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

Agnieszka Bzowska*, Ewa Kulikowska*, and David Shugar*,**

* Department of Biophysics, Institute of Experimental Physics, University of Warsaw, Zwirki i Wigury 93, 02-089 Warsaw, Poland

** Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Rakowiecka 36, 02-532 Warsaw, Poland

Z. Naturforsch. **45c,** 59–70 (1990); received May 19/September 7, 1989

Purine Nucleoside Phosphorylase, Mammalian, Bacterial, Specificity, Substrate Analogues

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_m) 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-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 O^6 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^6 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^1Ino, 1-methylinosine; m^1Guo, 1-methylguanosine; m^7Ado, 7-methyladenosine; m^7Ino, 7-methylinosine; m^7Guo, 7-methylguanosine; $m_2^{1,7}$Guo, 1,7-dimethylguanosine; $m_2^{7,9}$Ade, 7,9-dimethyladenine; P_i, orthophosphate; ribobenzimidazole, 1-β-D-ribofuranosylbenzimidazole; 6-thioGuo, 6-thioguanosine.

Reprint requests to Agnieszka Bzowska.

Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/90/0100-0059 $ 01.30/0

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^1Ino, m^7Ade, 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^7Ino was obtained from Cyclo (Los Angeles, Cal., U.S.A.) formycin B from Calbiochem (Zurich, Switzerland), and formycin A from Mejii Saika Kaishi (Kawasaki, Japan).

m^1Guo and $m_2^{1,7}$Guo were obtained from the corresponding nucleotides by dephosphorylation with alkaline phosphatase as described elsewhere [6]. Both m^7Ado 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 $NaNO_2$ 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 901C 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_{obs} and $\Delta\varepsilon(\lambda_{obs})$: m^7Guo – 260 nm (4.6×10^3); m^1Guo – 260 nm (4.0×10^3); $m_2^{1,7}$Guo – 263 nm (5.6×10^3); Guo – 258 nm (5.5×10^3); m^1Ino – 244 nm (1.34×10^3); m^7Ino – 280 nm (3.5×10^3); m^7Ado – 285 nm (2.55×10^3); ribobenzimidazole – 245 nm (1.74×10^3); 6-thioGuo – 275 nm (2.8×10^3).

With continuous monitoring, reactions with the mammalian enzymes were followed to completion. With good substrates (Ino, m^7Guo), the use of ~0.01 U/ml PNP, and an excess of xanthine oxidase with Ino, and 0.005 U/ml with m^7Guo, led to completion of the reaction in 15–20 min. With feebler substrates (m^7Ado, $m_2^{1,7}$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 μM [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]:

$$t = K_m/V_{max}\ln(c_o/c) + c_o/V_{max}(1 - c/c_o),$$

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

$$t = K_m/V_{max}\ln(c_o/c)$$

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^{7}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^{7}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^{7}Guo with both mammalian enzymes, product inhibition by ribose-1-phosphate and m^{7}Gua being insignificant under these conditions.

With the *initial velocity method*, Ino concentrations were in the range 10–500 μM, m^{7}Guo 20–700 μM, m^{1}Guo 10–100 μM, m^{1}Ino 25–120 μM, Guo 10–100 μM, m^{7}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^{7}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^{7}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^{7}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^{7}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^{7}Ado, purine riboside, benzimidazole and 7-deazainosine were also minitored by TLC. In all cases (except for 7-deazaIno) 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 I. R_f values for m^{7}Ade (silica gel plates), and other nucleosides and corresponding bases (cellulose plates); solvent (A) *n*-butanol:acetic acid:H_2O (5:2.5:2.5); solvent (B) chloroform:methanol (8.5:1.5); solvent (C) *sec.*-butanol:H_2O (upper layer) and solvent (D) 1 M ammonium acetate:ethanol (2:5).

