I. Friend Erythroleukemia Cell Culture. The cells employed in this work were Friend murine virus induced erythroleukemia cells of DBA/2J origin, established originally by Friend et al.,[30] and kindly supplied by Dr. N. Gabelman of Mount Sinai School of Medicine, N.Y. Cells were grown in suspension culture at 37 °C in 10% CO_2 humidified atmosphere by weekly passage of 10^5 cells/mL in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 units/mL of penicillin, and 100 μg/mL of streptomycin (GIBCO, Grand Island, N.Y.).

J. Induction Experiments and Determination of Hemoglobin. Cells in log phase were cultured at 37 °C at a level of 10^5 cells/mL in T-25 cm^2 Corning polyethylene flasks containing 10 mL of medium. Compounds were added at the time of seeding using a Hamilton syringe. Each compound was tested over a range of concentrations in two separate experiments employing twofold increases in concentration. Cultures incubated with Me_2SO (210 mM) served as a positive control. Water-insoluble agents were dissolved in either 50% ethanol or acetone; the final concentration of these solvents was kept severalfold below the level at which cell proliferation and differentiation were affected. After 3 days of incubation, the cell concentration was determined using a Coulter counter, Model ZBI. On day 6, the cell cultures were scored for the proportion of benzidine-positive cells using a benzidine peroxide stain as described by Orkin et al.[31] In those cultures in which benzidine-positive cells were formed, cells were collected by centrifugation at 350g for 10 min and washed twice with phosphate-buffered 0.9% NaCl, pH 7.4, and cell extracts were prepared by sonication and assayed for total hemoglobin content by measuring the absorbancy at 515 nm of the cell lysate which had been incubated with benzidine as described.[32]

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Stereoelectronic Factors in the Binding of Substrate Analogues and Inhibitors to Purine Nucleoside Phosphorylase Isolated from Human Erythrocytes

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Several aspects of the stereoelectronic requirements of substrates of human erythrocytic purine nucleoside phosphorylase (E.C. 2.4.2.1) were elucidated providing the following information: (a) the N^1 position cannot have a nonhydrogen substituent; (b) the 5′-OH group must be present for catalytic activity to be exhibited but is not an essential functional group for inhibitory action to be observed; (c) on the C^8 position groups larger than $-NH_2$ or $-Br$ cannot be accommodated; (d) the *syn*-glycosyl conformation (i.e., 8-bromoguanosine) is acceptable but may not be an absolute requirement for phosphorolysis; (e) among nucleic base inhibitors methylation at N^3, N^7, or N^9 vastly decreases the inhibitory properties as does a nitrogen in lieu of C–H in the 8 position. The results clearly indicate that this enzyme differs in its stereoelectronic requirements from the *Escherichia coli* enzyme.

Levene and Medigreceann first reported on enzymatic degradation of purine nucleosides.[1] Klein later recognized[2] that phosphate and arsenate activate the enzymatic splitting of the nucleoside glycosyl bond. Kalckar finally demonstrated that the process was accompanied by the uptake of inorganic phosphate and the formation of a

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hydrolytically labile phosphate ester, ribose 1-phosphate,[3] and proposed the following reaction for purine nucleoside phosphorylase (E.C. 2.4.2.1; purine nucleoside:orthophosphate ribosyltransferase, PNPase).

$$\text{purine nucleoside} + P_i \underset{\text{synthesis}}{\overset{\text{breakdown}}{\rightleftharpoons}} \text{purine base} + \text{ribose 1-phosphate}$$

For human erythrocyte purine nucleoside phosphorylase it was shown that the equilibrium favors nucleoside synthesis.[4,5] It is now generally accepted that this enzyme functions in purine salvage (since no 5′-kinases are known for guanosine and inosine, the substrates for the human erythrocyte enzyme[4,5]) probably as an initial step toward formation of 5′-IMP and 5′-GMP from the appropriate purine base and 5-phosphoribosyl-1-pyrophosphate. The enzyme is of probable importance for normal brain and lymphocyte function.[6,7]

We here report results of studies that were aimed at a delineation of the stereoelectronic factors involved in the mechanism of action of purine nucleoside phosphorylase from human erythrocytes. A very extensive study of a similar intent was recently published on the enzyme performing this function isolated from *Escherichia coli*.[8] The results of that study[8] differ significantly from ours and, hence, strongly suggest that the substrate specificities of the enzymes from the two sources are fundamentally different.

