Properties of Purine Nucleoside Phosphorylase (PNP) of Mammalian and Bacterial Origin

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Purine nucleoside phosphorylase (PNP), from calf spleen, human erythrocytes and *E. coli* have been examined with regard to structural requirements of substrates and inhibitors. Kinetic parameters ($K_{m}$, $V_{max}$, $K_{i}$) for a variety of N(1) and/or N(7)-methylated analogues of guanosine, inosine and adenosine have been evaluated for all three enzymes. The substrate and/or inhibitor properties of purine riboside, 1,6-dihydropurine riboside, some deazapurine nucleosides: 3-deaza- and 7-deazainosine, 1,3-dideoxapurine riboside (ribobenzimidazole), and a variety of acyclonucleosides, have been determined with mammalian and bacterial enzymes.

Overall results indicate distinct similarities of kinetic properties and structural requirements of the two mammalian enzymes, although there are some differences as well. The N(1) and O$^\beta$ of the purine ring are necessary for substrate-inhibitor activity and constitute a binding site for the mammalian (but not the bacterial) enzymes. Moreover, nucleosides lacking the N(3) undergo phosphorolysis and those lacking N(7) are inhibitors (but not substrates). Methylation of the ring N(7) leads to two overlapping effects: labilization of the glycosidic bond, and impediment to protonation at this site by the enzyme, a postulated prerequisite for enzymatic phosphorolysis. It is proposed that a histidine interacts with N(1) as a donor and O$^\beta$ as an acceptor. Alternatively N(1)–H and C(2)–NH$_2$ may serve as donors for hydrogen bonds with a glutamate residue.

The less specific *E. coli* enzyme phosphorolyses all purine ring modified nucleosides but 7-deazainosine which is only an inhibitor. On the other hand, the bacterial enzyme exhibits decreased activity towards N(7)-methylated nucleosides and lack of affinity for a majority of the tested acyclonucleoside inhibitors of the mammalian enzymes.

The foregoing results underline the fundamental differences between mammalian and bacterial enzymes, including variations in the binding sites for the purine ring.

Introduction

Purine nucleoside phosphorylase (purine nucleoside: orthophosphate ribosyltransferase, EC 2.4.2.1) catalyzes the reversible phosphorolysis of ribo- and deoxyribonucleosides of guanine and hypoxanthine, as well as of adenine in prokaryotes. Its deficiency results in selective cellular immunodeficiency [1, 2], so that inhibitors of the enzyme are considered to be potentially useful immunosuppressive agents for the chemotherapy of T-cell leukemia, autoimmune diseases and for suppression of the host-versus-graft reaction [3, 4]. They should also be useful in treatment of metabolic disorders, such as xanthine gout [3, 5], and might serve as biochemical modifiers in chemotherapy with purine nucleoside analogues, by minimizing their intracellular cleavage and inactivation [5].

This has stimulated structural and kinetic studies, largely with the purified enzyme from human erythrocytes. Less attention has been devoted to possible differences in specificity of PNP from various sources, of obvious relevance to the search for effective inhibitors. Our previous findings [6] on the importance of the purine ring N(1) as a binding site for the calf spleen, but not the *E. coli*, enzyme, as well as the known difference in specificity towards adenosine and adenine [4], pointed to dissimilarities in structural requirements between the

Abbreviations: PNP, purine nucleoside phosphorylase; m$^1$Ino, 1-methylinosine; m$^1$Guo, 1-methylguanosine; m$^1$Ado, 7-methyladenosine; m$^1$Ino, 7-methylinosine; m$^1$Guo, 7-methylguanosine; m$^1$Guo, 1,7-dimethylguanosine; m$^7$Ade, 7,9-dimethyladenine; P$_i$, orthophosphate; ribobenzimidazole, 1-β-D-ribofuranosylbenzimidazole; 6-thioguanosine, 6-thioguanosine.

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mammalian and bacterial phosphorylases. We have therefore examined the substrate and inhibitory specificities of the enzymes from human erythrocytes and calf spleen, as well as that from a bacterial source, *E. coli*. Our study involved nucleosides with various alterations in the purine aglycon (pyrimidine and imidazole ring modifications), and also acyclonucleoside analogues, with the sugar pentose ring replaced by an acyclic carbon chain. We have explored binding sites for the mammalian enzymes; and studied the role of N(7) methylation, which labilizes the glycosidic bond, but, in the case of guanosine as a substrate for calf spleen enzyme, does not alter substrate properties [6, 7].

A more detailed description of acyclonucleoside inhibitors of mammalian PNP is to be presented elsewhere [8].

