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 ribosides, 1,6-dihydropurine riboside, some deazapurine nucleosides: 3-deaza- and 7-deazainosine, 1,3-dideazapurine 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 O6 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 phosphorylase 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 phosphorylase. It is proposed that a histidine interacts with N(1) as a donor and O6 as an acceptor. Alternatively N(1)–H and C(2)–NH2 may serve as donors for hydrogen bonds with a glutamate residue.

The less specific E. coli enzyme phosphorylates 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 phosphorylation 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; m7Ino, 1-methylinosine; m7Guo, 1-methylguanosine; m7Ado, 7-methyladenosine; m7Ino, 7-methylinosine; m7Guo, 7-methylguanosine; m1,3,7Guo, 1,7-dimethylguanosine; m1,6Ado, 7,9-dimethyladenine; P, orthophosphate; ribobenzimidazole, 1-ß-D-ribofuranosylbenzimidazole; 6-thioGuo, 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'7 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 m'1,7 Guo were obtained from the corresponding nucleotides by dephosphorylation with alkaline phosphatase as described elsewhere [6]. Both m'7 Ado and m'2,7,9 Ade [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-deazaadenosine) 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].

Ayclonucleosides 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 901C 150-watt xenon source and a Hamamatsu 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 \( \lambda_\text{obs} \) and \( \Delta \varepsilon(\lambda_\text{obs}) \): m'7 Guo - 260 nm (4.6 x 10³); m'Guo - 260 nm (4.0 x 10³); m'2,7 Guo - 263 nm (5.6 x 10³); Guo - 258 nm (5.5 x 10³); m'1 Ino - 244 nm (1.34 x 10³); m'7 Ado - 280 nm (3.5 x 10³); m'7 Ado - 285 nm (2.55 x 10³); ribobenzimidazole - 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, m'7 Guo), the use of ~0.01 U/ml PNP, and an excess of xanthine oxidase with Ino, and 0.005 U/ml with m'7 Guo, led to completion of the reaction in 15–20 min. With feebler substrates (m'7 Ado, m'1,7 Guo), 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 imol 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]:

$$
t = \frac{K_m}{V_{max}} \ln\left(\frac{c_o}{c}\right) + \frac{c_o}{V_{max}}(1 - c/c_o),
$$
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 m'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 m'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 m'Guo with both mammalian enzymes, product inhibition by ribose-1-phosphate and m'Gua being insignificant under these conditions.

With the initial velocity method, Ino concentrations were in the range 10–500 μM, m'Guo 20–700 μM, m'Guo 10–100 μM, m'Ino 25–120 μM, Guo 10–100 μM, m'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 m'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 m'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 m'Guo) as standards, from the equation [20]:

$$
K_i = [I] (K_m^{app}/K_m - 1)^{-1}
$$

where $K_m$ is the Michaelis constant for Ino (or m'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, m'Ado, purine riboside, benzimidazole and 7-deazainosine were also monitored by TLC. In all cases (except for 7-deazaIno) products of phosphorolysis (the parent purine analogue) were identified against an authentic sample. The solvent systems and $R_f$ values are listed in Table I.

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

Emission spectra were corrected for the spectral sensitivity of the detector. Quantum yields for fluorescence of m7Ado and m7,9Ado 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 0 = 0.55 with λmax 366 nm [22]; anthracene in methanol, 0 = 0.30 with λmax 366 nm [22]; and m7Guo at pH 3 (H2SO4), 0 = 0.012 with λmax 281–300 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 m7Ado and m7Ino), as follows: m7Guo, λmax = 258 nm (ε = 13.4 x 103); m7,9Guo, λmax = 263 nm (ε = 11.2 x 103); m7Ino, λmax = 251 nm (ε = 10.0 x 103); Guo, λmax = 252.5 nm (ε = 13.6 x 103); Ino, λmax = 248 nm (ε = 12.3 x 103) [25, 26]; m7Ado, λmax = 271 nm (ε = 12.8 x 103) [11]; 7-deazaIno, λmax = 260 nm (ε = 10.8 x 103); benzimidazole riboside, λmax = 245 nm (ε = 6.8 x 103) [27]; 6-thioGuo, λmax = 257 nm (ε = 8 x 103). The extinction coefficient for 7-deazaIno at pH 7 was calculated relative to that in methanol λmax = 259 nm (ε = 9.7 x 103) [28]. For m7Guo and m7Ino, 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, m7Gua, λmin = 260 nm (ε = 3.9 x 103) [25] and m7Hx, λmax = 256 nm (ε = 8.5 x 103) at pH 7 [7]; for m7Guo at pH 2, λmax = 258 nm (ε = 10.0 x 103) [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 (Km = 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 Km = 4.5 μM, as compared to 100 μM with the human enzyme [38], but is also equally effectively inhibited by formycin A (Km = 5.5 μM), (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 Km values of 91 μM and 60 μM, respectively. Also methylation of O6 leads to a striking reduction in affinity.
Table II. Effects of some modifications of the purine ring on the binding and activity of nucleoside analogues with *E. coli* and calf spleen PNP. The signs + and − denote presence or absence of substrate or inhibitor activity, tested by TLC and/or spectrophotometrically (see Methods).

