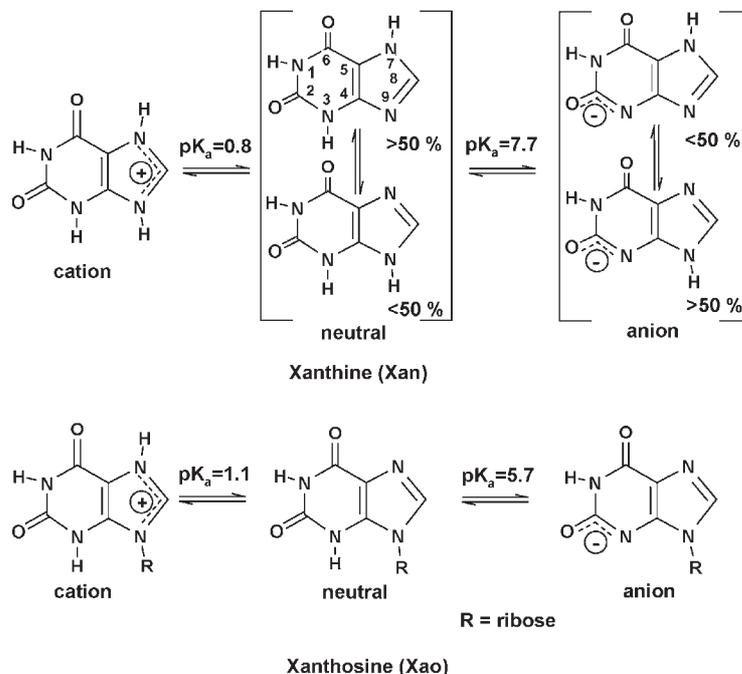
Scheme 1. Structures of the cationic, neutral and monoanionic forms of guanine (Gua) and hypoxanthine (Hyp), and guanosine (Guo) and inosine (Ino).

Note the 6-keto structures at physiological pH, and 6-enolate structures of the monoanions. Note also the N(7)-H N(9)-H prototropic tautomerism of the neutral and monoanionic forms.

multi-dimensional NMR spectroscopy of Xao in Me₂SO and in aqueous medium (Poznanski *et al.*, 2003).

Additional evidence for the 2,6-diketo form of neutral Xao was provided by Roy & Miles

the basis of the displacement of the C(8)-H signals in the NMR spectra of Xan and 1-methyl-Xan, that the monoanions of both of these may be a mixture of two tautomeric species with ionization of both the N(3)-H and



Scheme 2. Structures of the neutral and monoanionic forms of xanthine (Xan) and xanthosine (Xao).

Note that dissociation of the N(3)-H affects the prototropic tautomeric equilibrium N(9)-H ↔ N(7)-H, and that the monoanions of Xan and Xao are still 6-oxopurines, like the neutral forms of Gua and Guo, and Hyp and Ino (see Scheme 1).

(1983) from the virtual identity of the pH-dependent UV spectra of 1-methyl-Xao and 1,9-dimethyl-Xan, and the close resemblance of the spectrum of isocaffeine, 1,3,9-trimethyl-Xan, with those of the neutral forms of 1-methyl-Xao and XMP. The structure of Xao in the solid state is 2,6-diketo (Lesyng *et al.*, 1984), as is also N(1)-allyl-Xao, albeit the latter is in the *syn* conformation (Liaw *et al.*, 1992).

There are, however, conflicting reports as regards the nature of the monoanionic species, e.g. Albert & Brown (1954) reported a pK_a of 7.3 for Xan, postulated to be due to dissociation of the N(1)-H, as for Gua and Hyp (see Scheme 1). Christensen *et al.* (1970) implicitly assumed that the monoanions of both Xan and Xao are due to dissociation of the N(1)-H. Lichtenberg *et al.* (1971) proposed, on

N(7)-H. More recently, Sau *et al.* (2000) ascribed formation of the monoanion of 1-methyl-Xan, with a pK_a of 7.7, to dissociation of the N(9)-H.

We have compiled from literature sources the most probable pK_a values for monoanion (and dianion) formation in Xan, Xao, XMP, and related N-methyl and other derivatives, which are relevant to establishment of the nature of their monoanions, and hence of the relative substrate properties of the neutral and monoanionic species in various enzyme systems and metabolic pathways. These, some of which we have checked by spectrophotometric titration, are compiled in Table 1.

It should further be noted that, when reference is made throughout the text to the monoanion of a xanthosine nucleotide, e.g.

XMP, this refers to the monoanion of the xanthine moiety, and not to the phosphate group.

It was initially proposed by Cavalieri *et al.* (1954), on the basis of the pH-dependent UV absorption spectra, and pK_a values for monoanion formation, of a number of N-methyl de-

assigned to the N(7)-H by Lichtenberg *et al.* (1971). Dissociation of the N(3)-H in Xan and Xao has been unequivocally confirmed in several laboratories, e.g. by Roy and Miles (1983) with the aid of infrared spectroscopy, and the use of various synthetic analogues, including ^{18}O -labeled derivatives to identify the pres-

Table 1. Dissociation constants, pK_{a1} for monoanion formation, and pK_{a2} for dianion formation, and sites of dissociation, of xanthine, methylated xanthines, and related compounds^a

Compound	pK_{a1}	Site	pK_{a2}	Site
Xanthine	7.7	N(3)	11.65	N(7,9)
1-methyl-	7.7	N(3)	12.1	N(7,9)
3-methyl-	8.3	N(1)	11.6	N(7,9)
7-methyl-	8.35	N(3)	13 ^b	N(1)
9-methyl-	6.3	N(3)	10.5	N(1)
1,3-dimethyl-	8.8	N(7,9)	-	-
1,7-dimethyl-	8.6	N(3)	-	-
1,9-dimethyl-	6.3	N(3)	-	-
3,7-dimethyl-	9.9	N(1)	-	-
3,9-dimethyl-	10.5	N(1)	-	-
Xanthosine	5.7	N(3)	13 ^c	-
1-methyl-	5.85	N(3)	nd	-
O ⁶ -methyl-	6.15	N(3)	nd	-
5'-phosphate	5.7	N(3)	nd	-

^aRounded-out values (± 0.1) compiled from various sources, including Ogston (1935), Cavalieri *et al.* (1954), Lichtenberg *et al.* (1971), Bergmann & Dickstein (1955), Pfeleiderer & Nübel (1961), Christensen *et al.* (1970), Roy & Miles (1983), many of them checked by spectrophotometric titration in our laboratories. ^bThis compound is reported to decompose at highly alkaline pH (Lichtenberg *et al.*, 1971). ^cIt should be noted that, unlike the corresponding 9-methylxanthine, the ribose *cis*-hydroxyls of the ribosides also dissociate in this pH range, with $pK_a \approx 12.3$ (Remin *et al.*, 1976); nd, not determined.

derivatives of Xan, that monoanion formation in both Xan and Xao is due to dissociation of the N(3)-H, as shown in Scheme 2. Support for this was forthcoming from the demonstration by Mizuno *et al.* (1969) that, in crystals of the monoanion of Xan, it is the N(3)-H which is dissociated, although the authors assumed that the monoanion is represented by the N(9)-H prototropic tautomer, as earlier suggested for the monoanion in solution (Pfeleiderer & Nübel, 1961), but subsequently

ence or absence of band frequencies of the carbonyl groups. Suzuki *et al.* (2000) demonstrated the absence of an N-H signal in the ^1H NMR spectrum of 1-methyl-Xao in slightly alkaline medium, testifying to dissociation of the N(3)-H, subsequently extended and amply confirmed by multidimensional NMR spectroscopy of Xao over a broad pH range (Poznanski *et al.*, 2003). The identity of the pK_{a1} values for Xan and 1-methyl-Xan (Table 1) further suggests that the monoanion of

the latter is also due to dissociation of the N(3)-H. The closely similar pK_{a1} values for Xao (5.7) and 1-methyl-Xao (5.85), and for O^6 -methyl-Xao (6.15), where only N(3)-H dissociation can occur, is further evidence for dissociation of the N(3)-H in Xao, as well as in XMP (Roy & Miles, 1983), further supported by the results of *ab initio* quantum mechanical calculations (Poznanski *et al.*, 2003).

In a more recent theoretical study, Rogstad *et al.* (2003) applied quantum mechanical methods, using density functional theory with the B3LYP functional and the 6-31G++G** basis set, to calculate the microscopic dissociation constants, and the resultant pK_a , for xanthine, which was evaluated as 6.9, considered in good agreement with an experimental value of 7.44, taken from the older literature. The agreement, however, is less satisfactory, bearing in mind the established pK_a for xanthine, 7.7 (Table 1).

3.1. Thioxanthines and their nucleosides and nucleotides

Some of these compounds are of considerable interest, particularly as potential antimetabolites, described in various sections, below. For example, 6-thiopurines, such as 6-thiohypoxanthine and 6-thioguanine, are converted intracellularly by phosphoribosyl-transferases (Section 13) to their corresponding 5'-monophosphates, which inhibit several vital metabolic reactions, leading to induction of remission in patients with myelocytic and acute lymphocytic leukemia. Although of proven clinical utility, ongoing efforts are devoted to development of analogues with enhanced therapeutic efficacy, including thioxanthines, recently reviewed by Elgemeie (2003) and el Kouni (2003). It is of interest that 6-thioxanthine, the most extensively studied thioxanthine, was shown to be a promising candidate for gene therapy of cancer in combination with the *Escherichia coli gpt* gene (which encodes the enzyme xanthine-guanine phosphoribosyl transferase), claimed

to be a promising alternative to the combination of the *E. coli* thymidine kinase gene with Ganciclovir (Ono *et al.*, 1997).

The structures of the neutral and mono-anionic forms of 6-thioxanthine, and its N-methyl derivatives, were long ago described by Lichtenberg *et al.* (1972), with the aid of UV and NMR spectroscopy. As expected, the neutral form of 6-thioxanthine is 6-thione-2-keto. Shortly thereafter Twanmoh *et al.* (1973), implicitly assuming that the neutral forms of 2-thio-, 6-thio- and 2,6-dithio-xanthines are 2-thione-6-keto, 6-thione-2-keto, and 2,6-dithione, respectively, employed ^1H NMR spectroscopy for assignment of the N-H signals of these compounds in Me_2SO (and in Me_2SO following addition of water), found to be consistent with the foregoing structures. In both studies, ^1H NMR spectroscopy pointed to predominance of the N(7)-H prototropic tautomer at neutral pH.

3.2. Monoanions of thioxanthines

Particularly interesting are the mono-anionic forms of these compounds, relative to those of xanthine. As in the case of xanthine, 1-methylxanthine and 9-methylxanthine, monoanion formation is due to dissociation of the N(3)-H. And, as might have been anticipated, Lichtenberg *et al.* (1972) found that all pK_a values for monoanion formation in 6-thioxanthine and its N-methyl derivatives were appreciably lower than for the corresponding xanthines (see Table 2). It follows that the monoanionic species of the thioxanthines are much more predominant at neutral pH than those of the corresponding xanthines, e.g. xanthine (pK_a 7.7, Table 1) exists at physiological pH as about a 1:1 mixture of the neutral and monoanionic forms, whereas 6-thioxanthine (pK_a about 6.2) is > 95% as the monoanion.

The pK_a of 6-thioxanthosine has, to our knowledge, not been reported. However, for 9-methyl-6-thioxanthine, its pK_a of 4.9 indicates that the pK_a for 6-thioxanthosine should

be about 5, or even lower. This follows from the fact that the pK_a of 9-methylxanthine is 6.3 as compared to 5.7 for xanthosine (see Table 1). Consequently, at physiological pH (7.4), 6-thioxanthosine (and 6-thio-XMP) must exist almost *exclusively* as the monoanions.

Insofar as we are aware, the structures of 2-thioxanthine and, to our knowledge, the hitherto unknown 2-thioxanthosine, have not

Monny, 1966; Fikus & Shugar, 1969; Torrence *et al.*, 1977).

In aqueous medium the structure of poly(X) itself is rather complex and exhibits more than one ordered form, depending on the pH of the medium, and clearly related to ionization of the N(3)-H of the xanthine residues. The most comprehensive study of its ordered structures in aqueous solution was conducted

Table 2. Dissociation constants, pK_{a1} , for monoanion formation, and pK_{a2} for dianion formation, of 6-thioxanthine and its N-methyl analogues, and proposed sites of dissociation^a

Compound	pK_{a1}	Site	pK_{a2}	Site
6-Thioxanthine	6.2 ^b (7.7) ^c	N(3)	11.4 (11.6) ^c	N(7,9)
1-methyl-	6.7 (7.7)	N(3)	11.0 (12.7)	N(7,9)
3-methyl-	7.9 (8.3)	N(7,9)	11.2 (11.6)	N(1)
7-methyl-	6.8 (8.3)	N(3)	12.1 (13.1)	N(1)
9-methyl-	4.9 (6.3)	N(3)	12.6 (10.5)	N(1)
1,3-dimethyl-	8.2 (8.8)	N(7,9)	-	-
1,7-dimethyl-	7.5 (8.6)	N(3)	-	-
1,9-dimethyl-	5.3 (6.3)	N(3)	-	-
3,7-dimethyl-	8.8 (9.9)	N(1)	-	-
3,9-dimethyl-	8.8 (10.5)	N(1)	-	-

^aData from Lichtenberg *et al.* (1972); ^bStoychev *et al.* (2002) report a value of 6.5; ^cValues in brackets are for the corresponding parent xanthines, taken from Table 1.

been reported. Spectrophotometric titration demonstrated that the pK_a of 2-thioxanthine is 5.9 (Stoychev *et al.*, 2002), so that it exists largely as the monoanion at physiological pH, but its structure remains to be established.