**Materials**

Ino, Guo, m'Ino, m'Ade, Hepes, xanthine oxidase (1 U/mg) and calf spleen PNP (25 U/mg) were products of Sigma (St. Louis, Mo., U.S.A.). The source of enzyme from *E. coli* was a partially purified extract (0.4 U/mg) remaining following isolation of uridine phosphorylase [9], and kindly supplied by Dr. Alicja Drabikowska. Human erythrocyte PNP (98 U/mg at pH 7 and 30 °C with 500 μM Ino as substrate) was isolated by affinity chromatography, essentially as described by Osborne [10]. m'Ino was obtained from Cyclo (Los Angeles, Cal., U.S.A.) formycin B from Calbiochem (Zurich, Switzerland), and formycin A from Meiji Saika Kaishi (Kawasaki, Japan).

m'Guo and mG,7Guo were obtained from the corresponding nucleotides by dephosphorylation with alkaline phosphatase as described elsewhere [6]. Both m7Ado and mG,7Ade [11, 12] were kindly furnished by Prof. Tozo Fujii (Kanazawa University, Japan).

7-Deazainosine was prepared according to a known procedure [13] by deamination of tubercidin (7-dezaadenosine) with NaNO₂ in acetic acid.

1,6-Dihydropurine and its riboside were prepared by electrochemical reduction of purine and purine riboside as described by Smith & Elving [14].

Acyclonucleosides were synthesized as elsewhere described [8, 15].

**Ultra-violet absorption spectrophotometry** was performed with a Zeiss (Jena, G.D.R.) Specord UV-VIS M40 recording instrument, or a Zeiss VSU-2P, fitted with thermostatically controlled cell compartments.

**Fluorescence spectra** were recorded with an Aminco-Bowman model SPF spectrofluorimeter equipped with a Hanovia 901 C 150-watt xenon source and a Hammamatsu 1P28 photomultiplier.

Measurements and control of pH made use of a Mera-Elwro instrument with a combination semimicro electrode.

**Thin-layer chromatography**, on Merck (Darmstadt, G.F.R.) cellulose F-254 plates and silica gel F-254 plates, was carried out with solvent systems described in Methods.

**Calculations** were performed with a PDP 11 minicomputer, with the aid of a program previously described [16, 17] and available on request to us, or the BBA Data Bank (citing BBA/DD279/31851/786 (1984) 170).

**Methods**

**Enzyme assays**

Phosphorolysis was generally conducted in the presence of 50 mm phosphate buffer pH 7 at 25 °C, but a few measurements were performed at 37 °C. With Ino as substrate, the standard spectrophotometric assay, by coupling with xanthine oxidase [18], was employed. With other nucleoside substrates, direct spectrophotometry was used, with the following λ<sub>obs</sub> and Δε(λ<sub>obs</sub>): m7Guo - 260 nm (4.6 x 10³); m'Guo - 260 nm (4.0 x 10³); mG,7Guo - 263 nm (5.6 x 10³); Guo - 258 nm (5.5 x 10³); m'Ino - 244 nm (1.34 x 10³); m7Ino - 280 nm (3.5 x 10³); m7Ade - 285 nm (2.55 x 10³); ribo-benzimidazole - 245 nm (1.74 x 10³); 6-thioGuo - 275 nm (2.8 x 10³).

With continuous monitoring, reactions with the mammalian enzymes were followed to completion. With good substrates (Ino, m7Guo), the use of ~0.01 U/ml PNP, and an excess of xanthine oxidase with Ino, and 0.005 U/ml with m7Guo, led to completion of the reaction in 15–20 min. With feebler substrates (m7Ado, mG,7Guo), 0.1–1 U/ml PNP was necessary for complete phosphorolysis in 1 h.

With the initial velocity method, lower enzyme concentrations were employed, 0.002–0.02 U/ml
for the mammalian enzymes, and 0.007–0.02 U/ml for the bacterial PNP. Kinetic parameters for phosphorolysis by the *E. coli* enzyme were determined only by the initial velocity method, because of inhibition by the liberated ribose-1-phosphate with a $K_i = 170 \mu M$ [19].

One unit of PNP is the amount of enzyme that converts 1 μmol Ino to Hx per min at 25 °C in the presence of 50 mM phosphate at pH 7 and 500 μM Ino, and an excess of xanthine oxidase.

**Calculation of kinetic parameters**

With continuous monitoring, about 10–20 experimental points, taken from the curve representing the total course of phosphorolysis, were fitted by a weighted linear least-squares procedures [17] to the integrated form of the Michaelis-Menten equation for a one-substrate-one-product reaction [20, 21]:

$$t = \frac{K_m}{V_{max}} \ln\left(\frac{c_o}{c}\right) = c_o \left(1 - \frac{c}{c_o}\right)$$

or, when $c_o \ll K_m$, to the pseudo-first order equation:

$$t = \frac{K_m}{V_{max}} \ln\left(\frac{c_o}{c}\right)$$

where $c_o$ is initial substrate concentration, $c$ is the concentration at time $t$, and $K_m$ and $V_{max}$ are apparent values.