The questions we attempt to resolve include the following: what is the origin of the specificity of the enzyme for inosine and guanosine vis-à-vis the inactivity toward adenosine; does the enzyme accept substituents different from OH at the 5′ position; are there any perceptible glycosyl conformational requirements for enzyme activity, i.e., is the *syn*- or *anti*-glycosyl conformation of the substrate preferred; and finally, which base nitrogen atoms participate in the binding process.

Results and Discussion

Specificity of the Enzyme. As a control of our enzyme system we repeated some kinetic studies reported by others.[5] Table I confirms the specificity of the enzyme for guanosine and inosine and at most a very low phosphorolytic activity (0–2% compared to guanosine) toward adenosine. There are conflicting reports on this point in the literature. The most extensive study was performed by Zimmerman et al.[9] who reported a "V_{max} for ribosylation of hypoxanthine 274–1630 times greater than the V_{max} for ribosylation of adenine". Our results are not inconsistent with those of Zimmerman et al.[9] The difficulties encountered in eliminating all traces of adenosine deaminases continue to hinder performance of unequivocal experiments to settle this issue. The enzyme from *E. coli* exhibited activity toward adenosine.[8] Formycin B (**6**) is a competitive inhibitor but not a substrate, as was also reported before.[5]

5′-GMP (3) and 5′-Deoxyinosine (20). Within experimental error 5′-GMP exhibited very low, if any, phosphorolytic activity. Such low activity could be due to the impurities present in the 5′-GMP. Figure 1 demonstrates that 5′-GMP is a competitive inhibitor of guanosine phosphorolysis.

5′-Deoxyinosine (**20**) (generated in situ by the action of adenosine deaminase on 5′-deoxyadenosine) was found to have no substrate activity toward purine nucleoside phosphorylase. However, it did possess ~43% inhibitory action toward guanosine phosphorolysis. These two results both point out the fact that the presence of the 5′-OH is essential for full catalytic activity. The inhibition exhibited by both compounds is not surprising as the guanine and hypoxanthine base both act as inhibitors. Therefore, any derivative possessing the intact base can be anticipated to exhibit inhibitory properties.

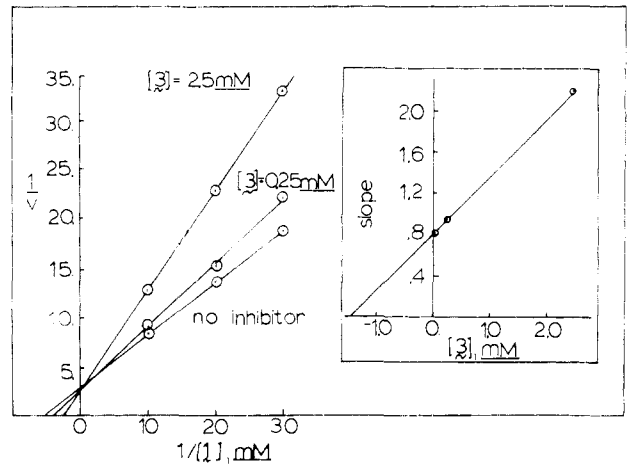


Figure 1. Competitive inhibition pattern exhibited by 5′-GMP (**3**). The inset shows the slope replot used in the calculation of K_I. The 1.0-mL reaction mixture contained 100 μmol of pH 6.0 acetate buffer, 50 μmol of arsenate, and 1.1 μg of protein. The initial velocity was measured by phenol reaction as ΔOD at 660 nm.