4. POLYXANTHYLIC ACID (poly(X))

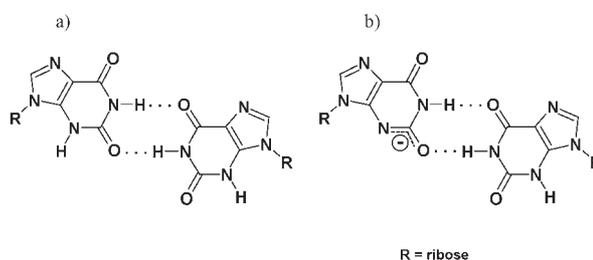
This polynucleotide, readily prepared enzymatically from XDP with the aid of polynucleotide phosphorylase, or from XTP with deoxynucleotidyl transferase, and available commercially, is of some interest because of its ability to form helical complexes with a broad range of potentially complementary polynucleotides (e.g. Michelson &

by Roy *et al.* (1979), with the aid of IR spectroscopy (to follow the C(2) and C(6) carbonyl band frequencies), and pH titration (with determination of the number of protons taken up or liberated per mole xanthine residues). This led, by a process of elimination of several hypothetical structures, to two proposed structures. One of these, in the pH range 6–9.5, was postulated to be a four-stranded helix, with bases linked by single hydrogen bonds, N(1)-H...O⁶, and the stability of which changes progressively with pH because of N(3)-H ionization, but with no major changes in helix geometry. At pH about 7, the polymer is half-titrated, with two negative charges per tetramer. At pH 8.3, titration of the N(3)-H is essentially complete, with uptake of one pro-

ton/xanthine residue, and the polymer exhibits only a single carbonyl band at 1660 cm^{-1} , identified as $\text{C}(6)=\text{O}$. This structure possesses four negative charges per tetramer, and electrostatic repulsion between monomers is screened by internal counterions. Below pH 5, a remarkably stable and quite different structure was observed, proposed to be a six-stranded helix in which both carbonyl oxygens and both N-H protons of each residue are hydrogen bonded.

Strikingly different conclusions were reached by Arnott *et al.* (1981), who succeeded in drawing fibres of the potassium salt of poly(X) at two pH values, 5.7 and 8. In both instances, the fibres exhibited good X-ray diffraction patterns, each fully consistent with its existence as an antiparallel duplex form. The fibres drawn at pH 5.7 occurred with nearest neighbour molecules spaced at 2.11 nm, an axial translation per residue of $h = 0.301\text{ nm}$, and a rotation per residue of $t = 36^\circ$. The intensity distribution of the diffraction pattern was quite close to that of A-RNA, for which $h = 0.281\text{ nm}$ and $t = 32.7^\circ$. By contrast, the fibres drawn at pH 8 exhibited a less compact, statistically disordered, crystal packing, with nearest neighbors separated by 2.35 nm, $h = 0.252\text{ nm}$ and $t = 32.7^\circ$, and an intensity distribution identical to that for A-DNA, for which $h = 0.256\text{ nm}$ and $t = 32.7^\circ$. Both duplexes exhibited the same symmetry as Watson-Crick base pairs, but with the $\text{C}(1') \cdots \text{C}(1')$ distances 0.2 nm greater. On the assumption that triple hydrogen-bonded base pairs would be highly favoured, and lead to suppression of ionization of the residues, it was concluded that the duplex formed at pH 5.7 involves pairing of the enolic form of xanthine residues. For the structure formed at pH 8, triple hydrogen-bonded base pairs were also considered to predominate, with hydrogen bonding to either a negatively charged N(3) or a neutral enolate O^2 of one base, hence only 50% of the residues in the form of monoanions. The authors did not exclude the possibility of existence of multi-

ple-stranded structures in aqueous solution. But, although the diffraction data provide compelling evidence for existence of poly(X) fibres as twin-stranded structures, the proposed existence of the enolic form of xanthine residues in base pairing is highly questionable, and quite inconsistent with the infrared data presented by Roy *et al.* (1979; 1983), demonstrating the total absence of the $\text{C}(2)=\text{O}$ in solution at alkaline pH. We propose, instead, the following base pairing patterns for the neutral and alkaline forms of duplex poly(X) fibres (Scheme 3).



Scheme 3. Proposed base pairing in the neutral and monoanionic forms of antiparallel duplex poly(X) fibres.

(a) Neutral form with non-ionized base pairs; (b) with 50% of the xanthine residues ionized, by dissociation of the N(3)-H, adapted from Arnott *et al.* (1981). But see text for details and discussion. Note that base pairing involves the xanthine $\text{C}(2)=\text{O}$ (left) and $\text{C}(6)=\text{O}$ (right) in each pair of molecules, but rotation of the xanthine rings on the left side of each pair by 180° about an axis through O^6 and $\text{N}(7)$ will lead to inverse autoassociates *via* the xanthine $\text{C}(6)=\text{O}$ and $\text{N}(1)=\text{H}$, respectively.

5. COMPLEXES OF poly(X) WITH POTENTIALLY COMPLEMENTARY POLYNUCLEOTIDES

Poly(X) has been reported to be extremely versatile in forming multi-stranded helical structures with a variety of synthetic polynucleotides (Michelson & Monny, 1966; Fikus & Shugar, 1969; Torrence *et al.*, 1977; Roy *et al.*, 1983). We limit ourselves here to complexes

with poly(U) and analogues of the latter containing 5-substituted uracil residues.

5.1. Complexes of poly(X) with poly(U) and poly(U) analogues

Formation of such complexes, initially reported for poly(X):poly(U) by Michelson and Monny (1966), was more extensively investigated by Fikus and Shugar (1969). Surprisingly, no evidence could be found for formation of a complex of poly(X) with poly(U) at pH 5.2, presumably due to the highly stable self-structure of poly(X) at acid pH. By contrast, addition of a molar equivalent of poly(U) to poly(X) at pH 7.8, where the xanthine residues of the latter are fully ionized, resulted in 20% hypochromicity at 255

nm, testifying to formation of a complex. The remarkably sharp melting profiles in the presence of various concentrations of Na^+ are displayed in Fig 1. Addition of an excess of poly(U) or poly(X) revealed the profile of the excess of one or the other superimposed on that of poly(X):poly(U). Similar profiles were exhibited by 1:1 complexes of poly(X) with poly(rT) (5-methyl-U), poly(EtU) (5-ethyl-U) and poly(FU) (5-fluoro-U), as shown in Fig. 1.

It will be noted that the melting temperature, T_m , for poly(X):poly(rT) is 12° higher than that for poly(X):poly(U), as previously observed for poly(A):poly(rT) relative to poly(A):poly(U) (Swierkowski *et al.*, 1965; Swierkowski & Shugar, 1969). And replacement of poly(X):poly(rT) by poly(X):poly(EtU) leads to a decrease of 10° in T_m , as earlier re-

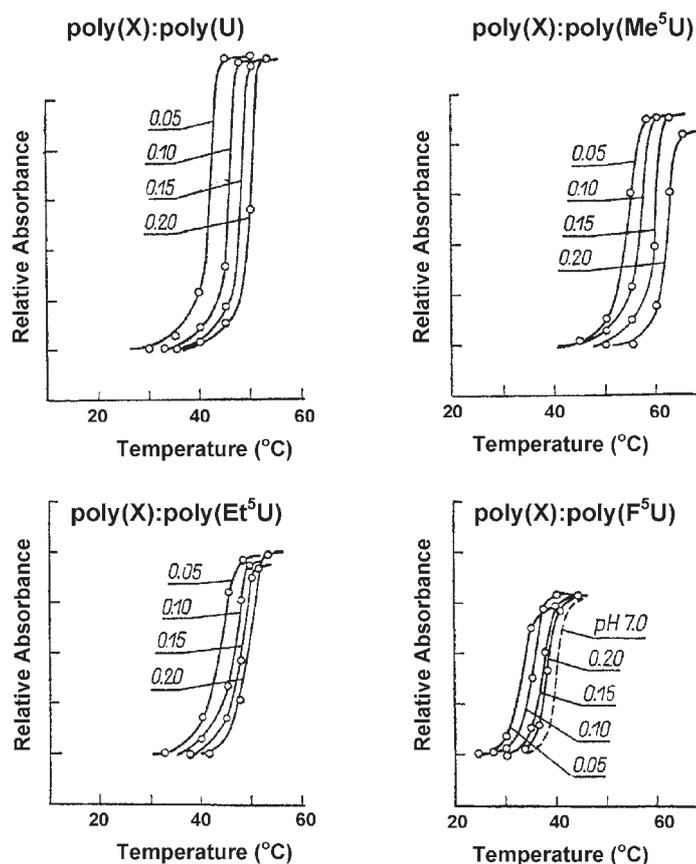
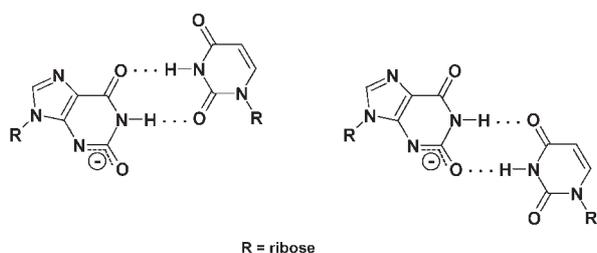


Figure 1. Melting profiles of 1:1 mixtures of poly(X) with poly(U), poly(rT) (5-methyl-U), poly(EtU) (5-ethyl-U) and poly(FU) (5-fluoro-U) in presence of various concentrations of Na^+ , in 0.01 M phosphate buffer pH 7.8.

In the case of poly(X):poly(FU), the pK_a of the FU residues is 7.8, so that half of them are ionized at this pH. Hence the profile of the complex in 0.2 M Na^+ was also run at pH 7, where only about 15% of the residues are ionized. Adapted from Fikus & Shugar (1969).

ported for poly(A):poly(EtU) relative to poly(A):poly(U) (Swierkowski & Shugar, 1969). Particularly interesting is poly(X):poly(FU), the T_m for which is about 10° lower than for poly(X):poly(U). The 5-fluorouracil residues of poly(FU), with a pK_a of 7.8, are a mixture of neutral and monoanionic forms at this pH. However, reduction of the pH to 7, where only about 15% of the FU residues are in the form of the monoanion, did not affect the shape of the melting profile, with only a small increase in the T_m (see Fig. 1). It follows that, as previously reported for poly(A):poly(FU) (Szer & Shugar, 1963), formation of the complex poly(X):poly(FU) is also accompanied by suppression of the ionization of the 5-fluorouracil residues in poly(FU).

Bearing in mind existence of the xanthine residues of poly(X) at pH 7.8 as monoanions, the mode of base pairing proposed in duplexes of poly(X) with poly(U) and its analogues is as shown in Scheme 4. Such base pairing has been observed in synthetic polynucleotides containing an isolated xanthine residue in one strand with a thymine residue in the complementary strand (see below).



Scheme 4. Two possible modes of base pairing in the 1:1 complexes of poly(X) with poly(U), and poly(U) analogues with 5-substituted uracil residues, in alkaline medium, where the xanthine residues are ionized, due to dissociation of the N(3)-H.

Rather puzzling, however, was the finding that, under the foregoing conditions, poly(X) was unable to form complexes with poly(dU) or poly(dT).

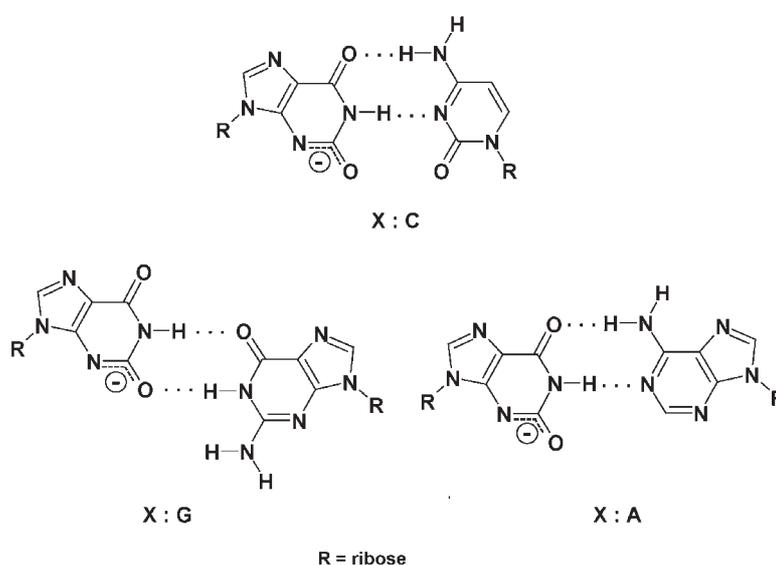
The interaction of poly(X) with poly(U) was subsequently investigated in more detail by Roy *et al.* (1983), who confirmed, as above, that the two combine in equimolar proportions, but pointed out, correctly, that this does not constitute proof that the actual combining proportion is 1:1, and not a multiple of this ratio, e.g. a four-stranded X_2U_2 , or a six-stranded X_3U_3 , complex. The X:U complex was found to exhibit a strikingly marked dependence of the melting temperature, T_m , on the nature of the metal counterion present in the solution, e.g. in the presence 0.1 M Li^+ the T_m was $33^{\circ}C$ at pH 7, whereas with 0.1 M Rb^+ the T_m was $56^{\circ}C$, a phenomenon not observed with two-stranded nucleic acid helices. An additional unusual property of the X:U complex was the increase in its stability with an increase in dissociation of the N(3)-H of the xanthine residues, attaining a plateau at pH 7 (where dissociation is complete, as shown by the total absence of the xanthine C(2)=O by infrared spectroscopy), and remaining constant beyond this point. Since ionization of the N(3)-H increases the charge density on the poly(X) chains, the added electrostatic repulsion should lead to a decrease, and not the observed increase, in T_m , indeed a strong, but not fully convincing, argument against a twin-stranded helix. This effect of various metal counterions is clearly deserving of further study of complexes of poly(X) with other polynucleotides. On the other hand, the mode of base pairing in the four-stranded helix proposed by Roy *et al.* (1983), involving single hydrogen bonds between bases, including the xanthine enolates, does not account for the specificity of base pairing proposed for a duplex (Scheme 4), also found to occur in synthetic oligonucleotides and nucleic acids containing xanthine residues, as described below. Further clarification of this problem is clearly predicated on the unequivocal establishment of the multiplicity of the poly(X):poly(U) complex, e.g. by sedimentation or low-angle X-ray scattering in solution.