For both Ino (when coupled with xanthine oxidase) and m7 Guo the calculated apparent $K_m$ and $V_{max}$ were independent of the initial substrate concentration in the range 50–120 μM for Ino, and 25–100 μM for m7 Guo, and were in good agreement with kinetic parameters obtained by the initial velocity method. Hence the foregoing procedure gives the real values of the parameters for Ino and m7 Guo with both mammalian enzymes, product inhibition by ribose-1-phosphate and m7 Gua being insignificant under these conditions.

With the initial velocity method, Ino concentrations were in the range 10–500 μM, m7 Guo 20–700 μM, m7 Guo 10–100 μM, m7 Ino 25–120 μM, Guo 10–100 μM, m7 Ino 10–1000 μM and 6-thioGuo 20–500 μM. The $K_m$ and $V_{max}$ were determined by linear regression analysis from Eadie-Hofstee plots of $v_o$ vs. $v_o/c_o$ [20]. However, in the case of the human PNP, which exhibits substrate activation [4] only the linear portion for low substrate concentrations was utilized.

Acyclonucleosides and 1,6-dihydropurine riboside were tested for inhibition of phosphorolysis of Ino, following independent confirmation that none of them affected xanthine oxidase activity used in the coupled assay. 7-Deazainosine is an inhibitor of xanthine oxidase, so its properties as inhibitor of PNP were tested with m7 Guo as a substrate. Formycins are also inhibitors of xanthine oxidase, but only at concentrations an order of magnitude higher than those employed for inhibition of bacterial PNP.

With mammalian enzymes phosphorolysis of Ino (and m7 Guo) with inhibitors was followed to completion. With the bacterial enzyme, the initial velocity method was employed. Inhibition constants $K_i$ were calculated, using kinetic parameters for Ino (or m7 Guo) as standards, from the equation [20]:

$$K_i = [I] \left(\frac{K_{m,app}}{K_{m}} - 1\right)^{-1}$$

where $K_m$ is the Michaelis constant for Ino (or m7 Guo), $K_{m,app}$ is the value in the presence of inhibitor and [I] is the inhibitor concentration.

**Thin-layer chromatography**

Substrate properties of Ado, m7 Ado, purine riboside, benzimidazole and 7-deazainosine were also monitored by TLC. In all cases (except for 7-deazalno) products of phosphorolysis (the parent purine analogue) were identified against authentic sample. The solvent systems and $R_f$ values are listed in Table I.

<table>
<thead>
<tr>
<th>Analogue</th>
<th>Solvent</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>m7 Ade</td>
<td>0.19</td>
<td>0.12</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>m7 Ado</td>
<td>0.05</td>
<td>0.00</td>
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<td></td>
<td></td>
</tr>
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<td>Ade</td>
<td>0.64</td>
<td></td>
<td>0.61</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ado</td>
<td>0.55</td>
<td></td>
<td>0.55</td>
<td></td>
<td></td>
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<tr>
<td>Purine</td>
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<td></td>
<td>0.65</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Purine riboside</td>
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<td></td>
<td>0.58</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Benzimidazole</td>
<td>0.75</td>
<td>0.58</td>
<td>0.83</td>
<td>0.76</td>
<td></td>
</tr>
<tr>
<td>Ribobenzimidazole</td>
<td>0.68</td>
<td>0.69</td>
<td>0.77</td>
<td>0.81</td>
<td></td>
</tr>
<tr>
<td>7-DeazaIno</td>
<td>0.54</td>
<td></td>
<td>0.45</td>
<td>0.72</td>
<td></td>
</tr>
</tbody>
</table>

Table 1. $R_f$ values for m7 Ade (silica gel plates), and other nucleosides and corresponding bases (cellulose plates); solvent (A) n-butanol:acetic acid:H2O (5:2.5:2.5); solvent (B) chloroform:methanol (8.5:1.5); solvent (C) sec-butanol:H2O (upper layer) and solvent (D) 1 M ammonium acetate:H2O (2:5).
Quantum yield measurements

Emission spectra were corrected for the spectral sensitivity of the detector. Quantum yields for fluorescence of m'Ado and m'2'Ado were determined at room temperatures (20 °C) at pH 1 (H₂SO₄) and pH 7 (0.02 M Hepes/NaOH buffer), using three different standards: quinine bisulphate in 1 N H₂SO₄, for which \( \phi = 0.55 \) with \( \lambda_{\text{exc}} = 366 \text{ nm} \) [22]; anthracene in methanol, \( \phi = 0.30 \) with \( \lambda_{\text{exc}} = 366 \text{ nm} \) [22]; and m'Guo at pH 3 (H₂SO₄), \( \phi = 0.012 \) with \( \lambda_{\text{exc}} = 281-300 \text{ nm} \) [23]. Corrections were made for differences in source intensity at various excitation wavelengths.

Protein concentrations

Protein concentrations of the enzymes were determined by the method of Lowry [24], with human serum albumin as standard.