Analogue	Solvent A	B	C	D
m^{7}Ade	0.19	0.12	–	–
m^{7}Ado	0.05	0.00	–	–
Ade	0.64	–	0.61	–
Ado	0.55	–	0.55	–
Purine	0.72	–	0.65	–
Purine riboside	0.64	–	0.58	–
Benzimidazole	0.75	0.58	0.83	0.76
Ribobenzimidazole	0.68	0.69	0.77	0.81
7-DeazaIno	0.54	–	0.45	0.72

Quantum yield measurements

Emission spectra were corrected for the spectral sensitivity of the detector. Quantum yields for fluorescence of m^7Ado and $m_2^{7,9}$Ade were determined at room temperatures (20 °C) at pH 1 (H_2SO_4) and pH 7 (0.02 M Hepes/NaOH buffer), using three different standards: quinine bisulphate in 1 N H_2SO_4, for which $\varnothing = 0.55$ with λ_{exc} 366 nm [22]; anthracene in methanol, $\varnothing = 0.30$ with λ_{exc} 366 nm [22]; and m^7Guo at pH 3 (H_2SO_4), $\varnothing = 0.012$ with λ_{exc} 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 m^7Guo and m^7Ino), as follows: m^1Guo, λ_{max} = 258 nm ($\varepsilon = 13.4 \times 10^3$); $m_2^{1,7}$Guo, λ_{max} = 263 nm ($\varepsilon = 11.2 \times 10^3$); m^1Ino, λ_{max} = 251 nm ($\varepsilon = 10.0 \times 10^3$); Guo, λ_{max} = 252.5 nm ($\varepsilon = 13.6 \times 10^3$); Ino, λ_{max} = 248 nm ($\varepsilon = 12.3 \times 10^3$) [25, 26]; m^7Ado, λ_{max} = 271 nm ($\varepsilon = 12.8 \times 10^3$) [11]; 7-deazaIno, λ_{max} = 260 nm ($\varepsilon = 10.8 \times 10^3$); benzimidazole riboside, λ_{max} = 245 nm ($\varepsilon = 6.8 \times 10^3$) [27]; 6-thioGuo λ_{max} = 257 nm ($\varepsilon = 8 \times 10^3$). The extinction coefficient for 7-deazaIno at pH 7 was calculated relative to that in methanol λ_{max} = 259 nm ($\varepsilon = 9.7 \times 10^3$) [28]. For m^7Guo and m^7Ino, which are mixtures of two ionic forms at pH 7 (pK_a = ~6.8 and 6.4 respectively [29–31]), the concentrations were determined, following completion of phosphorolysis, from the spectral constants of the products, m^7Gua, λ_{min} = 260 nm ($\varepsilon = 3.9 \times 10^3$) [25] and m^7Hx, λ_{max} = 256 nm ($\varepsilon = 8.5 \times 10^3$) at pH 7 [7]; for m^7Guo at pH 2, λ_{max} = 258 nm ($\varepsilon = 10.0 \times 10^3$) [25].

Results and Discussion

Role of purine ring N(1) and O^6 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 (K_m = 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 μM, as compared to 100 μM with the human enzyme [38], but is also equally effectively inhibited by formycin A (K_i = 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 O^6. 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 O^6 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).

	Enzyme *E. coli*		Calf spleen	
Analogue	Substrate activity (or V_{max}/K_m % rel. to Ino)	Inhibitor activity K_i [μM]	Substrate activity	Inhibitor activity K_i [μM]
Purine riboside	+	490[a]	−	−[b]
1,6-Dihydropurine riboside	+	~ 25[c]	−	−[d]
3-Deazainosine	+	[e]	+	[e]
Ribobenzimidazole	~1	[f]	−	[f]
7-Deazainosine	−	~ 85[g]	−	~60[g]
Formycin A	−	~ 5.5[f]	−	−[b,f]
Formycin B	−	~ 4.5[f]	−	100[h]
6-Deoxyacyclovir	[e]	[e]	−	−[i]

[a] From ref. [36]; [b] at 100 μM concentration; [c] with Ino as a substrate; [d] at 530 μM concentration; [e] not tested; [f] inhibitor of xanthine oxidase; [g] with m^{7}Guo as substrate; [h] from ref. [38]; [i] 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 O^6 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^{1}Guo and m^{1}Ino do not inhibit phosphorolysis of Ino. By contrast, m^{1}Guo and m^{1}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^{1}Ino is not a substrate and that m^{1}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 O^6 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 O^6 *via* hydrogen bonding. However, in contrast to the earlier proposal [46], we conclude that N(1) is the proton donor and O^6 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 O^6 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 m^{7}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 O^6 by S^6, 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 O^6 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.