6. BASE PAIRING OF XANTHINE IN DNA DUPLEXES

The properties of DNA duplexes containing a single xanthine residue in one strand were examined by Eritja *et al.* (1986), bearing in mind that xanthine may appear in DNA as a product of spontaneous deamination of guanine, or reaction with reactive nitrogen species such as NO or nitrous acid (see review by Wilson *et al.*, 2003). The duplexes were prepared by exploiting the ability of *Drosophila*

the base pairing proposed earlier for duplexes of poly(X) with poly(U) and some poly(U) analogues (Scheme 4). Proposed modes of pairing for X:A, X:G and X:C are shown in Scheme 5, the legend to which comments on the proposed pairing of X:C.

Kamiya *et al.* (1992) extended the findings of Eritja *et al.* (1986) by profiting from the fact that any point mutation at the second position of codon 12 of the c-Ha-ras gene leads to focus forming activation of the gene. They therefore introduced dXao into this position



Scheme 5. Proposed base pairing, in a synthetic duplex oligonucleotide, of a Xan residue in one strand with a cytosine, guanine or adenine in the complementary strand (Eritja *et al.*, 1986).

But note that for the proposed X:C pair, with the xanthine residue in the monoanionic form, due to dissociation of the N(3)-H, there will be electrostatic repulsion between the xanthine C(2)-O⁻ and the cytosine C(2)=O. However, rotation of the cytosine moiety by 180° about an axis through the cytosine C(6) and N(3) will lead to reversed Watson-Crick pairing, with an ionic hydrogen bond between the C(2)-O⁻ and the cytosine C(4)-NH₂.

DNA pol- α (devoid of proof-reading 3'-exonuclease activity) to incorporate natural dNTP substrates opposite a xanthine residue at a specific site in a nonadecanucleotide template. All four natural bases were found to undergo incorporation opposite xanthine, the relative rates being T > C >> A > G, but not correlated with the relative thermodynamic stabilities of the resulting duplexes, from measurements of the T_m of the melting profiles (X:T > X:G > X:A and X:C). Two proposed possible modes of pairing of X:T are similar to

in the synthetic gene, followed by transfection of the gene into NIH3T3 cells. The dX-containing gene exhibited high transforming activity, and an almost exclusive mutation of G to A by the base analogue, indicating that dTMP was incorporated at the site opposite the dX residue. The overall result, following generation of dXao by deamination of dGuo, was a mutation with a G \rightarrow A transition, which may be directly involved in focus-forming activation of the *ras* gene.

According to the proposal of Eritja *et al.* (1986) and Kamiya *et al.* (1992), the X:C base pair is formally similar to a Watson–Crick pair (Scheme 5), whereas the X:T pair is a wobble base pair, which may distort the duplex, hence not suitable for polymerization. However, because of destabilization of the X:C pair (by electrostatic repulsion between the Xan C(2)-O⁻ and the cytosine C(2)=O, see Scheme 5), dTMP, as mentioned above, exhibited a higher rate of incorporation than dCMP opposite dXMP *in vitro* (Eritja *et al.*, 1986), and was also readily incorporated in the mammalian system (Kamiya *et al.*, 1992).

One of several comprehensive studies on the possible modes of base pairing of base analogues, including xanthine, in a synthetic DNA duplex is that of Benner and coworkers (Geyer *et al.*, 2003), based on measurements of the T_m of the melting profiles for the following duplexes,



where N_a and N_b , each located in the interior of the helix to avoid end effects, represent the position of the appropriate base analogue. Since paired bases may have perturbed pK_a values, e.g. protonation of imino nitrogens at slightly acid pH, leading to modifications of H-bonding, T_m values were followed at both pH 7.9 and 5.4. This choice of pH values also proved appropriate for following the role of the monoanion of the xanthine ring in XMP, with a pK_a of 5.7, hence fully ionized at pH 7.9. Although not shown, all duplexes with different N s were reported to display smooth single-melting transitions, consistent with two-state melting. It would have been useful if the authors had included the half-breadths (ΔT_m) of the melting profiles, to permit better assessment of the transitions.

With duplexes containing natural bases, or various base analogues, paired with Xan in one of the strands, the T_m values were found

to increase by 2–5°C on going from pH 7.9 to 5.4, consistent with the assumption that a negative charge on Xan decreases the stability of the duplex at alkaline pH. This was reinforced by the finding that replacement of Xao by a dXao analogue, 7-deaza-2'-deoxyxanthosine (erroneously referred to as dX), which has a pK_a of 7.2 (Seela *et al.*, 1985), so that more of the neutral form is present at pH 7.9, led to increases in T_m values of 3–5°C. It should, however, be noted that, unlike xanthosine, it is not known whether ionization of 7-deazaxanthosine is due to dissociation of the N(1)-H or N(3)-H, which may well affect conclusions regarding its mode of base pairing.

Bearing in mind the ability of poly(X) to form helical structures in solution, and anti-parallel duplexes when drawn as fibres (Section 4), it is of interest that, in line with results for the foregoing, an X:X pair was 4° more stable at acid pH.

7. XANTHINE IN NATURAL NUCLEIC ACIDS

Xanthine residues in synthetic polynucleotides have been shown to be capable of base pairing with all four natural bases of DNA (see Section 6). Furthermore, XTP and dXTP are moderate to good substrates for some polymerases, examples of which are cited below. An extensive list of references to recognition of XTP and dXTP, both as substrates and as components of templates, by various RNA and DNA polymerases, will be found in a report by Rogstad *et al.* (2003). Nonetheless, xanthine is not a normal constituent of nucleic acids. One compelling reason for this is the existence of both XTP and dXTP at physiological pH with the Xan base predominantly in the form of a monoanion, and in equilibrium with a minor proportion of the neutral form, hence likely to affect fidelity of replication and transcription.

It has also been widely assumed that the glycosidic bond of dXao is inherently unstable, leading to rapid depurination, even at neutral pH (Lindahl, 1993; Suzuki *et al.*, 1997). This was recently questioned by two groups. Wuenschell *et al.* (2003) examined the rate of depurination of a Xan residue incorporated at position 10 in a single-stranded 34mer oligodeoxynucleotide at acid and neutral pH. As expected, depurination was very rapid at acid pH. But at pH 7 the rate of depurination was virtually identical with that for a guanine residue located at the same position. Concurrently, and independently, Vongchampa *et al.* (2003), reported that dXao itself has a half-life of about 50 days at pH >7 and 37°C. Its stability is further substantially enhanced when it is a constituent of single-stranded and duplex oligonucleotides. At pH 7 and 37°C, an incorporated dXao residue in a 30mer oligodeoxynucleotide was estimated to have a half-life of 2 years. Additional evidence for the stability of Xan residues was provided by the melting profile of a 30mer duplex oligodeoxynucleotide containing a single dXao residue in one strand. When paired with dCyd in the complementary strand, the melting profile exhibited a T_m of 73°C. Replacement of the dXao residue by dIno or dGuo gave T_m values of 74°C and 78°C, respectively. The small (5°C) decrease in duplex stability on changing from a G:C base pair to X:C was considered due to the negative charge on Xan residues (Vongchampa *et al.*, 2003). The authors did not follow the mobility of the oligodeoxynucleotides in a gel, which would have furnished additional evidence for their conclusions. It had, in fact, much earlier been demonstrated by Piccirilli *et al.* (1990) that, following incorporation of a Xan residue in a synthetic primer/template by the Klenow fragment of DNA polymerase I at physiological pH, the resulting product migrated faster on a gel than the analogous products containing Gua or Ade in place of Xan. A similar finding was subsequently reported by Suzuki *et al.* (1998).

There is, on the other hand, ample evidence that oxidative deamination of bases in DNA, including spontaneous deamination, which leads to conversion of dCyd to dUrd, and dAdo to dIno, also results in the formation of dXao from dGuo (Suzuki *et al.*, 1997). These deamination products are also produced by reactive nitrogen species generated by ionizing radiations, as well as endogenous genotoxins, especially nitric oxide (NO), which reacts readily with O₂ to produce nitrous anhydride, N₂O₃, a potent nitrosating agent which has been proposed to deaminate aromatic amines *via* an aryl diazonium ion intermediate (Caulfield *et al.*, 1998). Whereas dGuo is much less susceptible to hydrolytic deamination than dCyd and dAdo, deamination of Gua to Xan by N₂O₃ was reported to proceed at twice the rate of that for Cyt and Ade. On exposure of human lymphoblastoid TK6 cells to NO, the resulting level of Xan residues in DNA was found to be 40-fold higher than that from untreated control cells (Nguyen *et al.*, 1992).

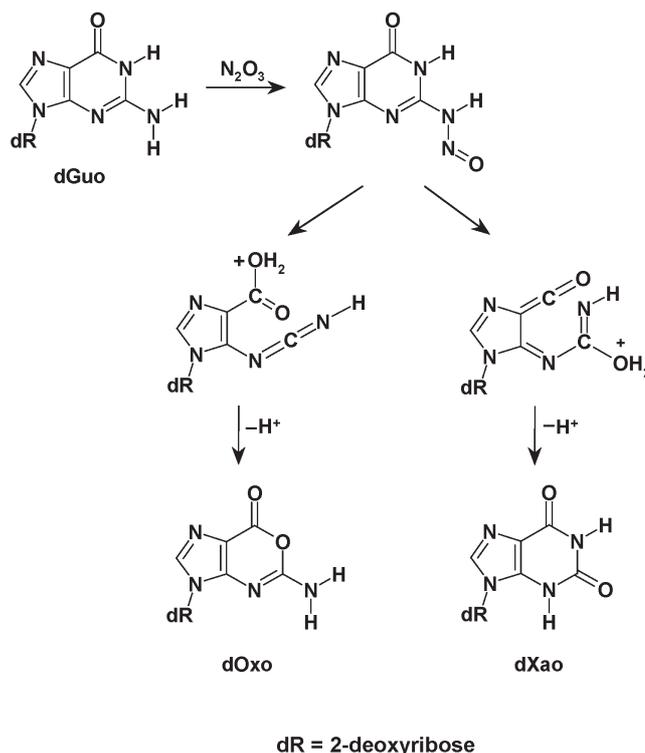
7.1. 2'-Deoxyoxanosine (dOxo)

Quite intriguing was the finding that the reaction of dGuo with nitrous acid or nitric oxide resulted in formation, in addition to the expected major product dXao, of an additional product, 2-deoxyoxanosine (dOxo), shown in Scheme 6 (Suzuki *et al.*, 1997). It should be noted that it is a formal structural analogue of 2-deoxyisoguanosine. The corresponding riboside, oxanosine (Oxo), had much earlier been isolated as a novel antibiotic from a strain of *Streptomyces capreolus*. Its deoxynucleoside dOxo was subsequently chemically synthesized and found to exhibit more potent antiviral and antitumor activities than the parent riboside (Suzuki *et al.*, 1997).

Incubation of an aerated solution of dGuo in the presence of NO at neutral pH also led to the appearance of dOxo. Since many cell types produce endogenous NO, it is to be ex-

pected that this would lead to generation, in addition to dXao, of dOxo from dGuo residues in DNA. In accordance with the earlier proposed mechanism for formation of such products (Caulfield *et al.*, 1998), cited above, Suzuki *et al.* (2000) succeeded in isolating, from a guanosine-HNO₂ reaction system, of a

dOxo residues (Suzuki *et al.*, 1997). This, in conjunction with the stability of these residues, and consequent lack of formation of apurinic sites, would exclude a base excision repair pathway for such lesions unless there existed a DNA glycosylase for the base moiety of dOxo residues.



Scheme 6. Formation of 2'-deoxyoxanosine (dOxo) and 2'-deoxyxanthosine (dXao) from the reaction of 2'-deoxyguanosine (dGuo) with nitrous acid.