Substrate concentrations

Substrate concentrations were determined spectrophotometrically (at pH 7, except for m'Ado and m'Ino), as follows: m'Guo, \( \lambda_{\text{max}} = 258 \text{ nm} \) (\( \varepsilon = 13.4 \times 10^3 \)); m'2'Guo, \( \lambda_{\text{max}} = 263 \text{ nm} \) (\( \varepsilon = 11.2 \times 10^3 \)); m'1Ino, \( \lambda_{\text{max}} = 251 \text{ nm} \) (\( \varepsilon = 10.0 \times 10^3 \)); Guo, \( \lambda_{\text{max}} = 252.5 \text{ nm} \) (\( \varepsilon = 13.6 \times 10^3 \)); Ino, \( \lambda_{\text{max}} = 248 \text{ nm} \) (\( \varepsilon = 12.3 \times 10^3 \)) [25, 26]; m'Ado, \( \lambda_{\text{max}} = 271 \text{ nm} \) (\( \varepsilon = 12.8 \times 10^3 \)) [11]; 7-deazaIno, \( \lambda_{\text{max}} = 260 \text{ nm} \) (\( \varepsilon = 10.8 \times 10^3 \)); benzimidazole riboside, \( \lambda_{\text{max}} = 245 \text{ nm} \) (\( \varepsilon = 6.8 \times 10^3 \)) [27]; 6-thioGuo \( \lambda_{\text{max}} = 257 \text{ nm} \) (\( \varepsilon = 8 \times 10^3 \)). The extinction coefficient for 7-deazaIno at pH 7 was calculated relative to that in methanol \( \lambda_{\text{max}} = 259 \text{ nm} \) (\( \varepsilon = 9.7 \times 10^3 \)) [28]. For m'Guo and m'Ino, which are mixtures of two ionic forms at pH 7 (pKₐ = ~6.8 and 6.4 respectively [29–31]), the concentrations were determined, following completion of phosphorolysis, from the spectral constants of the products, m'Gua, \( \lambda_{\text{min}} = 260 \text{ nm} \) (\( \varepsilon = 3.9 \times 10^3 \)) [25] and m'Hx, \( \lambda_{\text{max}} = 256 \text{ nm} \) (\( \varepsilon = 8.5 \times 10^3 \)) at pH 7 [7]; for m'Guo at pH 2, \( \lambda_{\text{max}} = 258 \text{ nm} \) (\( \varepsilon = 10.0 \times 10^3 \)) [25].

Results and Discussion

Role of purine ring N(1) and O6 in binding to PNP

It has been generally postulated that the major difference in specificity between mammalian and bacterial phosphorylases towards the purine base is the susceptibility to phosphorolysis or synthesis of adenosine. For mammalian PNP literature data are somewhat conflicting [4, 32, 33], while for the E. coli enzyme, both synthesis (Kᵢ = 40 μM) and phosphorolysis of Ado occur [36].

To test the affinity of mammalian PNP for Ado, experiments were run with an excess of enzyme, and reactions followed spectrophotometrically, as well as by TLC, with solvents A and C (see Methods), using the E. coli extract as a control. In contrast to the bacterial enzyme, there was no detectable phosphorolysis of Ado by both mammalian enzymes (see Table IV). The small decrease in UV absorbance noted with the calf spleen enzyme was found due to presence of traces of adenosine deaminase (further confirmed by TLC), and was eliminated by addition to the incubation medium of 5 μM 2'-deoxycoformycin, a potent tight-binding inhibitor of the deaminase [37].

The lack of affinity of human PNP for Ado is further supported by the known fact that the enzyme is inhibited by formycin B [38, 39] with a 7-keto group (corresponding to the 6-keto in a purine), but not by formycin A with a 7-amino. The bacterial enzyme is inhibited more effectively by formycin B, with \( K_i = 4.5 \mu M \), as compared to 100 μM with the human enzyme [38], but is also equally effectively inhibited by formycin A (\( K_i = 5.5 \mu M \)) (see Table II).

Lack of activity vs. Ado hence appears general for eukaryotic PNP. Even the enzyme from the malaria parasite Plasmodium falciparum exhibits no such activity [40]. In higher plants, phosphorolysis of Ado and Ino is due to adenosine nucleosidase (EC 2.2.2.7) and inosine nucleosidase (EC 3.2.2.2), respectively [41]. On the other hand, whereas PNP of the prokaryotes E. coli [36] and S. typhimurium [42] are active vs. Ado, B. cereus appears to possess a specific adenosine phosphorylase [43].

The mammalian and bacterial enzymes differ with regard to their recognition of the purine O6. Purine riboside is not a substrate (or inhibitor) of the calf spleen enzyme (Table II) and phosphorolysis of Ino by mammalian PNP is not inhibited by 6-deoxyacyclovir at a concentration of 300 μM, while the parent acyclovir inhibits the human erythrocyte [44] and calf spleen [8] enzyme with \( K_i \) values of 91 μM and 60 μM, respectively. Also methylation of O6 leads to a striking reduction in affinity
Table III. Effect on $K_i$ of modifications of the ring N(1) and O$^\circ$ of guanine and hypoxanthine with human erythrocyte PNP; and physical properties of substituents and their hydrogen bond with nitrogen.