Base	K_i[a] [μM] Modification none	$N(1)-CH_3$	S^6	both	van der Waals' radius[b] [Å] H	CH_3	Hydrogen bond length [Å] O···H−N	S···H−N
Hx	10	2500	73	190	1.2	2.1	2.9[c]	3.7[c]
Gua	22	1300	110	260			1.87[d]	2.34[d]

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

tution 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].

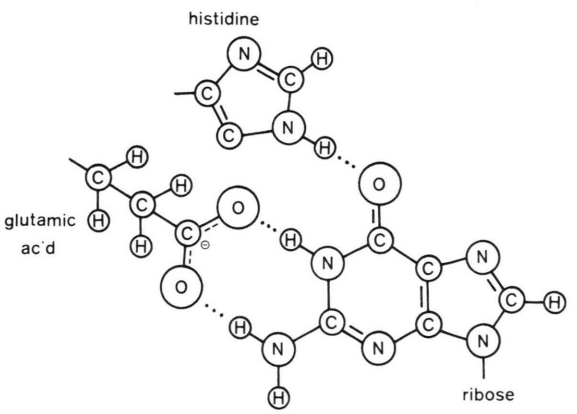


Fig. 1. Alternative model of the purine binding site of mammalian purine nucleoside phosphorylase (human erythrocyte and calf spleen).

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^7Ino, 6-thioGuo and phosphate) and initial velocities with the bacterial enzyme. Unless otherwise indicated (~), errors are ±15%.

Analogue	K_m [μM]			V_{max} [%]			V_{max}/K_m [%]			Substrate activation c [μM] with	
	human	calf	bact.	human	calf	bact.	human	calf	bact.	human	calf
Ino	~ 28[a]	13[b]	32	100	100	100	100	100	100	≥ 120	[c]
Ino at 37°	32	~ 23	–	215	300	–	190	170	–	≥ 120	[c]
m^1Ino	[d]	[d]	~ 53	[d]	[d]	~ 46	0	0	28		
m^7Ino	~ 370	~600	~270	~1310	~1350	86	65	29	10		
Guo	[e,f]	11[g]	~ 20	[e,f]	220[g]	67	80[e]	260[g]	110	[h]	[c]
m^1Guo	[h]	[d]	22	[h]	[d]	54	~ 0.03	0	790		
m^7Guo	~ 15[i]	15	~ 36	~ 130	320	28	240	280	25	≥ 300	[c]
$m_2^{1,7}$Guo	[h]	[h]	[h]	[h]	[h]	[h]	~ 0.1	~ 0.01	[h]		
6-thioGuo	167[k]	~ 35	[h]	[h]	~ 95	[h]	[h]	~ 35	[h]		
Ado	[d]	[d]	[h]	[d]	[d]	[h]	0	0	[h]		
m^7Ado	[h]	[h]	~110	[h]	[h]	~ 89	~ 0.2	~ 0.1	26		
P_i(Ino)	~ 910	~860	[h]	100	100	[h]	100	100	[h]	≥3000	[c]
P_i(m^7Guo)	~2040	~ 90	[h]	~ 240	~ 270	[h]	~107	~2580	[h]	≥7000	[c]

[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 25 °C a reported value of K_m is 46 M and V_{max} 90% that for Ino [44]; [f] at pH 7.5 and 30 °C K_m for Guo is 32 μM 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 μM and V_{max} = 104% that obtained by continuous monitoring method; [k] from ref. [49].

somewhat lower for the human erythrocyte PNP, K_i 330 μM [4]. For the *E. coli* enzyme, K_i 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^7Guo 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^7Guo 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^7Guo 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^7Ino [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^7Guo ≥ 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