Only the partial pathways are shown. See Suzuki *et al.* (1997) for full details.

short-lived intermediate ($t_{1/2}$ 6 min), tentatively identified as a diazotate of Guo. This intermediate underwent conversion *via* two independent pathways to oxanosine and Xao (Scheme 6) in the ratio 1:4. With dGuo, the products were dOxo and dXao in a similar ratio. Furthermore the N-glycosidic bond of a dOxo residue in a synthetic oligonucleotide was found to be as stable as that of dGuo, and much more so than that of dXao (Suzuki *et al.*, 1997).

However, melting profiles of duplexes such as d(T5OxoT6):d(A6NA5), where N = A, G, C or T), exhibited relatively low T_m values, pointing to poor base pairing properties of

An additional genotoxic mechanism of NO-induced DNA damage has been postulated from the finding that presence of an oxanine (Oxa) residue in DNA results in formation of DNA-protein cross-links (Nakano *et al.*, 2003). Incubation of duplex DNA containing an Oxa residue at a site-specific position with DNA binding proteins such as histone, high-mobility group proteins, or DNA glycosylases, led to formation of DNA-protein cross links, especially with DNA glycosylases, between Oxa and the side-chains of lysine and arginine. In line with this, a HeLa cell extract gave rise to two major DNA-protein products when incubated with DNA containing Oxa.

8. CELLULAR MECHANISMS FOR ELIMINATING XANTHINE FROM NUCLEIC ACIDS

8.1. (d)XTP pyrophosphohydrolases, (d)XTPase

It should be noted that intracellular oxidative deamination of DNA bases may occur not only in duplex DNA, but also in the free nucleotide pool. It is, in fact, most likely that oxidative damage of DNA bases will occur more frequently in the nucleotide pool than in chromosomal DNA, followed by misincorporation into nucleic acids, leading to mutagenesis. Since Xan residues are not found in native DNA, mechanism(s) must exist for preventing such incorporation.

In this context, it was long ago demonstrated by Wang and Morris (1974) that various tissues of the rabbit harbour an NTP pyrophosphohydrolase activity which, purified about 700-fold from rabbit liver, with an *m* about 37 kDa, exhibited a remarkable specificity for the non-canonical ITP, dITP and XTP. It was virtually inactive *vs* ATP (0.3%), and exhibited very low activity *vs* UTP, TTP, GTP, dGTP, CTP and dCTP (0.3–9%). It was proposed by the authors that the function of this enzyme is to prevent the incorporation of dITP and dXTP into DNA during replication, and of ITP and XTP into RNA during transcription. A rather unusual feature of this enzyme is its pH optimum, about 10, where it was 6-fold more active than at pH 7.4, but with the use of only ITP, and not XTP, as substrate (see also below).

The foregoing, which has hitherto attracted little attention, has now gained added significance following the isolation by Hwang *et al.* (1999) of a protein coded by a hypothetical gene, *Mj0226*, from the hyperthermophile *Methanococcus jannaschii*. The protein was crystallized, its 3D structure determined, and a search conducted for possible structural homologues in the Protein Data Bank. This led to identification of a number of homo-

logues, which shared the property of binding nucleotides and nucleotide analogues. On conducting such binding studies, the protein was found to weakly hydrolyze GTP to GMP + PP_i, suggesting it is an NTPase. This, in turn, led to analysis of its activity against a broad range of natural (d)NTPs and various analogues, culminating in the finding (Chung *et al.*, 2001) that the enzyme exhibits a remarkable specificity for XTP, ITP and dITP, with k_{cat}/K_m values more than two to four orders of magnitude higher than for 20 other NTPs and analogues. Although dXTP was not available, the similar k_{cat}/K_m values for ITP (650) and dITP (610) suggest that dXTP is an equally good substrate. The enzyme was, furthermore, shown to be devoid of endonuclease and 3'-exonuclease activities. Its pH optimum, but with only dITP as substrate, was about 10.5.

In line with its isolation from a hyperthermophile, the foregoing enzyme displayed an optimal temperature of 80°C, hence similar to the optimum temperature for growth. At 30°C, activity was 5-fold lower. However, the same authors (Chung *et al.*, 2001) prepared, by cloning and purification, two protein homologues of *Mj0226*, one from the hyperthermophile *Archaeoglobus fulgidus*, and a second from the mesophile *E. coli*. Both exhibited similar high specificities for XTP and dITP, with the *E. coli* enzyme activity followed at 37°C.

Further in line with the above, Chung *et al.* (2002) cloned, purified and characterized a hypothetical 21-kDa protein (ORF 0197) from *E. coli* K-12. This protein, denoted as *E. coli* Ec0197, shares 33.5% sequence identity with the NTPase from *M. jannaschii* identified by Hwang *et al.* (1999), a similar specificity for ITP, dITP and XTP (and presumably dXTP), and a similar optimum pH about 10–10.5, extending even to 11. At neutral pH, activity was about 40% that at pH 10. However, in contrast to other reports, dGTP and dUTP were reported to undergo hydrolysis at pH 10 at about 10% the rate for XTP.

Independently, Lin *et al.* (2001) identified the gene for a similar protein in human foetal brain. Cloning of its cDNA led to isolation of a 21.5-kDa protein with NTPase activity highly specific for ITP, dITP, dXTP, and again with an optimum pH of about 10. The gene encoding the cDNA sequence of the human enzyme, referred to as ITPA, was localized on the short arm of chromosome 20p, and its mRNA transcripts were found in all 24 human adult tissues examined. Furthermore, a BLAST search revealed the existence of similar sequences in more than 40 proteins, and putative protein sequences, all about 200 amino acids in length, and with similar structures in organisms ranging from bacteria to mammals.

Bearing in mind the existence of such activities in organisms as widely divergent as two hyperthermophiles, *E. coli*, and the rabbit (Wang & Morris, 1974), it would appear that, like the ubiquitous dUTPase, the function of which is to prevent the incorporation of uracil into DNA by hydrolysis of dUTP to dUMP, most or all organisms contain a (d)XTPase/(d)ITPase to prevent incorporation of Xan and Hyp residues into DNA and RNA. One other such enzyme known is the MutT protein of *E. coli*, an NTPase which hydrolyzes 8-oxo-dGTP, a highly mutagenic oxidized nucleotide, to 8-oxo-dGMP and PP_i (Bessman *et al.*, 1996; Sekiguchi & Tsuzuki, 2002).

One striking feature shared by all the foregoing enzymes is their unusual pH optimum, 10–11. At this strongly alkaline pH, the Xan base of (d)XTP (pK_a about 5.7) exists exclusively as the monoanion, with dissociation of the N(3)-H (see Scheme 2); and the Hyp base of (d)ITP (pK_a about 9) is more than 90% in the form of the monoanion, but in this case with dissociation of the N(1)-H (see Scheme 1). It should, however, be noted that in all instances the pH optimum was determined only with ITP or dITP as substrate. It would obviously be desirable to determine the pH optimum with (d)XTP.

8.2. Repair enzymes for xanthine lesions in DNA

Endonuclease V from *E. coli*, a product of the *nif* gene, which recognizes a broad spectrum of lesions in DNA, has been shown to recognize misincorporated dXao lesions (Schouten & Weiss, 1999; He *et al.*, 2000). Furthermore, under conditions where this enzyme recognizes dIno lesions in both single-stranded and double-stranded DNA, it proved to be active against dXao lesions only in duplex DNA, by cleavage of the strand containing the dXao lesion. Hence it appears to recognize the Xan residue as such, and not the accompanying mismatch with the base in the complementary strand (He *et al.*, 2000). Isoguanine lesions were also recognized, but poorly.

Terato *et al.* (2002) subsequently examined the repair ability of the DNA N-glycosylases from *E. coli* for dXao and dOxo lesions in DNA. Both AlkA (3-methyladenine DNA glycosylase II) and endonuclease VIII recognized the presence of Xan residues, substantiated by following directly the release of [³H]Xan from the DNA. Both enzymes exhibited a lower ability to excise the oxanine base of dOxo lesions.

The ability to repair Xan lesions in DNA by four enzymes of the base excision repair pathway was examined by Wuenschell *et al.* (2003), with the use of 34mer oligodeoxynucleotide duplexes containing a Xan residue in one strand opposite to each of the four natural bases in the complementary strand. AlkA protein was found to be the most effective for removal of Xan from X:C base pairs, with lower activity *vs* X:T, X:G and X:A pairs within the same sequence context. The human homologue of AlkA, the methylpurine glycosylase Mpg, also displayed good specificity for excision of Xan from X:C pairs.

9. METABOLIC ROLE OF MONOANION OF XANTHINE, ITS NUCLEOSIDES AND NUCLEOTIDES

We now consider the possible role of these monoanionic species, and related analogues, in various enzyme systems and metabolic pathways where their presence at high levels cannot be ignored. One of the simplest approaches would appear to be measurements of the pH-dependence of a given enzymatic reaction in which they may be involved. However, the complexities, and the numerous pitfalls inherent in the interpretation, of the mechanism of an enzyme-catalyzed reaction from pH-dependence of the kinetics are truly formidable, and have been frequently drawn attention to. The objectives of such studies include, amongst others, determination of pK_a values approximating those of individual ionizable groups, the rate constants for specific ionic forms of the reactants, as well as their modification during the course of ligand binding, a subject comprehensively reviewed, amongst many others, by Brocklehurst (1994). Other approaches must frequently be employed to assist in a proper interpretation, e.g. rapid reaction techniques, fluorescence emission, the use of structurally related ligand analogues.

Similar considerations apply when the ligands involved (substrates, inhibitors) may exist in different tautomeric forms, e.g. the protomeric N(7)-H / N(9)-H tautomers of the 6-oxopurines, the equilibrium of which is additionally dependent on whether the purine in question is in its neutral or anionic form (see Schemes 1 and 2).

10. XANTHINE OXIDASE

This enzyme and xanthine dehydrogenase, interconvertible (by proteolysis or disulfide formation) forms of the same gene product known as xanthine oxidoreductase, catalyzes the oxidation of many purines and related an-

alogues. In mammalian systems it catalyzes the last two steps of purine catabolism, the oxidation of Hyp to xanthine and of Xan to uric acid. Its mechanism of action, which is very complex, has been extensively studied, in many instances with the use of non-physiological substrates. The reaction is initiated at a molybdenum-pterin center, from which electrons are transferred, with the mediation of two Fe_2S_2 iron-sulphur centers, to the isalloxazine system of flavine adenine dinucleotide (FAD), and finally to NAD^+ or, with production of the superoxide anion and H_2O_2 , to molecular oxygen in the case of xanthine dehydrogenase and xanthine oxidase, respectively.

It is rather surprising that only very recently has attention been directed to the fact that, at physiological pH, xanthine (pK_a 7.7, see Table 1) is an approximately equilibrium mixture of the neutral and monoanionic species. In a very recent extensive theoretical study of the interaction of substrates and inhibitors with xanthine oxidase (Rastelli *et al.*, 1997), based on similarity concepts and molecular modelling, consideration was limited exclusively to possible involvement of putative tautomers of only the neutral forms of the physiological substrates Hyp and Xan.

Kim *et al.* (1996) first demonstrated that, with xanthine as substrate, the steady-state kinetic parameter k_{cat}/K_m exhibited a symmetrical bell-shaped dependence on pH with a maximum at pH 7, pointing to an ionizable group with pK_a about 6.6 in the active site of the enzyme, and a second ionizable group with pK_a about 7.4. In the latter pH range there is no known ionizable group in the enzyme, whereas the pK_a of 7.4 is close to the pK_a of the substrate Xan (7.7). This was taken to imply that the substrate must be in the protonated, i.e. neutral, form for catalysis to proceed, and considered consistent with the proposed reaction mechanism, involving proton abstraction from C(8) of xanthine, followed by hydroxylation (Hille, 1996). If the substrate were in the ionized form, its result-

ing negative charge would destabilize the accumulating negative charge on C(8) in the course of deprotonation at this site. With the non-physiological substrate lumazine, a similar bell-shaped pH dependence was observed, with pK_a values of 6.5 (hence similar to that with xanthine) and 7.8, the latter accounted for by the fact that the pK_a of lumazine is 0.3 pH units higher than that of xanthine.

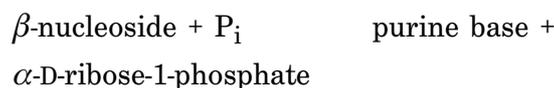
Subsequently Sau *et al.* (2000) reported that the pH-dependence of V_{max}/K_m for 1-methylxanthine exhibited a bell-shaped curve similar to that for xanthine, with corresponding pK_a values of 6.2 and 7.7, and concluded, like Kim *et al.* (1996), that only the neutral form of 1-methylxanthine is the substrate. Although Kim *et al.* (1996) did not identify the nature of the monoanionic form of xanthine, Sau *et al.* (2000) erroneously ascribed it to dissociation of the N(1)-H, and the monoanionic form of 1-methylxanthine to dissociation of the N(9)-H. However, as pointed out above (Section 3), the monoanionic species of both are due to dissociation of the N(3)-H, with almost identical pK_a values (see Table 1), and this probably accounts for the similar pH-dependent profiles of both substrates.