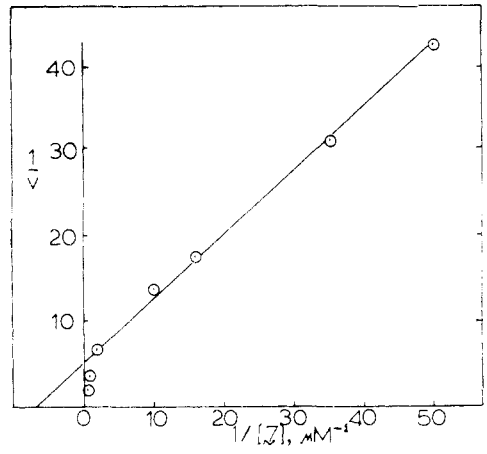
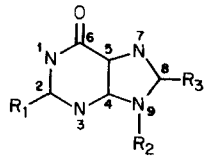


Figure 2. Lineweaver–Burk plot for N^7-methylguanosine (**7**). The 0.75-mL reaction mixture contained 100 μmol of pH 6.0 acetate buffer, 50 μmol of arsenate, and 1.1 μg of protein. The initial velocity was measured by orcinol reaction as ΔOD at 660 nm.

***N*^{1}-Methylguanosine** (**8**) is neither a substrate nor an inhibitor within our experimental detection limits. This compound clearly demonstrates the base specificity of the enzyme from human erythrocyte; i.e., the free lactam portion at N^1 and C^6 must be available for binding. This result complements the results on adenosine. Most importantly, the results on **8** are very different from results obtained from the *E. coli* enzyme[8] for which the closely related N^1-methylinosine served both as a substrate and as an inhibitor vis-à-vis guanosine phosphorolysis. It is also relevant to mention that in an earlier study on this same enzyme N^1-methylguanine and N^1-methylhypoxanthine were found to be very much weaker inhibitors than guanine and hypoxanthine in the synthesis of [^{14}C]inosine from [^{14}C]hypoxanthine.[10]

***N*^{7}-Methylguanosine** (**7**) has nearly equal V_{max} and a ~2–2.5 larger K_M than **1** (Figure 2). The double reciprocal plot is reminiscent of the one reported for **1**.[5] The imidazole ring in this substrate is positively charged (pK_a =

Table I. Alternate Substrate and Inhibitor Characteristics of Human Erythrocyte Purine Nucleoside Phosphorylase



compd[a]	R_1	R_2	R_3	other	act. toward[b] phosphorolysis, %, compared to 1 as 100%	K_M, M, if determined	% inhibn, vis-à-vis phosphorolysis of 1	K_I, M (type of inhibn obsd)
1	NH_2	ribose	H		100	8.3×10^{-5}		
2	NH_2	2′-deoxyribose	H		65			
3	NH_2	ribose 5′-phosphate	H		4		16[c]	1.5×10^{-3} (competitive)
4	H	ribose	H		100			
5	H	ribose	H	6-amino	~0–2			
6	H	ribose	H	8-aza-9-carbo	0		30[c]	2.0×10^{-3} (competitive)
7	NH_2	ribose	H	N^7-methyl	93	1.9×10^{-4}		
8	NH_2	ribose	H	N^1-methyl	0		undetectable	
9	NH_2	ribose	NH_2		10	3.1×10^{-2}	80[c]	2.9×10^{-4} (competitive)
10	NH_2	ribose	$NHCH_3$		0			
11	NH_2	ribose	$N(CH_3)_2$		0			
12	NH_2	ribose	Br		12	9.4×10^{-3}	undetectable	
13	NH_2	H	H				80[d]	5×10^{-6} (competitive)[f]
14	H	H	H				78[e]	
15	NH_2	CH_3	H				18[d]	
16	NH_2	H	H	N^7-methyl			20[d]	
17	NH_2	H	H	N^3-methyl			14[d]	
18	H	H	H	8-aza			14[e]	
19	NH_2	H	H	8-aza			16[e]	
20	H	5′-deoxyribose	H				42.8[e]	