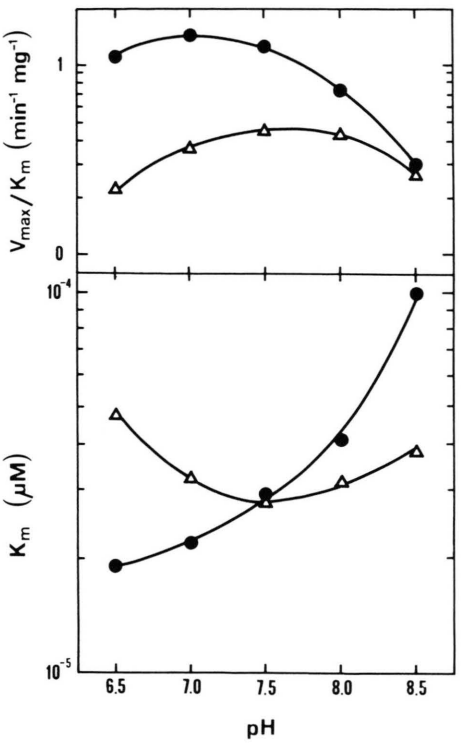


Fig. 2. pH-Dependence of K_m (lower frame) and V_{max}/K_m (upper frame) for phosphorolysis by human purine nucleoside phosphorylase of Ino (△) and m^{7}Guo (●). Measurements were carried out in the presence of 50 mM phosphate at 25 °C and the reactions were followed spectrophotometrically.

PNP, it will be seen from Table IV that m^{7}Ado 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 m^{7}Ado, 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 m^{7}Ade as a standard.

An analogous effect of N(7)-methylation on substrate properties prevails with m^{1}Guo, in that $m_2^{1,7}$Guo is very slowly phosphorolyzed by the calf enzyme ($V_{max}/K_m \sim 0.01\%$ the rate for Ino). With the human PNP the rate constant for $m_2^{1,7}$Guo is 3-fold that for m^{1}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 m^{7}Gua and $m_2^{7,9}$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 m^{7}Ado with the mammalian enzymes, it is readily phosphorolyzed by the *E. coli* PNP, $K_m \sim 110$ μM as compared to 32 μM for Ino, and V_{max} comparable to that for Ino (see Table IV).

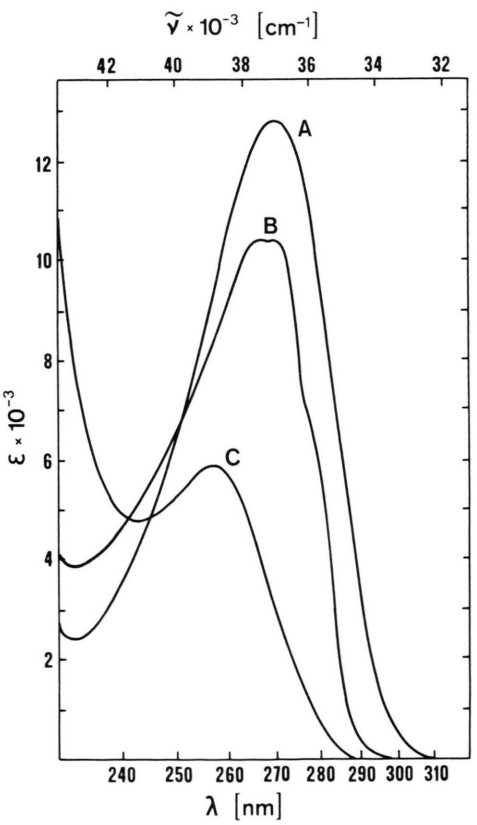


Fig. 3. Absorption spectra of m^{7}Ado prior to (A) and following (B) complete phosphorolysis by human purine nucleoside phosphorylase, and after 5 h at 37 °C (C) following imidazole ring opening.

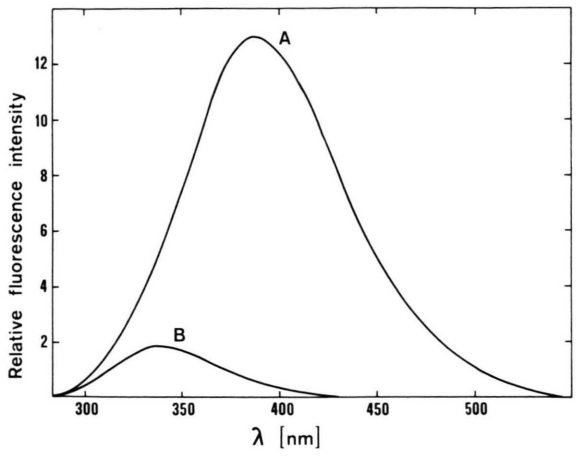


Fig. 4. Fluorescence spectra of m^7Ado (16 μM) prior to (A) and following complete phosphorolysis (B) by *E. coli* purine nucleoside phosphorylase; λ_{exc} = 275 nm.