<table>
<thead>
<tr>
<th>Base</th>
<th>$K_i$ [µM]</th>
<th>Modification</th>
<th>van der Waals' radius$^a$ [Å]</th>
<th>Hydrogen bond length [Å]</th>
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<tr>
<td></td>
<td></td>
<td>none</td>
<td>N(1)− CH$_3$</td>
<td>O− H− N</td>
</tr>
<tr>
<td>Hx</td>
<td>10</td>
<td>2500</td>
<td>73</td>
<td>2.9$^c$</td>
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<tr>
<td>Gua</td>
<td>22</td>
<td>1300</td>
<td>110</td>
<td>2.9$^c$</td>
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$^a$ From [45]; $^b$ from [55]; $^c$ from [53]; $^d$ mean H− O and H− S distance [54].

Role of other modifications of purine ring

In contrast to the purine ring N(1), the ring N(3) appears to be of lesser significance for binding and phosphorolysis, since 3-deazainosine is a relatively good substrate for both the $E. coli$ and calf spleen enzymes (Table II), and 3-deazaguanosine is a substrate ($K_m = 233$ µM) and inhibitor of human erythrocyte PNP [62]. Surprisingly, isoadenosine, 3-(β-D-ribofuranosyl)adenine, has been described as a substrate and inhibitor of the bacterial enzyme with $K_i \sim 1150$ µM [36].

On the other hand, methylation of the ring N(3) of the parent purines leads to a drastic decrease in affinity for the human erythrocyte PNP, the $K_i$ values for hypoxanthine, 3-methylhypoxanthine, xanthine and 3-methylxanthine being 10 µM, 1400 µM, 39 µM and 1400 µM, respectively [45].

Removal of both N(1) and N(3) does not fully eliminate susceptibility to phosphorolysis by the bacterial enzyme, since 1-(β-D-ribofuranosyl)-benzimidazole (1,3-dideazapurine riboside) is a weak substrate ($V_{max}/K_m \sim 1\%$ that for Ino, Table II), possibly due to the higher stability of the glycosidic bond relative to that of purine nucleosides [63]. As anticipated, it is not a substrate for the calf spleen PNP.

It appeared of interest to examine the behaviour of a reduced purine ring with different planarity and electron distribution. In fact, 1,6-dihydropurine riboside (Table II) proved to be a weak substrate, and a good inhibitor ($K_i \sim 25$ µM) of the $E. coli$, but not calf spleen, enzyme.

Role of the purine ring N(7)

Although 7-deazainosine is not a substrate for calf spleen, or $E. coli$, PNP, it exhibits reasonable affinity for both enzymes, with $K_i$ values of ~60 µM and ~85 µM, respectively (Table II), and...
Table IV. Kinetic parameters for phosphorolysis of some nucleosides by PNP from human erythrocytes, calf spleen and *E. coli*. All reactions at 25 °C and pH 7 in the presence of 50 mM phosphate were monitored spectrophotometrically, in the case of inosine by the coupled xanthine oxidase assay [18]. Kinetic parameters were determined by continuous monitoring with mammalian enzymes (except for m’Ino, 6-thioGuo and phosphate) and initial velocities with the bacterial enzyme. Unless otherwise indicated (~), errors are ±15%.

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<tbody>
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<td>Ino</td>
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<td>13</td>
<td>32</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
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<td>~120</td>
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<td>260</td>
<td>110</td>
<td>h</td>
<td>c</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>m’Guo</td>
<td>h</td>
<td>d</td>
<td>22</td>
<td>h</td>
<td>d</td>
<td>54</td>
<td>~0.03</td>
<td>0</td>
<td>790</td>
<td>h</td>
<td>c</td>
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<tr>
<td>m’Guo</td>
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<td>~36</td>
<td>~130</td>
<td>28</td>
<td>240</td>
<td>280</td>
<td>25</td>
<td>~300</td>
<td>c</td>
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<td>6-thioGuo</td>
<td>167</td>
<td>~35</td>
<td>~95</td>
<td>h</td>
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<td>h</td>
<td>h</td>
<td>~1</td>
<td>~0.01</td>
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<td>h</td>
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</tr>
<tr>
<td>m’Ado</td>
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<td>h</td>
<td>~110</td>
<td>h</td>
<td>h</td>
<td>~89</td>
<td>~0.2</td>
<td>~0.1</td>
<td>26</td>
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<tr>
<td>P’(Ino)</td>
<td>~910</td>
<td>~860</td>
<td>h</td>
<td>100</td>
<td>100</td>
<td>h</td>
<td>100</td>
<td>100</td>
<td>h</td>
<td>~3000</td>
<td>e</td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>P’(m’Guo)</td>
<td>~2040</td>
<td>~90</td>
<td>~240</td>
<td>~270</td>
<td>h</td>
<td>~107</td>
<td>~2580</td>
<td>h</td>
<td>~7000</td>
<td>c</td>
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</table>