[a] Abbreviations: 1 = guanosine; 2 = 2′-deoxyguanosine; 3 = 5′-GMP; 4 = inosine; 5 = adenosine; 6 = formycin B; 7 = N^7-methylguanosine; 8 = N^1-methylguanosine; 9 = C^8-aminoguanosine; 10 = C^8-*N*-methylaminoguanosine; 11 = C^8-*N*,*N*-dimethylaminoguanosine; 12 = C^8-bromoguanosine; 13 = guanine; 14 = hypoxanthine; 15 = N^9-methylguanine; 16 = N^7-methylguanine; 17 = N^3-methylguanine; 18 = 8-azahypoxanthine; 19 = 8-azaguanine; 20 = 5′-deoxyinosine. [b] The standard system consisted of 5 μmol of purine nucleoside, 50 μmol of arsenate, 100 μmol of pH 6.0 acetate buffer, and 1.1 μg of protein including purine nucleoside phosphorylase in a total volume of 1.0 mL. The enzyme activity was determined from the amount of ribose formed in 30 min at 37 °C according to the orcinol reaction. In the case of 3, phosphorolytic activity was assayed by determining the guanine formed via the phenol reaction. [c] The standard system consisted of 0.5 μmol of guanosine, 10 μmol of arsenate, 1.25 μmol of nucleoside analogue, 100 μmol of pH 6.0 acetate buffer, and 0.2 μg of protein containing purine nucleoside phosphorylase in a total volume of 1.0 mL. The percent inhibition is obtained by comparing the amount of ribose formed in the absence and presence of inhibitor. [d] The standard system consisted of 0.83 μmol of 1, 10 μmol of arsenate, 1.3 μmol of purine base, 97 μmol of pH 6.0 acetate buffer, and 0.2 μg of protein including the purine nucleoside phosphorylase in a total volume of 0.75 mL. [e] The standard system consisted of 2.5 μmol of guanosine, 10 μmol of arsenate, 4.0 μmol of purine base, 100 μmol of pH 6.0 acetate buffer, and 0.9 μg of protein containing purine nucleoside phosphorylase in a total volume of 0.75 mL. [f] B. K. Kim, S. Cha, and R. E. Parks, Jr., *J. Biol. Chem.*, **243**, 1763 (1968).

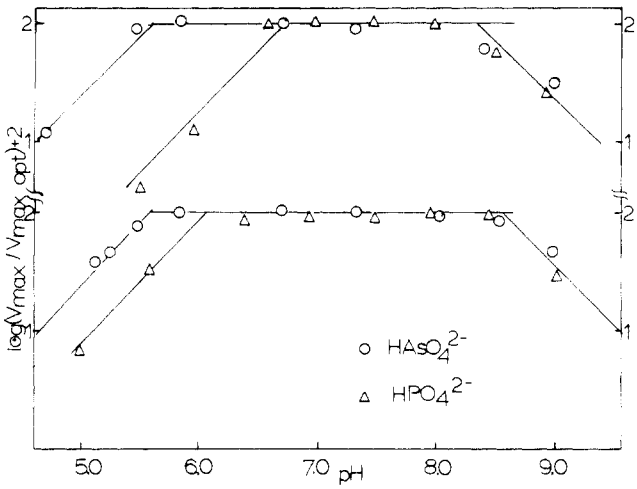
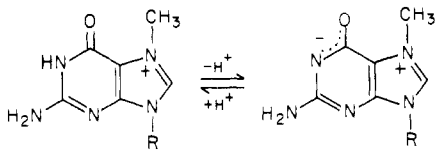


Figure 3. Log ($V_{max}/V_{max\ optimum}$) vs. pH profiles for **7** (lower data sets) and for inosine (phosphorolysis) and guanosine (arsenolysis) (the upper data). Theoretical lines of slope 1 and 0 are drawn through the experimental points. The kinetics of phosphorolysis with **7** were determined spectroscopically at 293 nm, as **7** and the product of phosphorolysis **16** differ in ϵ at this wavelength. The phosphorolysis of inosine was followed by the xanthine oxidase coupled enzyme assay.[3b] Arsenolysis reaction mixtures for **1** and **7** were assayed by the orcinol reaction.

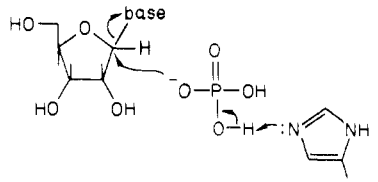
6.44 at 52 °C[11]) in the entire optimum pH range of the enzyme.