Spectral properties of m^7Ado

Phosphorolysis of m^7Ado 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^7Ade, 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 $k \sim 10^{-4}$ sec^{-1} at 37 °C, reflected in a decrease in absorbance in the range 245–300 nm (Fig. 3).

As might be anticipated, $m_2^{7,9}$Ade is also fluorescent, with a lower quantum yield, ~0.037, and an emission maximum at 370 nm, as compared to 395 nm for m^7Ado (see Table V). It is more resistant to ring opening, the rate constant being 10^{-3} sec^{-1} at pH 10.6 at 18 °C as compared to 2×10^{-3} sec^{-1} for m^7Ado at pH 8.5 at the same temperature.

The emission properties of m^7Ado and $m_2^{7,9}$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^7Ade 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^7Ado at pH 1–7 (see Table V). For Ade and m^7Ade, the maximum is in the range 362–365, hence similar to that observed in the present study for $m_2^{7,9}$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.* 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].

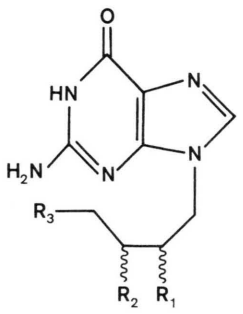
As we now show, there are differences in affinity between the mammalian and *E. coli* enzymes for acyclonucleosides.

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

Table V. Spectral data for absorption and fluorescence emission of m^7Ado and $m_2^{7,9}$Ade.

Compound	pH	Absorption λ_{max} [nm]	ε_{max} ($\times 10^{-3}$)	λ_{exc} [nm]	Emission λ_{max}^{fluor}	∅ ($\times 10^2$)
m^7Ado	7.0	271	12.8[a]	260–285	395	6.7[b], 5.5[c], 4.2[d]
	1.0	271	12.9[a]	260–285	395	6.8[b], 4.1[d]
$m_2^{7,9}$Ade	7.0	269	12.1[e]	265–285	370	4.5[b], 3.9[c], 2.9[d]
	1.0	268	11.9[e]			

[a] Data from [11]; [b] with quinine bisulphate in 1 N H_2SO_4 as standard (∅ = 0.55) [22]; [c] with anthracene in ethanol as standard (∅ = 0.30) [22]; [d] with m^7Guo in pH 3 (H_2SO_4) as standard (∅ = 0.012) [23]; [e] data from [12].



	R_1	R_2	R_3	Enantiomer	Ki (μM) calf	Ki (μM) human
1.	H	OH	OH	(S)	16	11.4
2.	H	OH	OH	(R)	24	~21
3.	H	OH	NH_2	(R,S)	~30	18
4.	H	H	OH	–	77	~52
5.	H	OCH_3	OH	(R,S)	~120	~100
6.	H	CH_2OH	OH	–	21	36
7.	H	F	OH	(R,S)	150	~100
8.	OH	OH	OH	(D threo)	>200	>100
9.	OCH_3	OH	OH	(±erythro)	2.5	2.8
10.	CH_3	OH	OH	(2R:3R,S)	4.2	1.8

Fig. 5. Inhibition constants for some acycloguanosines with human erythrocyte and calf spleen purine nucleoside phosphorylase: effect of substituents on the acyclic chain.

analogues 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.