The foregoing conclusions that only the neutral forms of xanthine and 1-methylxanthine are substrates of xanthine oxidase are at variance with a much earlier report by Bergmann and Levene (1976) on the relative rates of oxidation of a series of purines and thiopurines by milk xanthine oxidase. The objective was to obtain information about the "active" structures recognized by the enzyme, based in part on the arbitrary assumption of binding of substrates in rare tautomeric forms. All reactions were conducted at the optimal pH 8. It is, consequently, of interest that the relative V_{max} values for Hyp (108), xanthine (100) and 6-thioxanthine (57) are similar, as are their K_m values, 3.3, 5.2 and 4.6 μM , respectively. Since at pH 8 the population of the anions of Hyp (pK_a about 9), xanthine (pK_a about 7.7) and 6-thioxanthine (pK_a about 6.2) are about 10%, 60%, and > 95%, respectively,

it would appear that the enzyme effectively accepts both the neutral and monoanionic forms as substrates. These findings would have been more convincing if reaction rates had been followed as a function of pH.

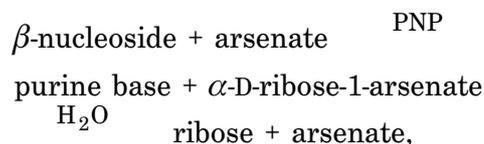
11. PURINE NUCLEOSIDE PHOSPHORYLASES (PNP)

These ubiquitous enzymes catalyze the cleavage (phosphorolysis) of the glycosidic bond of purine ribo- and 2'-deoxyribo-nucleosides in the presence of inorganic phosphate (P_i), a reaction reversible with natural and many synthetic substrates, as follows:



In intact cells, phosphorolysis is the predominant reaction, due to coupling with guanase and xanthine oxidase, leading to stepwise formation of xanthine (Xan) and, finally, urate (Bzowska *et al.*, 2000). PNP is one of the major enzymes operating in the so-called purine salvage pathway, whereby purines liberated by phosphorolysis are converted by phosphoribosyltransferases (HGPRtase) to the corresponding nucleoside 5'-phosphates (see Section 13).

Replacement of P_i by arsenate (AsV) in the above reaction renders the reaction non-reversible, as follows:



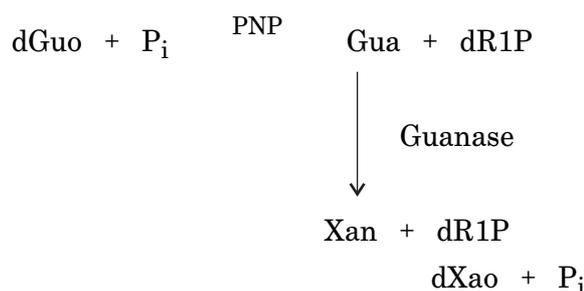
due to the high instability of ribose-1-arsenate. Arsenolysis is frequently employed in studies on the reaction mechanisms of PNPs (e.g. Kline & Schramm, 1993). But it has very recently been shown to also be of potential biological significance, as described below (Sec-

tion 11.3). The enzyme has been isolated, purified and characterized from a wide range of mammalian and bacterial sources, revealing the existence of two major forms (Bzowska *et al.*, 2000; Pugmire & Ealick, 2002): (a) homotrimers, with a subunit molecular mass of about 31 kDa, which accept the 6-oxopurines Hyp and Gua, and their ribo- and 2'-deoxyribo-nucleosides and, in some instances, Xan and (d)Xao (see below), but not 6-aminopurines; (b) homo-hexamers, with a subunit molecular mass of about 26 kDa, which accept the 6-amino Ade and (d)Ado in addition to the 6-oxopurines. The mammalian enzymes are usually trimers, whereas the bacterial ones are largely hexamers. Some reported exceptions to the foregoing are described by Bzowska *et al.* (2000) and Pugmire and Ealick (2002). In particular, the trimeric structure of the mammalian enzymes in the crystal has recently been confirmed for the human enzyme in solution by low-angle X-ray scattering (de Azevedo *et al.*, 2003). By contrast, analytical ultracentrifugation suggests that the *E. coli* PNPI (which is a hexamer, a trimer of dimers, in the crystal) is, in solution, an equilibrium mixture of the hexamer and probably the dimer (see Bzowska *et al.*, 2000), of obvious relevance to interpretation of kinetic data.

11.1. Mammalian PNPs

Numerous reports have described Xan and Xao as weak substrates of PNP of mammalian origin, but in all instances at only a single arbitrarily selected pH, thus overlooking the presence of the monoanionic forms of these substrates at physiological pH. Friedkin (1952) long ago reported that phosphorolysis of dGuo at pH 7.4 by a partially purified PNP from rat liver, known to contain guanase, led to appearance of the then unknown 2'-deoxyxanthosine (dXao), as shown by the reaction sequence in the next paragraph.

Phosphorolysis of Guo by the same enzyme led to appearance of Xao, subsequently also



reported by Giorgelli *et al.* (1997), who were apparently unaware of the results of Friedkin. The dXao isolated by Friedkin (1952) was shown to be a substrate for phosphorolysis, at a rate about 2% that for dGuo at pH 7.4.

The first comprehensive study of the substrate properties of Xan and Xao, with the mammalian enzymes (from human erythrocytes and calf spleen), over a broad range of pH, 3.5–8.5, is that of Stoychev *et al.* (2002). The optimum pH for these was found to be in the range 5–6, as compared to a range of 7–8 for Guo and Gua, and Ino and Hyp. This pH-dependence of the substrate properties of Xan and Xao pointed to both the neutral and anionic species as substrates, but with a very marked preference for the neutral forms.

Both the neutral and monoanionic forms of Xao were very poor inhibitors of phosphorolysis of Guo by human PNP, as was also Xan. In striking contrast, Gua was an excellent competitive inhibitor of phosphorolysis of Xao with a K_i about 2 μM . Furthermore, Xan was a good inhibitor of the reverse reaction with Hyp (IC_{50} about 20 μM) (Stoycker *et al.*, 2000), consistent with the finding of Krenitsky *et al.* (1968) that the reverse reaction for Hyp with human PNP is inhibited by Xan with a K_i about 40 μM .

11.2. *E. coli* PNPII (Xao phosphorylase, Ino-Xao phosphorylase)

In many instances, particularly microorganisms, there is more than one PNP, with different specificities (see Bzowska *et al.*, 2000).

Most interesting in this respect is *E. coli*, the PNP of which, referred to as PNPI, a hexameric product of the *deoD* gene, accepts as substrates the 6-oxo Gua and Hyp, and the 6-amino Ade, and their nucleosides. Koszalka *et al.* (1988) claimed that an apparently homogeneous PNPI from *E. coli* slowly cleaves Xao with a V_{\max} about 4% that for Ino at neutral pH. However, our homogeneous preparation of PNPI (by affinity chromatography) proved to be totally inactive *vs* Xao in the forward reaction, as well as Xan in the reverse synthetic reaction, over a broad pH range (Stoychev *et al.*, 2002).

Cultivation of *E. coli* in the presence of Xao (but no other nucleoside) leads to appearance of a second hexameric PNP, referred to as Xao phosphorylase, a product of the *xapA* gene, the substrates of which include the 6-oxo Hyp, Gua and Xan and their nucleosides, with a preference for Xan and Xao, but not the 6-amino Ade and Ado (Hammer-Jespersen *et al.*, 1980; Bezirjian *et al.*, 1986). Koszalka *et al.* (1988) named this enzyme Ino-Guo phosphorylase. We consider it more appropriate to refer to it as PNPII.

The Swiss-Prot database refers to PNPII as inosine-xanthosine (Ino-Xao) phosphorylase. Its sequence bears no similarity to that of *E. coli* PNPI, for which Xan and Xao are not substrates (see above), but is similar to that of mammalian PNPs (Bzowska *et al.*, 2000). But, notwithstanding its sequence similarity to mammalian PNPs (Seeger *et al.*, 1995) which are trimers, PNPII has been shown to be a hexamer, although two groups have reported that gel filtration reveals the presence of a second minor peak identified as an active trimer (Hammer-Jespersen *et al.*, 1980; Bezirjian *et al.*, 1986). We, and others (G. Dandanell, personal communication), find that an apparently homogeneous PNPII exhibits only a single active peak identified as a hexamer.

In contrast to calf and human PNP, for which Xao is a weaker substrate than Guo (Stoychev *et al.*, 2002), the pH-dependence of

the apparent V_{\max} values for Guo and Xao with PNPII (our unpublished data) show that both are comparable substrates, with a shift of the pH profile towards acidic pH for both Xao phosphorylase and the reverse reaction with Xan. Furthermore, V_{\max}/K_m values for Guo and Xao with PNPII at pH 7 (Koszalka *et al.*, 1988) are comparable, but the optimal pH range for enzyme activity is narrower for PNPII (Bezirjian *et al.*, 1986) than for mammalian PNPs (Stoychev *et al.*, 2002).

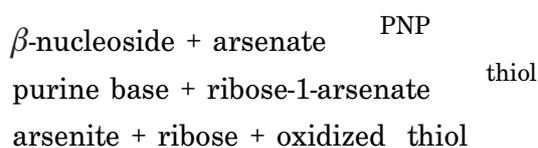
Xao (pK_a 5.7) is an excellent substrate for PNPII, and a moderate one for mammalian PNPs (Stoychev *et al.*, 2002) in the pH range 7–8, where it exists almost exclusively in the monoanionic form. By contrast, substrate activities of Guo and Ino are markedly reduced at $pH > pK_a$. Bearing in mind that the active site of PNPII is similar to that of mammalian PNPs, with an Asn residue interacting with the N(7) and O⁶, and the Glu carboxylate hydrogen bonding to the N(1)-H, dissociation of the purine N(1)-H in Guo and Ino leads to electrostatic repulsion with the negative charge of the Glu carboxylate, resulting in lack of substrate activity. With Xao, for which dissociation occurs at the N(3)-H (Section 3), its monoanionic form, a 6-oxopurine, is capable of interacting with the active site, and is in fact a good substrate.

11.3. PNP and arsenate reductase

Although not directly pertinent to the subject of this review, the following is worthy of note. The ubiquity of arsenic in the environment, and its associated highly deleterious effects on health, has prompted extensive studies on the biochemistry and toxicology of inorganic arsenic. Inorganic arsenate (AsV) and arsenite (AsIII) are present in carcinogenic concentrations in the drinking water of millions of people world-wide, and has led to the evolution of arsenic defense mechanisms in all organisms from *E. coli* to humans. Three families of arsenate reductase enzymes have now been extensively charac-

terized in prokaryotes and yeast; but, to date, no mammalian counterparts of these have as yet been unequivocally identified (see Rosen, 2002).

Recently Radabaugh *et al.* (2002) reported a putative enzyme activity in human liver that led to reduction of arsenate to the more toxic arsenite, initially referred to as arsenate reductase. The activity was identified as PNP which, in the presence of a nucleoside, arsenate and a thiol (the naturally occurring dihydrolipoic acid, DHLP), led to the following reaction sequence:



The proposal that this reaction may be of physiological importance *in vivo* is belied by the fact that cellular levels of DHLP are only in the micromolar range (see Nemeti *et al.*, 2003). Waalkes and Liu (2002) have proposed that PNP may be a *fortuitous* intracellular arsenate reductase. From the reaction sequence described above, it appears that PNP is not *a priori* an arsenate reductase; it simply releases the highly labile ribose-1-arsenate which reacts non-enzymatically with an appropriate thiol to reduce liberated toxic arsenate to the more toxic arsenite. Further research is required to clarify the mechanism of the *in vitro* PNP-catalyzed reduction of As (V) to As (III), and its possible *in vivo* role.

12. XANTHINE NUCLEOTIDE-SELECTIVE G PROTEINS

GTP-binding proteins (G proteins possessing GTPase activity), which are heterotrimeric ($\alpha\beta\gamma$) structures, mediate transmembrane signaling transfer between receptors and effectors. The large superfamily of G proteins includes also monomeric small G

proteins, such as Ras, Ran and Rab. Activated receptor promotes dissociation of GDP from $G\alpha$, the rate-limiting step of the G protein cycle, followed by receptor-catalyzed binding of GTP to $G\alpha$. This induces the active conformation of the G protein, leading to dissociation of the heterotrimer to $G\alpha$ -GTP and the $\beta\gamma$ complex, both of which are capable of modulating effector enzymes and ion channels. $G\alpha$ possesses GTPase activity, which hydrolyzes GTP to GDP, thereby deactivating the G protein, following which $G\alpha$ -GDP and $\beta\gamma$ reassociate to complete the G-protein cycle. A single receptor can activate more than one type of heterotrimer, while activated $G\alpha$, and the $\beta\gamma$ subunit, can interact with multiple effectors. Moreover, cross-talk between different G protein-regulated pathways further contributes to the complexity of these networks (Neer, 1995).

One approach widely adopted to analysis of this complex network is to specifically activate a selected $G\alpha$ protein *in vivo* to discern its function without interference from other G proteins, e.g. by switching, by means of mutation(s), its specificity from Gua nucleotides to one with another base moiety, the most widely preferred base being Xan. Although XMP is an intermediate in the biosynthesis of GMP from IMP (Section 14), the steady-state intracellular concentrations of XDP and XTP are very low. However, with the use of permeabilized cells, to allow uptake of XDP or XTP, it appears feasible to specifically activate the mutant protein (Yu *et al.*, 1997). Availability of an array of xanthine-nucleotide $G\alpha$ proteins should therefore complement gene knockout and other approaches to delineate the function of an individual $G\alpha$ protein in intact cell systems (Gille *et al.*, 2003), recently extensively reviewed by Gille and Seifert (2004).