* a by initial velocity method $K_m$ = 28 µM and $V_{max}$ = 112% that obtained by continuous monitoring method; 
  b by initial velocity method $K_m$ = 11 µM and $V_{max}$ = 105% that obtained by continuous monitoring method; 
  c no substrate activation; d not a substrate; e at pH 7.5 and 30 °C $K_m$ for Guo is 32 µM and $V_{max}$ 50% that for Ino [68]; f by initial velocity method; g not determined; h by initial velocity method $K_m$ = 18.5 µM and $V_{max}$ = 104% that obtained by continuous monitoring method; i from ref. [49].

somewhat lower for the human erythrocyte PNP, $K_m$ 330 µM [4]. For the *E. coli* enzyme, $K_m$ values of 120 µM and 250 µM for 7-deazaadenosine and 7-deazainosine (both non-substrates) have been reported [36]. Hence N(7) is probably not a binding site, but rather serves as a site for protonation by the enzyme, leading to labilization of the glycosidic bond [6, 64], as in the case of non-enzymatic hydrolysis of purine nucleosides [65].

From Table IV it will be seen that m’Guo is an excellent substrate of the human PNP, with $V_{max}/K_m$ 2.5-fold that for Ino. This is due to a simultaneous increase in $V_{max}$ and decrease in $K_m$ (15–19 µM compared to 26–28 µM for Ino). Since m’Guo at pH 7 is a mixture of two ionic forms, the cation and the zwitterion, with pK_a = 6.8 [29], the measured kinetic parameters are the resultants for the two forms. The calf enzyme exhibits a preference for the cation, in which N(1) is protonated, a conclusion based on the pH-dependence of phosphorolysis [7]. With the human enzyme the rate of phosphorolysis of m’Guo in the pH range 6.5–8.5 differs from that for Ino (see Fig. 2), decreasing with increase in pH (while for Ino maximum activity is at pH 7.5), thus pointing to a preference for the cationic form, as for the calf enzyme.

The second fluorescent substrate of the calf PNP, m’Ino [7], exhibits similar properties with the human enzyme (see Table IV), viz. a high $V_{max}$ relative to the parent Ino, and a correspondingly higher value for $K_m$, due to its low pK_a value (hence a lower cation concentration), such that the resultant rate constant is only slightly lower than for Ino.

With the bacterial PNP, on the other hand, both N(7)-methylated nucleosides are phosphorolyzed at lower rates (Table IV).

A highly significant difference between the two mammalian enzymes is the phenomenon of substrate activation [4], observed only for the human PNP at concentrations of Ino ≥ 120 µM, of m’Guo ≥ 300 µM and phosphate ≥ 3 mM (Table IV). However, with the calf enzyme, such substrate activation has been reported with alternative substrates, dIno and dGuo [66].

While Ado is not a substrate for mammalian enzymes, activation has been reported with alternative substrates, dIno and dGuo [66].
PNP, it will be seen from Table IV that m7Ado is very slowly phosphorolyzed by both mammalian enzymes with rate constants 0.2% and 0.1%, respectively, that for Ino. Because of the unusual nature of m7Ado, including its lability even at neutral 

phosphate at 25 °C and the reactions were followed spectrophotometrically.

An analogous effect of N(7)-methylation on substrate properties prevails with m1'Guo, in that m1,7'Guo is very slowly phosphorolyzed by the calf enzyme ($V_{\text{max}}/K_m \sim 0.01\%$ the rate for Ino). With the human PNP the rate constant for m1,7'Guo is 3-fold that for m1'Guo.

Hence, in general, N(7)-methylation, which introduces a positive charge on the imidazole ring, confers weak substrate properties with the mammalian enzymes on compounds not detectably phosphorolyzed or enhances several-fold the rate constant or $V_{\text{max}}$. With the calf spleen enzyme, however, it was shown [6] that a positive charge on the imidazole ring does not enhance affinity for the enzyme (comparable inhibitor properties of m'Gua and m2,7'Gua). It follows that the role of methylation at N(7) may be labilization of the glycosidic bond [65].

Under conditions of non-enzymatic hydrolysis, N(7)-methylation is known to labilize the glycosidic bond by several orders of magnitude [65]. It has been suggested [6] that such methylation may have also a reverse effect viz. impediment to protonation of N(7) by the enzyme, a proposed prerequisite for enzymatic phosphorolysis [64].

In contrast to the extremely feeble substrate properties of m7Ado with the mammalian enzymes, it is readily phosphorolyzed by the *E. coli* PNP, $K_m \sim 110 \mu M$ as compared to 32 $\mu M$ for Ino, and $V_{\text{max}}$ comparable to that for Ino (see Table IV).