7$^+$ **7**$^\pm$

Complete log V_{max} vs. pH profiles were determined for both arsenolysis and phosphorolysis of this compound and for **1** and **4** for the sake of comparison (Figure 3). The log V_{max} vs. pH profile for inosine phosphorolysis reproduces data reported by Huennekens et al.[12] and Kim et al.[13] From such log V_{max} vs. pH plots, according to Dixon and Webb,[14] one can deduce the ionization constants of the enzyme–substrate complex. While a precise interpretation of such data is difficult, two factors are easily gleaned from Figure 3.

On the acid side arsenolysis always leads to a lower apparent pK than phosphorolysis (the pK is given by the intersection of the linear portions of the curves). The difference between the activity pH profile for phosphorolysis and arsenolysis had been noted before.[13] We propose the following explanation consistent with our data. If the HPO_4^{2-} (or $HAsO_4^{2-}$) form of phosphate is the catalytically active one, then the ascending acid side of the log V_{max} vs. pH curves would represent gradual deprotonation of $H_2PO_4^-$ (and $H_2AsO_4^-$). The pK_a of $H_2AsO_4^-$ is 0.4 unit lower than that of $H_2PO_4^-$ (6.77 vs. 7.21[15]). The lowering of these pK_a values in the enzyme substrate complex by perhaps as much as 1 pK unit would be feasible in the presence of a nearby positive charge (our unpublished results strongly imply the requirement of an arginine at the phosphate binding site). In a recent study on bovine brain purine nucleoside phosphorylase Lewis and Glantz[16] reported log V_{max} vs. pH plots which provided apparent pK_a values of 5.3 and 7.7. They suggested that the pK_a at 5.3 was due to a histidine in the ES complex. Their direct histidine modification employing dye-sensitized photooxidation yielded an apparent pK of at least 6.6, probably higher.[16] We suggest that as phosphorolysis and arsenolysis provide different apparent pK_a values, HPO_4^{2-} (or $HAsO_4^{2-}$) is responsible for the acid pK in the ES complex as reflected by the log V_{max}–pH plots. In our hands dye-sensitized photooxidation of human erythrocytic purine nucleoside phosphorylase yielded a pH-dependent inactivation profile characteristic of histidine modification and an apparent pK_a of 6.5 employing arsenolysis in the assay (unpublished from this laboratory). This value is significantly higher than the pK_a obtained for the ES in the arsenolysis of **1** (Figure 3). It is tempting to speculate that the acid side of the log V_{max}–pH profile represents a synergistic effect, i.e.



such that the histidine enhances the nucleophilic character of phosphate (HPO_4^{2-} is a superior nucleophile to $H_2PO_4^-$). Lewis and Glantz[16] claimed that their apparent pK (on the basic side) of 7.3 in the log K_M vs. pH plot reflects inorganic phosphate ionization. This would imply that the enzyme reaction utilizes $H_2PO_4^-$ rather than HPO_4^{2-}, not very likely on account of the differences in basicities and nucleophilicities of the two species.

On the alkaline side of the log V_{max}–pH profiles the arsenolysis and phosphorolysis data are very similar for **7**. The apparent pK of the ES complex for **7** is slightly displaced (0.2 unit to higher pH) compared to **1**. A possible explanation for this shift is the following. The basic side of the log V_{max}–pH profile may reflect the pK_a of a cysteine residue in the ES complex (as also suggested by Lewis and Glantz[16] based on earlier chemical modification studies[17,18]). If the role of this RSH group is to hydrogen bond to the C^6-keto group, then the dipolar ion **7**$^\pm$ with a negative charge at the N^1–C^6 region (compared to **1** with an uncharged ring at this pH) should enhance protonation of RS^- and, hence, increase its pK. This explanation invokes an effect of the alternate substrate **7** on the pK_{app} of the ES complex which reflects on the ionization characteristics of the cysteine residue that is near the substrate. This model would further suggest that the failure of **8** to bind is primarily due to steric factors. In fact, the log V_{max}–pH profiles of **1** and **7** clearly rule out the possibility that the substrate is responsible for the pK on the alkaline side of the profiles. Were this pK due to substrate ionization **7**$^\pm$ with its dipolar character above pH 6.44 should have a very different profile than **1** which is deprotonated to **1**$^-$ (at N^1) at pH 9.2.