<table>
<thead>
<tr>
<th>Analogue</th>
<th>Enzyme <em>E. coli</em></th>
<th>Calf spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Substrate activity (or $V_{\text{max}}/K_m$ % rel. to Ino)</td>
<td>Inhibitor activity $K_i$ [μM]</td>
</tr>
<tr>
<td>Purine riboside</td>
<td>+</td>
<td>490&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>1,6-Dihydropurine riboside</td>
<td>+</td>
<td>− 25&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>3-Deazainosine</td>
<td>+</td>
<td>e</td>
</tr>
<tr>
<td>Ribobenzimidazole</td>
<td>~1</td>
<td>f</td>
</tr>
<tr>
<td>7-Deazainosine</td>
<td>−</td>
<td>85&lt;sup&gt;g&lt;/sup&gt;</td>
</tr>
<tr>
<td>Formycin A</td>
<td>−</td>
<td>~ 5.5&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>Formycin B</td>
<td>−</td>
<td>~ 4.5&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>6-Deoxyacyclovir</td>
<td>e</td>
<td>e</td>
</tr>
</tbody>
</table>

<sup>a</sup> From ref. [36]; <sup>b</sup> at 100 μM concentration; <sup>c</sup> with Ino as a substrate; <sup>d</sup> at 530 μM concentration; <sup>e</sup> not tested; <sup>f</sup> inhibitor of xanthine oxidase; <sup>g</sup> with m'Guo as substrate; <sup>h</sup> from ref. [38]; <sup>i</sup> at 200 μM concentration.

for PNP from human erythrocytes, the $K_i$ for 2-amino-6-methoxypurine being 520 μM as compared to 22 μM for guanine [45].

By contrast, the presence of an O6 is not a prerequisite for the *E. coli* PNP, since purine riboside is a substrate (Table II), consistent with an earlier report [36] on phosphorolysis of 2-aminopurine riboside and purine riboside, and their inhibitory activities with $K_i$ values of 120 μM and 490 μM, respectively.

We had previously found [6] that methylation of the ring N(1) of Guo and Ino abolishes susceptibility to phosphorolysis by the calf spleen enzyme, and that m'Guo and m'Ino do not inhibit phosphorolysis of Ino. By contrast, m'Guo and m'Ino are good substrates of the bacterial enzyme ([6, 36] and Table IV). Similar experiments now performed with the human erythrocyte PNP showed that m'Ino is not a substrate and that m'Guo is only barely detectably phosphorolyzed (see Table IV).

It follows that a fundamental difference between the mammalian and bacterial enzymes is the requirement of the former for O6 and N(1) of the purine base moiety. The results with the human erythrocyte and calf spleen enzymes are consistent with a proposed model for the action of calf thyroid PNP [46], *viz.* interaction of the imidazole of a histidine residue with the purine ring N(1) and O6 via hydrogen bonding. However, in contrast to the earlier proposal [46], we conclude that N(1) is the proton donor and O6 the acceptor, in accordance with the tautomeric forms of Ino and Guo in aqueous medium [47].