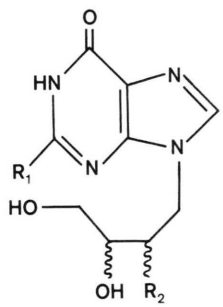
The importance of an amino group at C(2) of the base appears to be similar for both enzymes, in that acycloguanosines exhibit K_i values several-fold lower than the corresponding acycloinosines (Fig. 5), in contrast to Guo and Ino, which have comparable Michaelis constants. A similar, even more pronounced, 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 μM, 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_{max}/K_m or V_{max}, which, for compounds not detectably phosphorolyzed (m^1Ino, Ado), confers weak substrate properties. Both enzymes also exhibit a pref-



	R_1	R_2	Enantiomer	Ki (μM) calf	Ki (μM) human
11.	H	N_3	(±threo)	~11	~35
12.	NH_2	N_3	(±threo)	~3.6	5.1
13.	H	F	(±threo)	11.5	~25
14.	NH_2	F	(±threo)	2.7	3.5
15.	NH_2	F	(+erythro)	2.2	3.2
16.	NH_2	F	(-erythro)	26	29

Fig. 6. Inhibition constants for some acyclonucleosides with human and calf purine nucleoside phosphorylase: effect of the base.

erence for the cationic form of m^7Guo, 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^6, N(1) and C(2)$-NH_2$ 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^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 phosphorolyse Ado. It is clear that both the calf and bacterial enzymes degrade 3-deazainosine and bind, but do not phosphorolyse, 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′) and C(3′) of the sugar ring, 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 O^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

We are indebted to Dr. Tozo Fujii (Japan) and Dr. N. G. Johansson (Medivir, Sweden) for gifts of m^7Ado 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).

[1] E. R. Giblett, A. J. Ammann, D. W. Wara, R. Sandman, and L. K. Diamond, Lancet **1,** 1010–1013 (1975).
[2] A. J. Ammann, Ciba. Found. Symp. **68,** 55–75 (1978).
[3] R. E. Parks, Jr., J. D. Stoeckler, C. Cambor, T. M. Savarese, G. W. Crabtree, and S.-H. Chu, Molecular actions and targets for cancer chemotherapeutic agents (A. Sartorelli, J. S. Lazo, and J. R. Bertino, eds.), pp. 229–252, Academic Press, New York 1981.
[4] J. D. Stoeckler, Developments in Cancer Chemotherapy (R. E. Glazer, ed.), pp. 35–60, CRC Press, Boca Raton, Fla. 1984.
[5] J. D. Stoeckler, C. Cambor, V. Kuhns, S.-H. Chu, and R. E. Parks, Jr., Biochem. Pharmac. **31,** 163–171 (1982).
[6] A. Bzowska, E. Kulikowska, E. Darzynkiewicz, and D. Shugar, J. Biol. Chem. **19,** 9212–9217 (1988).
[7] E. Kulikowska, A. Bzowska, J. Wierzchowski, and D. Shugar, Biochim. Biophys. Acta **874,** 55–363 (1986).
[8] A. Bzowska, E. Kulikowska, N. G. Johansson, C. Bing-yi, B. Lindborg, and D. Shugar, in preparation.
[9] A. Vita and G. Magni, Anal. Biochem. **133,** 153–156 (1983).
[10] W. R. A. Osborne, J. Biol. Chem. **255,** 7089–7092 (1980).
[11] T. Fujii and T. Saito, Heterocycles **17,** 117–123 (1982).
[12] T. Fujii, F. Tanaka, K. Mohri, T. Itaya, and T. Saito, Tetrahedron Letters **49,** 4873–4876 (1973).
[13] I. Iwai, T. Nishimura, and B. Shimizu, in: Synthetic Procedures in Nucleic Acids Chemistry (W. W. Zorbach and R. S. Tipson, eds.), **Vol. 1,** 135–155 (1968).
[14] D. L. Smith and P. J. Elving, J. Amer. Chem. Soc. **84,** 1412–1420 (1962).
[15] A. Larson, K. Stenberg, A.-C. Ericson, U. Haglund, W. A. Yisak, N. G. Johansson, B. Oberg, and R. Datema, Antimicrob. Ag. Chemother. **30,** 598–605 (1986).
[16] J. Wierzchowski and D. Shugar, Z. Naturforsch. **38c,** 67–73 (1983).
[17] J. Wierzchowski, P. Lassota, and D. Shugar, Biochim. Biophys. Acta **786,** 170–178 (1984).
[18] J. D. Stoeckler, R. P. Agarwal, K. C. Agarwal, and R. E. Parks, Jr., Methods Enzymol. **51,** 530–538 (1978).