**Fig. 2.** pH-Dependence of $K_m$ (lower frame) and $V_{\text{max}}/K_m$ (upper frame) for phosphorolysis by human purine nucleoside phosphorylase of Ino ($\triangle$) and m7Guo ($\bullet$). Measurements were carried out in the presence of 50 mM phosphate at 25 °C and the reactions were followed spectrophotometrically.

**Fig. 3.** Absorption spectra of m7Ado prior to (A) and following (B) complete phosphorolysis by human purine nucleoside phosphorylase, and after 5 h at 37 °C (C) following imidazole ring opening.
Fig. 4. Fluorescence spectra of m7Ado (16 μM) prior to (A) and following complete phosphorolysis (B) by E. coli purine nucleoside phosphorylase; λ<sub>exc</sub> = 275 nm.

Spectral properties of m7Ado

Phosphorolysis of m7Ado is accompanied by only a small decrease in absorbance in the region 260–290 nm (Fig. 3), but with virtual disappearance of fluorescence in the region 300–500 nm (Fig. 4), since its quantum yield at neutral pH is about 0.055, whereas that of the base, m7Ade, is about 0.001 (see Table V). It is not, however, a convenient fluorescent substrate since, even at neutral pH (and more so at pH >7), the imidazole ring undergoes opening with a rate constant k ~ 10<sup>-4</sup> sec<sup>-1</sup> at 37 °C, reflected in a decrease in absorbance in the range 245–300 nm (Fig. 3).

As might be anticipated, m27-9Ade is also fluorescent, with a lower quantum yield, ~0.037, and an emission maximum at 370 nm, as compared to 395 nm for m7Ado (see Table V). It is more resistant to ring opening, the rate constant being 10<sup>-3</sup> sec<sup>-1</sup> at pH 10.6 at 18 °C as compared to 2 × 10<sup>-3</sup> sec<sup>-1</sup> for m7Ado at pH 8.5 at the same temperature.

The emission properties of m7Ado and m27-9Ade are similar to those reported by Knighton et al. [68], who demonstrated that the emission in acid medium of Ado is due to protonation of N(7), of m7Ade to protonation of N(9), and of Ade to protonation of both N(7) and N(9). For Ado in acid medium, the emission maximum was at 392 nm, close to that for m7Ado at pH 1–7 (see Table V). For Ade and m7Ade, the maximum is in the range 362–365, hence similar to that observed in the present study for m27-9Ade.

Inhibition of enzymes by acyclonucleosides

The enzymes from human erythrocytes [69] and E. coli [36] exhibit similar tolerance towards substrates with a modified sugar ring, e.g. deoxyribonucleosides are also substrates; but modifications at C(2') and C(3') drastically reduce or eliminate substrate properties. By contrast the steric configuration, or the absence, of the 5'-OH is of no significance for substrate binding, since the E. coli enzyme phosphorolyses α-L-lyxosyladenine [36], while the human erythrocyte PNP phosphorolyses 5'-deoxy- and 2',5'-dideoxyinosines and α-L-lyxosylhypoxanthine [69].

As we now show, there are differences in affinity between the mammalian and E. coli enzymes for acyclonucleosides.

Both mammalian enzymes are similarly inhibited by the members of a series of acyclonucleoside

Table V. Spectral data for absorption and fluorescence emission of m7Ado and m27-9Ade.

<table>
<thead>
<tr>
<th>Compound</th>
<th>pH</th>
<th>Absorption λ&lt;sub&gt;max&lt;/sub&gt; [nm]</th>
<th>Emission λ&lt;sub&gt;max&lt;/sub&gt; [nm]</th>
<th>λ&lt;sub&gt;exc&lt;/sub&gt; [nm]</th>
<th>λ&lt;sub&gt;fluor&lt;/sub&gt; [nm]</th>
<th>Φ (&lt;sup&gt;×10&lt;sup&gt;-2&lt;/sup&gt;&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>m7Ado</td>
<td>7.0</td>
<td>271</td>
<td>395</td>
<td>260–285</td>
<td>12.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.7&lt;sup&gt;b&lt;/sup&gt;, 5.5&lt;sup&gt;c&lt;/sup&gt;, 4.2&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>271</td>
<td>395</td>
<td>260–285</td>
<td>12.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.8&lt;sup&gt;b&lt;/sup&gt;, 4.1&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>m27-9Ade</td>
<td>7.0</td>
<td>269</td>
<td>370</td>
<td>265–285</td>
<td>12.1&lt;sup&gt;e&lt;/sup&gt;</td>
<td>4.5&lt;sup&gt;b&lt;/sup&gt;, 3.9&lt;sup&gt;e&lt;/sup&gt;, 2.9&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>268</td>
<td>370</td>
<td>265–285</td>
<td>11.9&lt;sup&gt;e&lt;/sup&gt;</td>
<td></td>
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</tbody>
</table>

<sup>a</sup> Data from [11]; <sup>b</sup> with quinine bisulphate in 1 N H<sub>2</sub>SO<sub>4</sub> as standard (Φ = 0.55) [22]; <sup>c</sup> with anthracene in ethanol as standard (Φ = 0.30) [22]; <sup>d</sup> with m7Guo in pH 3 (H<sub>2</sub>SO<sub>4</sub>) as standard (Φ = 0.012) [23]; <sup>e</sup> data from [12].
analalogues of guanosine and inosine, in which the pentose ring is replaced by aliphatic chains, with 4 or more carbons, in most instances with substituents on the carbons. The $K_i$ values for several inhibitors, with Ino as substrate, are listed in Fig. 5.