$G\alpha$ consists of the *ras*-like domain, structurally similar to small GTP-binding proteins, and the α -helical domain unique to $G\alpha$, both embedding the nucleotide-binding pocket. Particularly important for guanine selectivity

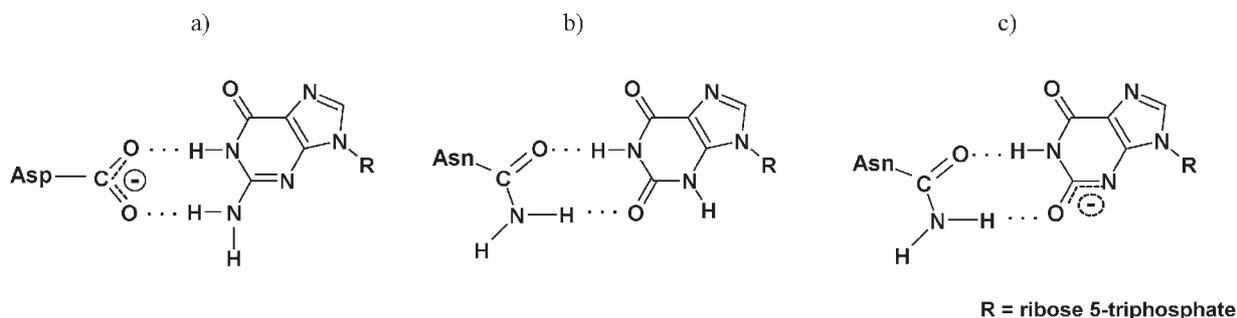
is an aspartate residue, belonging to the NKXD motif, highly conserved among small GTP-binding proteins and $G\alpha$. Exchange of this aspartate for asparagine (Asp/Asn mutation) in small GTP-binding proteins was found to switch base selectivity from Gua to Xan, and such mutants have been successfully employed to study the properties of a given specific small GTP-binding protein in complex systems containing multiple GTP-binding proteins (Yanachkov *et al.*, 1997).

Similar applications may be envisaged for G proteins, but, unexpectedly, Asp/Asn mutants of these were found to be inactive. However, additional exchange of a conserved glutamine for leucine, resulting in enhanced GDP affinity, which was found to be critical for functional expression of xanthine nucleotide-selective G protein mutants, led to the desired active $G\alpha$ with xanthine nucleotide specificity (Yu *et al.*, 2000; see Gille *et al.*, 2003).

Clearly relevant to the foregoing is the mode of binding of XTP by a mutant G protein, bearing in mind that, at physiological pH, the xanthine ring of XTP is > 95% in the monoanionic form. The crystal structures of the α -subunits of transducin and G_i both exhibit nearly identical binding pockets for the Gua nucleotide, similar to that in the crystal

structures of Ras and EF-Tu. One of the conserved features is the interaction between a specific $G\alpha$ amino-acid residue and the guanine ring, *viz.* hydrogen bonds from the side-chain carboxyl of a conserved aspartic acid (Asp-268 in transducin) to the ring N(1) and the C(2)-NH₂ of the guanine ring (Scheme 7a). Asp-268 of transducin belongs to the conserved motif, NKXD, found in all members of the GTPase superfamily, and it has been shown that characteristic hydrogen bonds formed with the Asp residue determines the specificity of nucleotide binding in other GTP-binding proteins, e.g. Ras and EF-Tu. Mutation of Asp to Asn at this position in other GTP-binding proteins results in active proteins regulated by xanthine, but not guanine, nucleotides (see Gille & Seifert, 2004).

It is generally assumed that the mode of binding of XTP in the Asp mutants is *via* hydrogen bonding of the carboxamide side-chain of asparagine to the ring N(1) and the C(2) carbonyl of the xanthine ring (see Scheme 7b). This overlooks the fact that, at the pH of the reaction, the N(3)-H of the xanthine ring is virtually fully ionized, such that an even stronger, ionic, hydrogen bond would occur, as shown in Scheme 7c. And, in fact, kinetic studies demonstrated that the



Scheme 7. Modes of binding of GTP and XTP by wild-type and mutant G proteins.

(a) Mode of binding of the guanine ring of GTP by an essential Asp residue (Asp268 in transducin) of a small GTP binding protein, as observed in several crystal structures; (b) proposed mode of binding of XTP when the Asp is replaced by Asn (see Yu *et al.*, 1997); (c) alternative proposed mode of binding of XTP by the Asn mutant.

D333N mutant of *E. coli* adenylosuccinate synthetase uses XTP as an energy source much more effectively than the wild type with GTP, ascribed to stronger bonding of the xanthine ring with the Asn333 of the mutant enzyme (Kang *et al.*, 1994). It should, however, be noted that, with the wild type proteins, there is also an ionic hydrogen bond from the GTP amino group to an Asp carboxylate.

An interesting departure from the foregoing is the GTP-binding protein of the chloroplast outer envelope membrane, *Arabidopsis* Toc33, which was found to bind GTP, GDP and XTP (but not ATP) with similar efficiencies. D217N and D219N mutations within the nucleotide-binding domain did not affect nucleotide specificity or GTPase activity, nor Toc33 functionality *in vivo* (Aronsson *et al.*, 2003). The unusual nucleotide-binding properties of this protein remain to be elucidated.

13. PURINE PHOSPHORIBOSYLTRANSFERASES (PRTases)

Purine phosphoribosyltransferases (PRTases), enzymes active in the purine salvage pathway, catalyse the Mg^{2+} -dependent reversible transfer of the 5-phosphoribosyl moiety of α -D-5-phosphoribosyl-1-pyrophosphate (PRPP) to N(9) of a purine base, yielding purine nucleotide and inorganic pyrophosphate:



Free living organisms can parallelly synthesize purine nucleotides *via de novo* pathways, but protozoan parasites lack the enzymes for these routes, so that their salvage enzymes have long been considered as chemotherapeutic targets for parasitic diseases (Wang, 1984; el Kouni, 2003).

In humans, the main substrates of these enzymes are the 6-oxopurines Gua and Hyp for hypoxanthine-guanine phosphoribosyltransferase (HGPRTase) and the 6-aminopurine

Ade for adenine phosphoribosyltransferase (APRTase). The crystal structures of PRTases specific for Hyp and Gua exhibit an interaction between the O⁶ and a conserved, positively charged lysine (Lys165 in the human enzyme), thereby discriminating against 6-amino purines (Eads *et al.*, 1994).

The 6-oxopurine Xan is salvaged by human HGPRTase at an extremely low level, and is converted to uric acid and excreted. By contrast, some bacteria and, especially, parasitic protozoa, possess PRTases with an elevated preference for Xan relative to Gua, and even so-called xanthine phosphoribosyltransferases (XPRTases) which preferentially catalyse the salvage of Xan and, inefficiently, Gua and Hyp (Craig & Eakin, 2000; el Kouni, 2003), hence attractive targets for chemotherapy.

X-Ray structures of enzymes that accept Xan, when compared with those of the HGPRT class (Somoza *et al.*, 1996; Shi *et al.*, 1999a; 1999b; Vos *et al.*, 1998; Héroux *et al.*, 1999a; 1999b), in efforts to determine how an enzyme discriminates between the C(2)-NH₂ in Gua and the C(2)=O in Xan, reveal that there is no common mechanism for binding of Xan. Whereas the modes of binding of Hyp and Gua in the forward reaction, and IMP and GMP in the reverse reaction, have been reasonably well assigned, the situation is much less clear for the modes of binding of Xan and XMP (Vos *et al.*, 1997). The efficient use of Xan by some PRTases is also dependent on secondary structural features, e.g. a chimeric human HGPRTase, in which about 50 N-terminal residues distal to the active site were replaced with the corresponding segment from *P. falciparum* HGXPRTase, exhibited enhanced specificity for Xan (Subbaya *et al.*, 2000).

Crystallography, and kinetic experiments, have usually been performed at pH about 7 to 9, where Xan (pK_a 7.7) is a mixture of neutral and monoanionic forms, and the Xan ring of XMP (pK_a about 5.7) is predominantly, or even exclusively, an anion. But the possible

involvement of these anionic forms, if at all, as substrates and/or as inhibitors, has been totally ignored. Moreover, at the resolutions hitherto attained, X-ray diffraction is unable to distinguish between a C(2)=O and a C(2)-O⁻, notwithstanding that the latter is a better acceptor. It would be helpful, in this context, to examine the pH-dependence of the substrate properties of Xan and Gua, and their mutual competition, with an enzyme that utilizes both substrates. Such pH-dependencies have been reported for enzymes that utilize Gua and Hyp (see below).

Molecular dynamics simulations were applied to this problem by Pitera *et al.* (1999) in attempts to explain the ability of the protozoan parasite *Trichomonas foetus* HGXPRTase to accept xanthine as a substrate, as against the human HGXPRTase, which has a 100-fold lower affinity for xanthine relative to Gua and Hyp. The two enzymes exhibit only about 30% sequence identity. Their active sites, however, are very similar in structure, but do not explain why only the parasite enzyme recognizes xanthine and XMP. Calculations were based on splitting the nucleotides GMP and XMP into two parts, the base + sugar (treated as a single *neutral* species), and the phosphate group, taken as a *singly* charged anion. The nucleotides GMP and XMP were therefore each considered as carrying a charge of -1. Since the reactions occur at physiological pH, both assumptions are open to question. The phosphate group, with a $pK_a \approx 6.7$, would be predominantly a dianion under these conditions. As concerns the base in the case of XMP, the authors erroneously "considered only the 2-oxo tautomer, as enolic tautomers of the nucleobases are rarely populated in solution", hence ignoring the fact that the xanthine ring in XMP is an anion under these conditions. Nonetheless, calculations led to excellent predictive findings, including the design of two active mutants. One conceivable, but rather unusual, interpretation is that the enzyme binds only

the neutral form, and that the anionic species is totally inert.

Relevant to the foregoing are reports on substrate properties of the monoanion of Hyp, which binds to, and is a substrate of, *T. crusi* HGXPRTase (Canyuk *et al.*, 2001), although in this case monoanion formation (pK_a 8.9) results from dissociation of the N(1)-H, with a negative charge at O⁶, and not of N(3)-H, with a charge at O². The rates of IMP and GMP formation revealed an increase of HGXPRTase activity at alkaline pH, with a maximum or plateau at about pH 10 (Olsen & Milman, 1974; Johnson & Nash, 1983; Xu & Grubmeyer, 1998; Canyuk *et al.*, 2001). This reflects the ionic state, not only of the enzyme side chains, but also of the purine substrate, but not of the cofactor PRPP (Canyuk *et al.*, 2001). Increasing reaction rates with Hyp at pH > 8 were displayed also by three, weakly active, *T. crusi* HGXPRTase mutants which possessed chemically different, non-ionizable side-chains at position 137, in place of Asp, a residue previously proposed as a general catalytic acid/base (Xu & Grubmeyer, 1998), but subsequently questioned by Canyuk *et al.* (2001). This common characteristic of the *T. crusi* HGXPRTase mutants suggests alkaline dissociation of Hyp rather than that of an enzyme residue (Canyuk *et al.*, 2001), in line with an earlier report that Chinese hamster HPRTase utilizes the monoanion of Hyp (Olsen & Milman, 1974).

Gua and Hyp PRTase activities in extracts of the archeobacterium *Metanococcus voltae* have been characterized by Bowen *et al.* (1996). These activities, both optimal at pH 9, were not inhibited by Xan, nor by a variety of other purine base analogues, with the exception of 8-azaGua and 8-azaHyp (which were not substrates, and were bactericidal) and 2-thioxanthine. The latter is of particular interest, in that it inhibited only the Hyp, but not the Gua, PRTase activity, and, furthermore, did not inhibit growth of the organism, suggesting that it is a substrate, although not

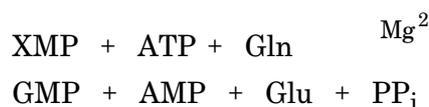
shown directly. Moreover, since its pK_a is 5.9 (Stoychev *et al.*, 2002), and reactions were conducted at pH 8, it would appear that its monoanion may be the inhibitor, by virtue of its being a substrate. The possible substrate properties of 2-thioxanthine warrant further examination, but bearing in mind that the putative product, 2-thio-XMP, is hitherto unknown, hence not characterized. The inhibitory properties of 8-azaGua and 8-azaHyp are intriguing in that, unlike the parent Gua and Hyp, they undergo dissociation by loss of a proton from the triazole ring and, furthermore, with a $pK_a \approx 6.5$ (Wierzchowski *et al.*, 1996), hence are exclusively monoanions at pH 9, but with the pyrimidine rings identical to those of Gua and Hyp, respectively, which may account for their ability to bind to, and inhibit, the enzymes.