[19] K. F. Jensen, Eur. J. Biochem. **61,** 377–386 (1976).
[20] I. H. Segal, Enzyme Kinetics, John Wiley and Sons, New York 1975.
[21] J. Wierzchowski, H. Sierakowska, and D. Shugar, Biochim. Biophys. Acta **828,** 109–115 (1985).
[22] C. A. Parker, Photoluminescence of Solutions, Ch. 3 L, Elsevier, London 1968.
[23] Y. Nishimura, T. Yamamoto, and M. Tsuboi, Nucleic Acids Res., Special Publication **3,** 85–88 (1977).
[24] O. H. Lowry, N. J. Rosenbrough, A. L. Farr, and B. J. Randall, J. Biol. Chem. **193,** 265–275 (1951).
[25] R. M. C. Dawson, D. C. Elliott, W. H. Elliott, and K. M. Jones (eds.), Data for Biochemical Research, Oxford University Press, Oxford 1969.
[26] J. H. Lister, Fused pyrimidines, Part II: Purines (D. J. Brown, ed.), John Wiley & Sons, New York 1971.
[27] Z. Kazimierczuk, L. Dudycz, R. Stolarski, and D. Shugar, Z. Naturforsch. **35c,** 30–35 (1980).
[28] F. Seela and D. Hasselman, Chem. Ber. **113,** 3989–3993 (1980).
[29] S. Hendler, E. Furer, and P. R. Srinivasan, Biochemistry **9,** 4141–4152 (1970).
[30] M. Leng, F. Pochon, and H. Michelson, Biochim. Biophys. Acta **169,** 338–349 (1968).
[31] Y. Nishimura, S. Takahashi, T. Yamamoto, M. Hattori, K. Miura, K. Yamaguchi, S. Ohtani, and T. Hata, Nucleic Acids Res. **8,** 1107–1119 (1980).
[32] R. P. Agarwal and R. E. Parks, Jr., J. Biol. Chem. **246,** 3763–3768 (1971).
[33] T. P. Zimmerman, N. Gersten, and R. P. Miech, Proc. Amer. Ass. Cancer Res. **11,** 87–95 (1970).
[34] T. P. Moyer and A. G. Fischer, Arch. Biochem. Biophys. **174,** 622–629 (1976).
[35] K. F. Jensen and P. Nygaard, Eur. J. Biochem. **51,** 253–265 (1975).
[36] J. Doskocil and A. Holy, Collection Chechoslov. Chem. Commun. **42,** 370–383 (1977).
[37] R. P. Agarwal, Pharmacol. Ther. **17,** 399–429 (1982).
[38] M. R. Sheen, B. K. Kim, and R. E. Parks, Jr., Mol. Pharmac. **4,** 293–299 (1968).
[39] R. E. Parks and R. P. Agarwal, The enzymes, 3rd ed., **Vol. 7** (P. D. Boyer, ed.), pp. 483–514, Academic Press, New York 1972.
[40] P. E. Daddona, W. P. Wiesmann, W. Milhouse, J.-W. Chern, L. B. Townsend, M. S. Hershfield, and H. K. Webster, J. Biol. Chem. **261,** 11667–11673 (1986).
[41] A. Guranowski, Plant Physiol. **70,** 344–349 (1982).
[42] B. C. Robertson and P. A. Hoffee, J. Biol. Chem. **248,** 2040–2043 (1973).
[43] S. Senesi, G. Falcone, U. Mura, F. Sgarella, and P. L. Ipata, FEBS Lett. **64,** 353–357 (1976).
[44] J. V. Tuttle and T. A. Krenitsky, J. Biol. Chem. **259,** 4065–4069 (1984).
[45] T. A. Krenitsky, G. B. Elion, A. M. Henderson, and G. H. Hitchgins, J. Biol. Chem. **243,** 2876–2881 (1968).
[46] J. D. Carlson and A. G. Fisher, Biochim. Biophys. Acta **571,** 21–34 (1979).
[47] W. Saenger, Principles of Nucleic Acids Structure, Springer Verlag, New York 1984.
[48] R. P. Agarwal and R. E. Parks, Jr., J. Biol. Chem. **244,** 644–647 (1969).