Such a model is supported by other data, *e.g.* involvement of a histidine was deduced from the pH-dependence of the kinetic parameters for phosphorolysis of Ino [4, 48]; the good substrate and inhibitory activities of analogues with O6 replaced by S or Se ([38, 45, 49, 50] and Table IV) with similar electronic properties; the much better substrate properties of the cation of m7 Guo (with a proton on N(1)), relative to the zwitterionic form ([7]; see also below); and the following (see Table III): the only moderate decrease (5- to 7-fold) in affinity of Gua and Hx for the erythrocyte enzyme on replacement of O6 by S6, the very marked (60- to 250-fold) decrease in inhibitory potency of the N(1)-methylated bases [45], and the decrease in substrate and inhibitory properties of nucleosides methylated at N(1) (see Table IV); as well as the only moderate (12- to 19-fold, see Table III) increase in $K_i$ values for the bases with both the foregoing modifications [45]. Methylation of N(1) not only eliminates its ability as a donor in hydrogen bonding, but also sterically hinders the propensity of O6 as an acceptor, the overall result being a lack of affinity for N(1)-methylated analogues. Substi-
Table III. Effect on $K_i$ of modifications of the ring N(1) and O$^6$ 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$^b$ [Å]</th>
<th>Hydrogen bond length [Å]</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>none N(1)−CH$_3$</td>
<td>both</td>
<td>O−H−N</td>
</tr>
<tr>
<td>Hx</td>
<td>10</td>
<td>2500</td>
<td>73</td>
<td>190</td>
</tr>
<tr>
<td>Gua</td>
<td>22</td>
<td>1300</td>
<td>110</td>
<td>260</td>
</tr>
</tbody>
</table>

$^a$ From [45]; $^b$ from [55]; $^c$ from [53]; $^d$ mean H−O and H⋯S distance [54].

The substitution of S$^6$ for O$^6$ results in only a small decrease in acceptor properties [51, 52]; but, possibly more important, in an increase in length of the hydrogen bond by 0.5−0.8 Å (see Table III) [53, 54]. The latter effect permits formation of at least one hydrogen bond.

On the other hand, two hydrogen bonds formed by N(1)−H and O$^6$, with the imidazole of a histidine residue would have rather unfavourable angles at the hydrogen atoms. A distinct preference for this angle is to be near 180° [56]; for example the angle N−H⋯O for a C=O⋯N−H hydrogen bond is rarely less than 140° [57], while its mean value is 161° [58]. Hence, probably only one hydrogen bond is formed with a histidine residue (with O$^6$ of the purine ring as acceptor), while two others are formed with a glutamate residue (see Fig. 1), in accordance with preliminary crystallographic data [59, 60] and the known observation that guanine and guanosine analogues usually bind more strongly than the corresponding hypoxanthines [8, 60, 61].

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 $\sim 60$ µM and $\sim 85$ 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 m7Ino, 6-thioGuo and phosphate) and initial velocities with the bacterial enzyme. Unless otherwise indicated (\( \sim \)), errors are ±15\%.

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<tbody>
<tr>
<td>Ino at 37°C</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
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</tr>
<tr>
<td>m7Ino</td>
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a: By initial velocity method \( K_m = 28 \mu M \) and \( V_{max} = 112\% \) that obtained by continuous monitoring method; b: by initial velocity method \( K_m = 11 \mu M \) and \( V_{max} = 105\% \) that obtained by continuous monitoring method; c: no substrate activation; d: not a substrate; e: at pH 7.5 and 25°C a reported value of \( K_m \) is 46 mM and \( V_{max} \) 90\% that for Ino [44]; f: at pH 7.5 and 30°C \( K_m \) for Guo is 32 \( \mu M \) and \( V_{max} \) 50\% that for Ino [68]; g: by initial velocity method; h: not determined; i: by initial velocity method \( K_m = 18.5 \mu M \) and \( V_{max} = 104\% \) that obtained by continuous monitoring method; k: from ref. [49].

somewhat lower for the human erythrocyte PNP, \( K_m = 330 \mu M \) [4]. For the E. coli enzyme, \( K_m \) values of 120 \( \mu M \) and 250 \( \mu M \) 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 m7Guo 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 \( \mu M \) compared to 26–28 \( \mu M \) for Ino). Since m7Guo at pH 7 is a mixture of two ionic forms, the cation and the zwitterion, with \( K_a = 6.8 \) [29], the measured kinetic parameters are the resultant 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 m7Guo 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, m7Ino [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 \( \geq 120 \mu M \), of m7Guo \( \geq 300 \mu M \) and phosphate \( \geq 3 \mu M \) (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
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 m7Gua and m2'7Gua). 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, $K_m \approx 110 \mu M$ as compared to 32 $\mu M$ for Ino, and $V_{max}$ comparable to that for Ino (see Table IV).

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 pH ([11] and see below), phosphorolysis in this instance was confirmed by TLC with solvents A and B, using authentic m7Ade as a standard.