The importance of an amino group at C(2) of the base appears to be similar for both enzymes, in that acycloguanosines exhibit $K_i$ values several-fold lower than the corresponding acycloinosines (Fig. 5), in contrast to Guo and Ino, which have comparable Michaelis constants. A similar, even more pronounced, effect has been noted for inhibition of human erythrocyte PNP by the 8-amino-9-benzyl analogues of guanine and hypoxanthine [61]. All these support the idea [69] that the binding of acyclonucleosides differs from that of the parent nucleosides.

The bacterial enzyme was not inhibited by compounds 2, 6, 8, 12, and 16, at concentrations several-fold higher than their $K_i$ values for inhibition of the mammalian enzymes (Fig. 5, 6). Only compound 15 inhibited the *E. coli* PNP, with $K_i = 13 \mu M$, a value still 5-fold higher than that for the mammalian enzymes.

**Concluding Remarks**

The foregoing findings are relevant to the similar physicochemical properties of the two mammalian enzymes. Both are trimers, with subunits of 30 kDa, similar CD spectra, hence similar secondary structure [71]. Histidine [4, 48], cysteine [4, 48], and arginine [72, 73] residues have been implicated in the catalytic activity of both enzymes. But the two enzymes differ in some kinetic properties, e.g. substrate activation at high concentrations. This may be related to the fact that the molecular weights for the human and calf enzymes differ (91 kDa and 86 kDa) and the amino acid compositions, while similar, are not identical [71].

The present results do underline some kinetic similarities between the two mammalian enzymes, e.g. inability to phosphorolyse Ado; effects of N(1) methylation, resulting in a dramatic decrease or total absence of activity; effects of N(7)-methylation, leading to a several-fold increase in $V_{\text{max}}/K_m$ or $V_{\text{max}}$, which, for compounds not detectably phosphorolyzed (m²Ino, Ado), confers weak substrate properties. Both enzymes also exhibit a pref-
ence for the cationic form of m7 Guo, relative to the zwitterion. And the acycloguanosine analogues are better inhibitors than the corresponding acyclonucleosines.

The foregoing results are consistent with similar binding by the active centre of each enzyme to the O6, N(1) and C(2)–NH2 of the purine base; and in accord with the catalytic mechanism proposed for phosphorolysis by the calf thyroid enzyme [48], viz. binding of a histidine residue to O6 and N(1) (but with reverse donor and acceptor properties) or the alternative mechanism referred to above, involving histidine and glutamic acid residues, followed by phosphorolysis of the glycosidic bond via protonation of the imidazole ring N(7), as for acid-catalyzed depurination of purine nucleosides [65].

Quite impressive is a comparison of the mammalian and bacterial enzymes, hitherto delineated only by the ability of the latter to phosphorolysyr. It is clear that both the calf and bacterial enzymes degrade 3-deazainosine and bind, but do so at C(5'). More striking are the differences. In contrast to the mammalian enzymes, the E. coli enzyme phosphorolyses N(1)-methyl nucleosides, purine ribosides lacking the O6, nucleosides with a reduced pyrimidine ring such as 1,6-dihydropurine riboside, or lacking the N(1) and N(3), such as ribobenzimidazole. Finally, some acyclonucleosides which exhibit high affinity for both mammalian enzymes are not bound by the bacterial enzyme.

The overall results not only provide a series of criteria for distinguishing between mammalian and bacterial PNP, but underline the structural differences between the two. It will be of interest to extend these studies to PNP from other bacterial sources, as well as to undertake a search for selective inhibitors of the bacterial enzymes which do not affect those from mammalian sources. Research in progress has already led to identification of one such inhibitor, viz. formycin A.

Acknowledgements

We are indebted to Dr. Tozo Fujii (Japan) and Dr. N. G. Johansson (Medivir, Sweden) for gifts of m7Ado and acyclonucleosides, to Dr. Elżbieta Bojarska for preparation of 1,6-dihydropurine riboside, and to Mrs. Lucyna Magnowska for excellent technical assistance. This investigation profited from the support of the Ministry of Higher Education (R.P.II. 13.1.8), the Polish Cancer Research Program (C.P.B.R. 11.5-109) and the Polish Academy of Sciences (C.P.B.R. 3.13).