[49] J. D. Stoeckler and R. E. Parks, Jr., Methods Pharmacol., **Vol. 6** (D. M. Paton, ed.), pp. 147–162, Plenum Publishing Corporation 1985.
[50] A. F. Ross, K. C. Agarwal, S.-C. Chu, and R. E. Parks, Jr., Biochem. Pharmac. **22,** 141–154 (1973).
[51] A. R. Fersht, Trends Biochem. Sci. **12,** 301–304 (1987).
[52] S. J. Weiner, P. A. Kollman, D. A. Case, U. C. Singh, C. Ghio, G. Alagona, S. Profeta, and P. Weiner, J. Amer. Chem. Soc. **108,** 765–784 (1984).
[53] J. D. Rawn, Biochemistry, p. 112, Harper & Raw Publishers, New York 1983).
[54] G. A. Jeffrey, H. Maluszynska, and J. Mitra, Int. J. Biol. Macromol. **7,** 336–348 (1985).
[55] L. Pauling, The Nature of Chemical Bond, Cornell University Press, Ithaca 1966.
[56] E. N. Baker and R. E. Hubbard, Prog. Biophys. Molec. Biol. **44,** 97–179 (1984).
[57] J. Olovsson and P. G. Jonsson, The Hydrogen Bond, **Vol. 2** (P. Shuster, G. Zundel, and G. Sanorfy, eds.), pp. 393–456, North-Holland, Amsterdam 1976.
[58] R. Taylor and O. Kennard, Acta Crystallogr. **B 39,** 133–138 (1983).
[59] S. E. Ealick, T. J. Greenhough, Y. S. Babu, D. C. Carter, W. J. Cook, C. E. Bugg, S. A. Rule, J. Habach, J. R. Helliwell, J. D. Stoeckler, S. F. Chen, and R. E. Parks, Jr., Ann. N.Y. Acad. Sci. **451,** 311–312 (1985).
[60] J. D. Stoeckler, S. E. Ealick, C. E. Bugg, and R. E. Parks, Jr., Fed. Proc. **45,** 2773–2778 (1986).
[61] D. S. Schewach, J.-W. Chern, K. E. Pillote, L. B. Townsend, and P. E. Daddona, Canc. Res. **46,** 519–523 (1986).
[62] L. B. Townsend, B. L. Cline, R. P. Panzica, P. E. Fagerness, L. W. Roti, J. D. Stoeckler, G. W. Crabtree, and R. E. Parks, Jr., in: Lectures in Heterocyclic Chemistry, **Vol. 4** (R. N. Castle and J. Lalezari, eds.), Hetero Corp., Orem, Utah, S79 1978.
[63] M. O. Oivanen, H. Lonnberg, Z. Kazimierczuk, and D. Shugar, Nucleosides & Nucleotides **8,** 133–144 (1989).
[64] J. D. Carlson and A. G. Fisher, Biochim. Biophys. Acta **566,** 259–265 (1979).
[65] J. A. Zoltewicz, D. F. Clark, T. W. Sharpless and G. Grahe, J. Amer. Chem. Soc. **92,** 1741–1750 (1970).
[66] K. C. Agarwal, R. P. Agarwal, J. D. Stoeckler, and R. E. Parks, Jr., Biochem. **14,** 79–84 (1975).
[67] T. Fujii, T. Saito, and I. Inoue, Heterocycles **16,** 909–913 (1981).
[68] W. B. Knighton, G. O. Glskaas, and P. R. Callis, J. Phys. Chem. **86,** 49–55 (1982).
[69] J. D. Stoeckler, C. Cambor, and R. E. Parks, Jr., Biochemistry **19,** 102–107 (1980).
[70] J. M. Stein, J. D. Stoeckler, S. Y. Li, R. L. Tolman, M. MacCoss, A. Chen, J. D. Karkas, W. T. Ashton, and R. E. Parks, Jr., Biochem. Pharmac. **36,** 1237–1244 (1987).
[71] J. D. Stoeckler, R. P. Agarwal, K. C. Agarwal, K. Schmid, and R. E. Parks, Jr., Biochemistry **17,** 278–284 (1978).
[72] F. Jordan and A. Wu, Arch. Biochem. Biophys. **190,** 699–704 (1978).
[73] S. J. Salamone and F. Jordan, Biochem. **21,** 6382–6387 (1982).