An analogous effect of N(7)-methylation on substrate properties prevails with m1'Guo, in that m2'7Guo is very slowly phosphorolyzed by the calf enzyme ($V_{max}/K_m \approx 0.01\%$ the rate for Ino). With the human PNP the rate constant for m2'7Guo 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 $V_{max}$. 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 m7Gua and m2'7Gua). It follows that the role of methylation at N(7) may be labilization of the glycosidic bond [65].

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Spectral properties of m'Ado

Phosphorolysis of m'Ado 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, m' Ade, 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 \( k \approx 10^{-4} \text{ sec}^{-1} \) at 37 °C, reflected in a decrease in absorbance in the range 245–300 nm (Fig. 3).

As might be anticipated, m_{27}^{7,9} Ade is also fluorescent, with a lower quantum yield, \( \approx 0.037 \), and an emission maximum at 370 nm, as compared to 395 nm for m'Ado (see Table V). It is more resistant to ring opening, the rate constant being \( 10^{-3} \text{ sec}^{-1} \) at pH 10.6 at 18 °C as compared to \( 2 \times 10^{-3} \text{ sec}^{-1} \) for m'Ado at pH 8.5 at the same temperature.

The emission properties of m'Ado and m_{27}^{7,9} Ade 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 m'Ade 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 m'Ado at pH 1–7 (see Table V). For Ade and m'Ade, the maximum is in the range 362–365, hence similar to that observed in the present study for m_{27}^{7,9} Ade.

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. deoxyribo-nucleosides 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 \( \alpha\)-l-lyxosyladenine [36], while the human erythrocyte PNP phosphorolyses 5'-deoxy- and 2'-5'-dideoxyinosines and \( \alpha\)-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 derivatives.

Table V. Spectral data for absorption and fluorescence emission of m'Ado and m_{27}^{7,9} Ade.

<table>
<thead>
<tr>
<th>Compound</th>
<th>pH</th>
<th>Absorption ( \lambda_{\text{max}} ) [nm]</th>
<th>( \varepsilon_{\text{max}} ) ( \times 10^{-3} ) [nm]</th>
<th>Emission ( \lambda_{\text{max}} ) [nm]</th>
<th>( \varnothing ) ( \times 10^{2} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>m'Ado</td>
<td>7.0</td>
<td>271</td>
<td>12.8(^a)</td>
<td>260–285</td>
<td>395</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>271</td>
<td>12.9(^a)</td>
<td>260–285</td>
<td>395</td>
</tr>
<tr>
<td>m_{27}^{7,9} Ade</td>
<td>7.0</td>
<td>269</td>
<td>12.1(^e)</td>
<td>265–285</td>
<td>370</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>268</td>
<td>11.9(^e)</td>
<td></td>
<td></td>
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</tbody>
</table>

\(^a\) Data from [11]; \(^b\) with quinine bisulphate in 1N H₂SO₄ as standard (\( \varnothing = 0.55 \)) [22]; \(^c\) with anthracene in ethanol as standard (\( \varnothing = 0.30 \)) [22]; \(^d\) with m'Guo in pH 3 (H₂SO₄) as standard (\( \varnothing = 0.012 \)) [23]; \(^e\) data from [12].
anallogues 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 (m$^1$Ino, Ado), confers weak substrate properties. Both enzymes also exhibit a pref-
ference for the cationic form of m\textsuperscript{7} Guo, relative to the zwitterion. And the acycloguanosine analogues are better inhibitors than the corresponding acycloinosines.

The foregoing results are consistent with similar binding by the active centre of each enzyme to the O\textsuperscript{6}, N(1) and C(2)–NH\textsubscript{2} 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 O\textsuperscript{6} 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 phosphorolysy Ado. It is clear that both the calf and bacterial enzymes degrade 3-deazainosine and bind, but do not phosphorolysy, 7-deazainosine, pointing to absence of binding at N(3) and involvement, presumably by protonation, of N(7) [64]. Furthermore, both the human erythrocyte and the bacterial enzymes do not tolerate steric modifications at C(2\textsuperscript{'}) and C(3\textsuperscript{'}) of the sugar ring, but do so at C(5\textsuperscript{'}).

More striking are the differences. In contrast to the mammalian enzymes, the E. coli enzyme phosphorolyses N(1)-methyl nucleosides, purine ribosides lacking the O\textsuperscript{6}, 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

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