14. IMP DEHYDROGENASE AND GMP SYNTHETASE

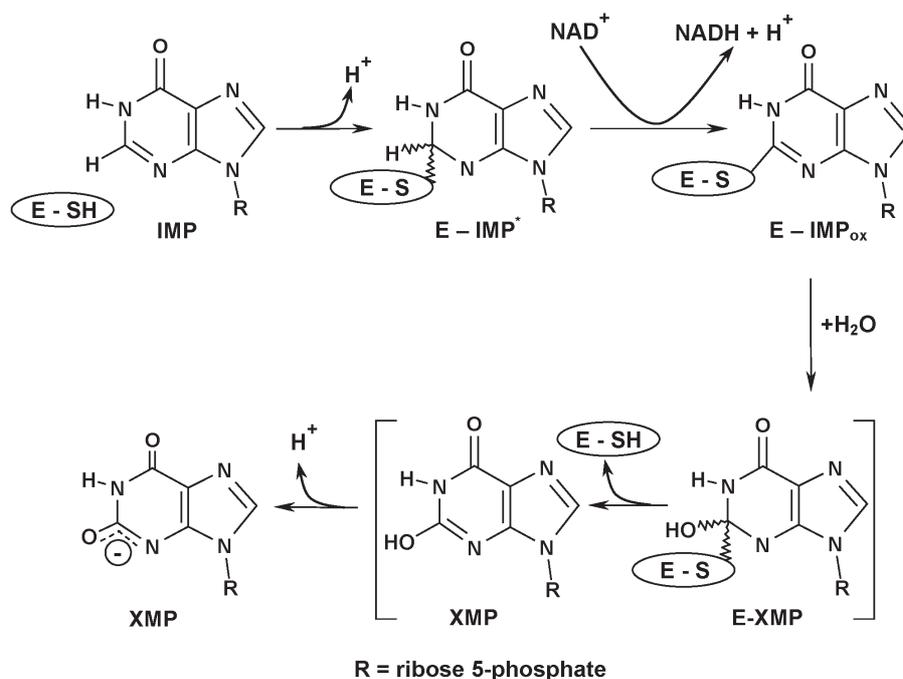
IMP dehydrogenase catalyzes the NAD^+ -dependent oxidation reaction that converts IMP

to XMP, the rate-limiting step in guanine nucleotide biosynthesis. The reaction, shown in Scheme 8, appears to follow a random-in ordered-out mechanism, where the IMP substrate and the cofactor NAD^+ bind to the active site of the enzyme, followed by nucleophilic attack at the C(2) of IMP by an active site cysteine (Cys331 in human type II IMP dehydrogenase) to form an E-IMP* covalent intermediate. Hydride transfer from this intermediate to NAD^+ leads to formation of the intermediate E-IMP_{ox} and NADH. A water molecule in the active site then intervenes to hydrolyze this covalent intermediate to E-XMP, with dissociation of NADH from the active site, followed by release of XMP.

XMP, in turn, is converted to GMP by GMP synthetase, with glutamine as donor of the amino group, and ATP as energy source, as follows:



In an extensive study of the pH-dependence of the mechanism of the IMP dehydrogenase

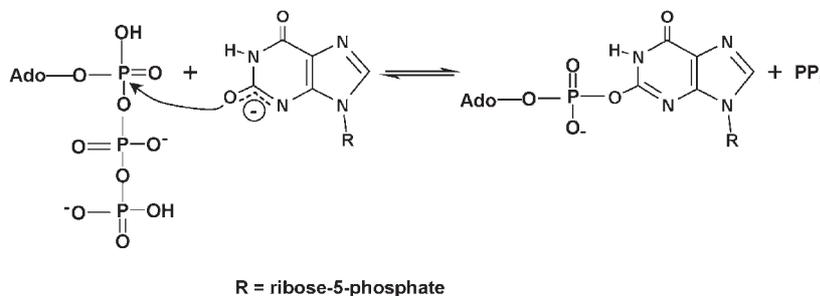


Scheme 8. NAD^+ -dependent oxidation of IMP to XMP catalyzed by IMP dehydrogenase.

reaction, Markham *et al.* (1999) are the only authors to have recognized that, at physiological pH, the final released product must be the C(2)-enolate of XMP. However, their suggestion that this is preceded by release of the C(2)-enol, which is then converted to the enolate, is, at best, problematical. The rate-limiting step is known to occur after hydride transfer, and could be hydrolysis of E-IMP_{ox} or XMP release, the latter being favoured by demonstration of the absence of a deuterium isotope effect on k_{cat} .

As regards the subsequent conversion of released XMP to GMP by GMP synthetase, kinetic data, and positional isotope exchange indicated that the kinetic mechanism involves an ordered addition of substrates, with MgATP binding first, followed by XMP to form an adenylyl-XMP ester intermediate (von der Saal *et al.*, 1985). The mechanism of this step, involving a nucleophilic attack by the C(2) oxygen of XMP at the α -phosphate of ATP, with displacement of PP_i (Scheme 9), clearly suggests that it is the monoanion of

contrast to the known cytotoxic effect of 6-thiopurines on mammalian cells, the action of 6-thioxanthine on the parasite *Toxoplasma gondii* is uniquely parasitostatic. The compound proved to be a substrate of the parasite HGPRTase, leading to formation of 6-thio-XMP, but not 6-thio-GMP. The absence of the latter product accounted for the lack of a parasitocidal effect, since incorporation of 6-thio-dGTP into nucleic acids is the mechanism for the lethal effect of 6-thiopurines on mammalian cells. Extracellular parasites that had accumulated a high concentration of 6-thio-XMP, as well as control parasites, efficiently used xanthine to make guanine nucleotides. This suggested that IMP dehydrogenase was being inhibited, while GMP synthetase was not, further confirmed by demonstrating directly in soluble extracts of *T. gondii* that 6-thio-XMP was an inhibitor of IMP dehydrogenase, with an IC₅₀ about 100 μ M. Inhibition was assumed to be competitive, in line with an earlier report that XMP is a competitive inhibitor of the human



Scheme 9. Formation of an adenylyl-XMP ester intermediate, *via* nucleophilic attack by the C(2) oxygen of the monoanion of XMP, catalyzed by GMP synthetase.

XMP which is the substrate, a fact generally overlooked. Glutamine then donates its amino group to C(2) of the ester intermediate, followed by release of the adenylate, with formation of GMP. Both the foregoing enzymes, especially IMP dehydrogenase, have long been considered as favourable chemotherapeutic targets (Franchetti & Grifantini, 1999; Pankiewicz, 2001).

Particularly intriguing was the observation of Pfefferkorn *et al.* (2001) that, in striking

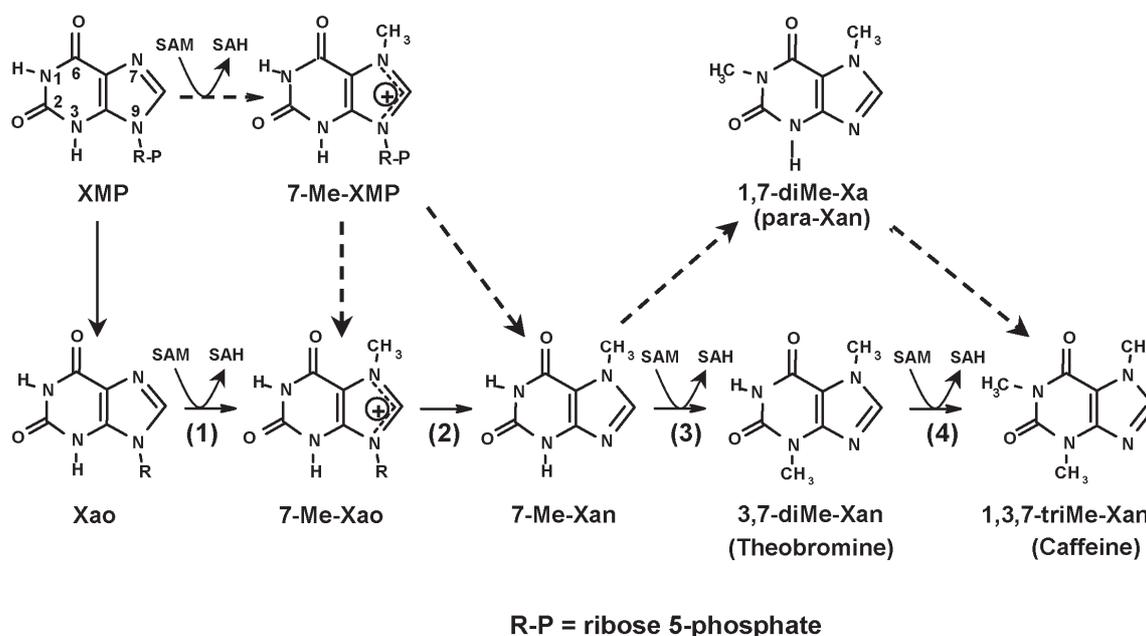
enzyme, with K_i 85–136 μ M (Yamada *et al.*, 1990). It follows that 6-thioxanthine is only parasitostatic because of inhibition of synthesis of guanine nucleotides.

15. BIOSYNTHESIS OF CAFFEINE

Caffeine, a member of a group of compounds known collectively as purine alkaloids, is present in high concentrations in cof-

fee, tea and a few other plant species. The pathway(s) for its biosynthesis, especially in coffee and tea plants, has long been under investigation. This has been stimulated, in part, by the belief that ingestion of coffee may have adverse effects on health, with a consequent increased demand for decaffeinated

Scheme 10, which also displays two minor alternative routes for which there is some experimental evidence (see Ashihara & Crozier, 2001; Mizuno *et al.*, 2003; Uefuji *et al.*, 2003). Note the absence of any reference to the ionic forms of the various substrates, discussed below.



Scheme 10. Proposed pathways for biosynthesis of caffeine in coffee plants.

The major pathway, indicated by solid lines, is according to Mizuno *et al.* (2003) and Uefuji *et al.* (2003). Alternative routes in coffee plants and in tea plants (dashed lines) have been proposed by Schulthess *et al.* (1996), and Kato *et al.* (1996). For the major pathway the enzymes involved, with *S*-adenosyl-L-methionine (SAM) as the methyl donor, are: (1) Xao *N*(7)-methyltransferase, (2) enzymatic cleavage of 7-Me-Xao to 7-Me-Xan (see text for details); (3) 7-Me-Xan *N*(3)-methyltransferase, (4) 3,7-diMe-Xan *N*(1)-methyltransferase. **Note:** see text for discussion of the actual structures of the substrates, and their relevance to the enzymes involved.

beverages, as well as the potential ecological role of caffeine. These aspects, and the impressive progress made during the past few years to elucidate the pathway for caffeine biosynthesis, have been recently reviewed by Ashihara and Crozier (2001), Ashihara and Suzuki (2004) and Kato and Mizuno (2004).

It is now generally accepted that the major pathway for synthesis of caffeine, starting from xanthosine (Xao), or its 5'-phosphate (XMP), proceeds *via* four steps, three of which are catalysed by *N*-methyltransferases, with *S*-adenosyl-L-methionine (SAM) as the methyl donor, as shown by steps 1, 3 and 4 in

The genes coding for the three *N*-methyltransferases have recently been identified, cloned and expressed in *E. coli*, and the resultant recombinant enzymes purified and characterized, including their substrate specificities (Ogawa *et al.*, 2001; Ashihara & Crozier, 2001; Mizuno *et al.*, 2003; Uefuji *et al.*, 2003). Mizuno *et al.* (2003) have also reported the isolation, from *Coffea arabica* L., of a dual-functional caffeine synthase gene, which codes for a protein that catalyses both steps 3 and 4, similar to an enzyme previously isolated by the same authors from tea plants. As pointed out by Ashihara and Crozier (2001),

such developments open up the possibility of producing long desired transgenic caffeine-free tea and coffee plants.

Uefuji *et al.* (2003) have successfully employed the three individual recombinant *N*-methyltransferases, in combination with an *E. coli* extract to cleave 7-Me-Xao to 7-Me-Xan (step 2 in Scheme 10), to achieve the biosynthesis of caffeine, starting from Xao, *in vitro*, in line with the proposed major pathway *in vivo* shown in Scheme 10, and in accord with that much earlier proposed in tea leaf extracts by Negishi *et al.* (1985).

In the experiments of Uefuji *et al.* (2003), it was assumed that step 2, the cleavage of 7-Me-Xao to 7-Me-Xan, was due to a "non-specific" purine nucleoside phosphorylase (PNP) activity in the *E. coli* extract employed. But, as elsewhere shown, the PNP of wild-type *E. coli*, known as PNPI, does not cleave Xao (Stoychev *et al.*, 2002). It is, however, not known whether it will cleave 7-Me-Xao. It would be useful to repeat the *in vitro* biosynthesis, with the use of purified *E. coli* PNPI in place of the bacterial extract. Alternatively, one could use PNPII of *E. coli*, an enzyme induced by cultivation of the bacteria in a medium containing Xao, and for which Xao is an excellent substrate (see Section 11.2). It is, of course, conceivable that the *E. coli* extract contains a nucleosidase (nucleoside hydrolase) which catalyses step 2. Hammer-Jespersen (1983) long ago excluded existence of a ribonucleoside hydrolase in *E. coli*. More recently Petersen & Moller (2001) demonstrated the presence of three ribonucleoside hydrolases in *E. coli*, one of which, coded for by the *rihC* gene, is active against Xao, but the levels of expression of these genes were very low. By contrast, Negishi *et al.* (1985) reported the isolation, but only partial purification, of an enzyme from tea leaves, referred to as methylxanthine nucleosidase, which catalyses the hydrolysis of 7-Me-Xao to 7-Me-Xan. Similar activities have been reported by several observers in extracts of coffee plants (e.g. Roberts & Waller,

1979; Schulthess *et al.*, 1996). It would clearly be desirable to purify and characterize the specificity of these enzymes, in part because of the unusual structure of 7-Me-Xao at physiological pH (see below). Genes containing the characteristic fingerprint motifs for nucleoside hydrolases have been identified in a variety of organisms, including parasites and plants, a subject recently reviewed by Versees and Steyaert (2003). Surprisingly, neither nucleoside hydrolase activity, nor the associate encoding genes, have hitherto been detected in mammals, so that, e.g., parasitic nucleoside hydrolases should be excellent targets for chemotherapeutic intervention.

Reverting to the proposed scheme for biosynthesis of caffeine (Scheme 10), it is indeed astonishing that all the xanthine ring derivatives are implicitly assumed throughout the synthetic pathway to be uniquely in the neutral diketo form! We therefore redirect attention to Scheme 2 and Table 2 on the nature of the neutral and ionic forms of xanthine, methylated xanthines, and their nucleosides and nucleotides, leading to the following comments:

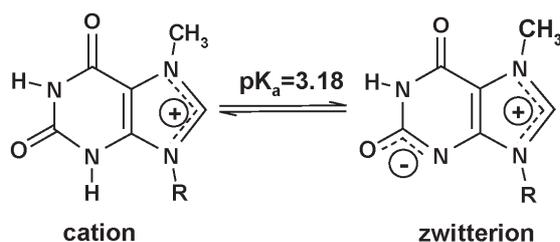
Step 1, catalysed by Xao *N*(7)-methyltransferase. The pH optimum for this reaction *in vitro* has been reported as 7 (Ashihara & Crozier, 2001). At this pH, the substrate Xao (pK_a 5.7) exists about 95% as a monoanion, due to dissociation of the N(3)-H. The acid-base properties of the product, 7-Me-Xao, have not hitherto been characterized; but, by analogy with 7-Me-Ino and 7-Me-Guo, we would expect it to have a pK_a at least ≤ 7 for dissociation of the N(1)-H or N(3)-H, and at pH 7 would exist as an equilibrium mixture of the cation and one of these zwitterions.

However, in the absence of experimental data for 7-Me-Xao, a good evaluation of its structure is forthcoming from a study of Pfleiderer (1961) on the synthesis and properties of purine betaines, including that of xanthine. It was there shown that 7,9-diMe-Xan, which is a formal structural analogue of

7-Me-Xao, exhibited an unusually low pK_a of 3.18 for dissociation of the N(1)-H or N(3)-H. Additional methylation at N(1), so that only the N(3)-H could dissociate, left this pK_a unchanged, whereas methylation at N(3) led to a pK_a of 7.0 for dissociation of the N(1)-H. It follows that, at physiological pH, 7,9-diMe-Xan is uniquely a zwitterion with dissociation of the N(3)-H (Scheme 11). The same would ap-

16. ALKYLXANTHINES: PHARMACOLOGICAL ASPECTS

The marked pharmacological and toxicological effects of caffeine are known to be highly relevant to such phenomena as the blockade of G-protein-coupled adenosine receptors, inhibition of cyclic nucleotide phosphodiesterases, sensitization to calcium of the cyclic



R = CH₃ or ribose

Scheme 11. The cationic and zwitterionic forms of 7,9-diMe-Xan, and, by analogy, of 7-Me-Xao.

Note the low pK_a of 3.2, so that these compounds are virtually zwitterions at physiological pH.

ply to 7-Me-Xao, the zwitterion of which is the product of step 1, and the substrate of step 2 in Scheme 10. Step 3, catalysed by 7-Me-Xan N(3)-methyltransferase, with a very narrow pH optimum at pH 8.5 *in vitro* (Ashihara & Crozier, 2001). At this pH, the substrate 7-Me-Xan (pK_a 8.35) exists as an approximate 1:1 mixture of the neutral and anionic forms. The product, 3,7-diMe-Xan (pK_a 9.9) exists predominantly, > 95%, as the neutral diketo form, and is the substrate in the final step 4.

The *in vitro* synthesis of caffeine from Xao (steps 1 to 4 of Scheme 10) described by Uefuji *et al.* (2003) was performed at pH 8. At this pH, Xao is virtually 100% a monoanion, 7-Me-Xao 100% a zwitterion, 7-Me-Xan a 1:1 mixture of the neutral and monoanionic species, and 3,7-diMe-Xan in the neutral form. It is clearly desirable to establish to what extent the neutral and ionic species are involved in the various steps, e.g. by examining the pH-dependence of each substrate with its appropriate enzyme, and/or the efficiency of the *in vitro* pathway as a function of pH.

ADP ribose-modulated calcium-release channels associated with intracellular stores of calcium, inhibition of GABA_A receptors. The effects of the natural methylxanthines, caffeine and theophylline, on the cardiovascular, respiratory, renal and nervous systems has thus led to the synthesis of an enormous number of alkylated xanthines with a view to obtaining more selective and potent agents, both as tools for elucidation of the mechanisms involved, and as potential chemotherapeutic agents, e.g. antiasthmatics, bronchodilators, cardiac stimulants, treatment of renal failure, etc., recently comprehensively reviewed by Daly (2000).

Most of the synthetic compounds hitherto reported are alkylated at both the ring N(1) and N(3), thus precluding their predominance as anions at physiological pH (see Table 1). Many of these are additionally alkylated at N(7) or N(9), hence exist only as the neutral species. There are, however, instances of mono- and di-N-alkyl analogues where this is not the case, and furthermore, the acid-base

properties of which have been totally disregarded in structure-activity relationship (SAR) studies. We limit ourselves to several typical examples.

One such is a report by Foley *et al.* (2003a) on analogues of 3-alkyl-1,8-dibenzylxanthines which are good GTP-competitive inhibitors of phosphoenolpyruvate carboxykinase, which catalyzes the rate-limiting step in gluconeogenesis, inhibition of which would be expected to lead to a decrease in fasting blood glucose levels in diabetic patients. In this series of compounds, the imidazole ring of the neutral form is protonated. As a 1,3-dialkylxanthine, its pK_a for dissociation of the imidazole proton would be in the vicinity of 9 (see Table 1), if we disregard the possible minor effects of the C(8)-benzyl substituent. It follows that, at physiological pH, the predominant species should be the neutral form, consistent with the conclusion of the authors, on the basis of an SAR study, that the N(7)-H was essential for activity. This was further supported by the demonstration (Foley *et al.*, 2003b) that, in the crystal structure of the enzyme with an inhibitor, the N(7)-H of the latter is a donor, *via* a water molecule, to Trp516 and Trp527 in the active site.

A very active field has been the development of adenosine receptor antagonists as pharmacological tools, and as potential drugs, exemplified by a recent study on a series of 1,8-disubstituted xanthines as potent and selective antagonists of the human A_{2B} adenosine receptor (Hayallah *et al.*, 2002), with potential therapeutic applications in asthma, type II diabetes and cystic fibrosis. These compounds, surprisingly, were found to be equipotent, or even more potent, at A_{2B} receptors than 1,3,8-trisubstituted xanthines. The most selective was 1-propyl-8-*p*-sulphophenylxanthine, with a $K_i \approx 50$ nM at the human A_{2B} adenosine receptors, and an almost 200-fold selectivity *vs* the human A_1 adenosine receptors. Two additional analogues exhibited K_i values at the A_{2B} receptors as low as 1 nM, with an up to 100-fold selectivity *vs*

human A_1 , and > 400-fold selectivity *vs* human A_{2A} and A_3 , receptors. These compounds were initially developed for use in elucidation of the function of the A_{2B} receptor. However, as in all such studies, no consideration was given to the fact that all of them are formally analogues of 1-methylxanthine, with a pK_a for dissociation of the N(3)-H of 7.7, hence with a $\approx 1:1$ population of the neutral and anionic species at physiological pH, clearly relevant to their mode of binding, and use for delineation of receptor function. This also applies to an earlier study by Shamim *et al.* (1989) in which 1-methylxanthine was found to be relatively potent and selective for the human platelet A_2 adenosine receptor, whereas 1-propyl-8-phenylxanthine proved to be a highly potent (K_i 20–70 nM), albeit non-selective at both A_1 and A_2 receptors. It would, of course, be desirable to determine to what extent, if any, the pK_a is affected by 1- and 8-substituents other than a methyl, particularly when such substituents bear a charge, e.g. analogues with the above-mentioned highly negatively charged 8-sulphophenyl moiety (which additionally affects ability to penetrate membranes). Yan and Muller (2004) demonstrated that a nitrophenyl ester of 1-propyl-8-*p*-sulphophenylxanthine, referred to above as a selective A_{2B} antagonist, undergoes cleavage of the nitrophenyl group by a rat liver homogenate, presumably enzymatically, suggesting potential use of the ester as a peroral prodrug. It would be of interest to determine the pK_a of the N(3)-H in this analogue prior to, and following, cleavage of the nitrophenyl moiety. In an earlier study, which included mono- and di-substituted xanthines, Muller *et al.* (1993) noted that analogues with a substituent on the ring N(9), e.g. 9-methylxanthine and 9-ethylxanthine, as well as 1,9-dimethylxanthine, were virtually inactive. This may also be ascribed, at least in part, to the fact that an N(9)-substituent lowers the pK_a to about 6.3, so that these compounds are predominantly anions at physiological pH. The observed inactivity of 7,9-dimethylxan-

thine, on the other hand, is most likely related to its very low pK_a of 3.2, so that it is exclusively a zwitterion at $pH \geq 6$ (see below).

The stimulatory effects of caffeine on skeletal, cardiac and smooth muscle are known to be due largely to increases in intracellular calcium, the putative target being a cyclic ADP-ribose-mediated ryanodine-sensitive calcium-release channel, where calcium increases the sensitivity of the channel to activation by Ca^{2+} . Various alkylxanthine analogues have been employed in SAR studies on calcium-invoked calcium release through such channels, all of them largely empirical, and with no regard to the acid-base properties of the analogues employed. In one such investigation (Rousseau *et al.*, 1988), using methylxanthines, four dimethylxanthines, 1,3-, 1,7-, 3,7- and 3,9- were found to be equally, or even more, effective than caffeine in enhancement of the Ca^{2+} efflux rate from the channels. But no attention was drawn to the fact that all four have a $pK_a \approx 8.6$ (see Table 1), so that their neutral forms predominate at physiological pH. In striking contrast, 1,9-dimethylxanthine, with a $pK_a \approx 6.3$ (see Table 1), due to dissociation of the N(3)-H, and hence predominantly a monoanion at physiological pH, was totally inactive. The authors also refer to earlier reports that 7,9-dimethylxanthine is equally inactive, but they were apparently unaware that this analogue is a purine betaine, with a positive charge on the imidazole ring, and, furthermore, with an unusually low $pK_a \approx 3.2$ for dissociation of the N(3)-H, and is therefore exclusively a zwitterion at physiological pH (see Section 15).

17. 7-METHYLXANTHOSINE-5'-DIPHOSPHATE (m^7XDP) AND mRNA CAP

Nuclear transcribed mRNA possesses a 5'-terminal "cap", m^7GpppN , in which 7-methylguanosine-5'-triphosphate (m^7GTP)

is linked to the 5'-position of the first nucleoside N. This cap structure is required for several functions, including optimal mRNA translation, which is mediated by the protein initiation factor 4E (eIF4E). Consequently the mode of binding of the cap with eIF4E is of some interest, the more so in that m^7Guo at physiological pH exists as a 1:1 equilibrium mixture of the cation, with a positive charge on the imidazole ring, and the zwitterion, due to dissociation of the ring N(1)-H ($pK_a \approx 7.2$).

In the crystal structures of eIF4E complexed with m^7GDP or m^7GpppG , the 7-methylguanine moiety is sandwiched between Trp56 and Trp102, the N(1)-H and the N²H are hydrogen bonded to the Glu103 carboxylate, while the O⁶ accepts a hydrogen from the NH of the Trp102 backbone (Marcotrigiano *et al.*, 1997; Niedzwiecka *et al.*, 2002a). This implies that it is the cation which is bound by eIF4E. But, surprisingly, measurements in solution of the pH-dependence of the association constant for binding displayed a broad plateau between pH 6.6 and 7.6, with only a surprisingly slow decrease in affinity both below and above this pH range (Niedzwiecka *et al.*, 2002a). However, subsequent measurements of the van't Hoff and calorimetric enthalpy values for binding pointed to the cation as the active form (Niedzwiecka *et al.*, 2002b).

The foregoing directed our attention to a report by Shatkin and coworkers (Adams *et al.*, 1978) on the inhibition of reovirus mRNA binding to wheat germ ribosomes at pH 7.6 by m^7GDP and some structurally related analogues, including m^7XDP . It was found that m^7IDP was only 50% as effective as m^7GDP , conceivably due in part to absence of the NH_2 donor at C(2) of the hypoxanthine ring, thus reducing affinity. Moreover, the pK_a for dissociation of the N(1)-H of m^7IDP is at least 0.6 units below that for the N(1)-H of m^7GDP (Kulikowska *et al.*, 1986), so that, at pH 7.6, m^7IDP exists to a much larger extent as the zwitterion, with the consequent absence of

an N(1)-H for binding to the Glu103 carboxylate. Finally, m⁷XDP was found to exhibit only minimal inhibition, if at all. The pK_a of the xanthine ring of m⁷XDP has not been reported, but its structural analogue, 7,9-dimethylxanthine, is highly acidic, with a pK_a ≈ 3.2, for dissociation of the N(3)-H, so that at physiological pH it, and, by analogy, m⁷XDP, would exist exclusively as the zwitterion, and, furthermore, with the N(1) protonated (see Section 15), hence capable of bonding to the Glu103 carboxylate. Nonetheless, the zwitterion proved essentially inactive, most likely due to electrostatic repulsion between the negatively charged O²⁻ and the Glu103 carboxylate, hence in accord with the proposal that the cation of m⁷GDP is the active species.

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