C^8-Substituted guanosine nucleosides were employed as both substrates and possible inhibitors. The motivation for this was the finding that the A-1 type acid hydrolytic mechanism for purine nucleoside glycosyl hydrolysis[11] is subject to a very large steric factor.[19] The syn conformers **11** and **12** were found to undergo hydrolysis perhaps 1000 times faster than **9** or **10**,[19] both of which exist as flexible syn–anti mixtures.[20] The results (see Figure 4 for representative data on **9** and Table I) demonstrate that a large steric bulk at the C^8 position is not tolerated by the active site. **9** and **12** appear to have similar V_{max} values, both ~10 times slower than **1**. As the group van der Waals radii of $-NH_2$ and $-Br$ are probably similar,[21] the results on **9** and **12** do not appear to be electronic in origin; rather they are conformational. The K_M values for **9** and **12** are difficult to interpret. These K_M values may imply that the syn **12** is better bound to the enzyme than the anti conformer of

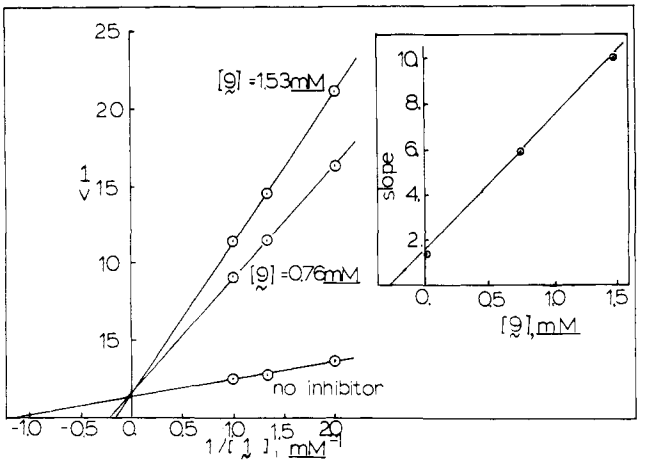


Figure 4. Competitive inhibition pattern exhibited by 8-aminoguanosine (**9**). The inset shows the slope replot used in the calculation of K_I. The 1.0-mL reaction mixture contained 100 μmol of pH 6.0 acetate buffer, 50 μmol of arsenate, 1.1 μg of protein, and variable quantities of I and **9**. The initial velocity was measured by the phenol reaction as ΔOD at 660 nm.

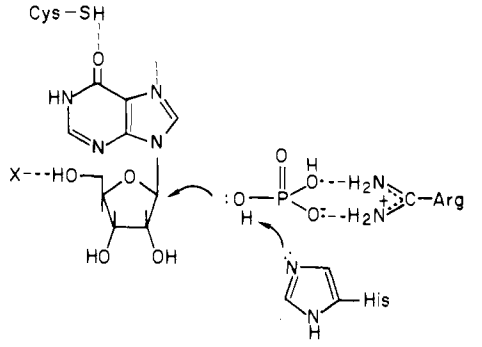
9. Due to the very limited solubility of compounds **9–12**, the Michaelis constants are subject to large errors. Therefore, the exact glycosyl conformational requirements of the enzyme cannot be defined. The fact that **12** is phosphorylated at all clearly demonstrates that the syn conformation is acceptable to the enzyme, as numerous studies, including X-ray, have shown that this molecule always exists in the syn conformation.[20,22]

Since formycin B (**6**) has also been suggested to exist as a syn conformer,[23] our proposal has further support. Whether or not the substrate must exist in the syn conformation for phosphorolysis cannot be concluded from our studies since the "good" substrates **1**, **2**, **4**, and **9** can exist as rapidly equilibrating syn–anti mixtures in solution. As the relaxation time for this conformational interconversion[24] (10^{-8} s) is very likely shorter than the half-life of the turnover, the appropriate conformer could always be utilized by the enzyme. The human erythrocyte enzyme appears to be different from the *E. coli* one in this respect also; the other enzyme[8] exhibits *no* phosphorolytic activity toward any syn substrates.

Base Analogues as Inhibitors. Compounds **13–19** are perhaps most interesting for their relative inhibitory effects. Krenitsky et al. have already reported on the inhibitory properties of some of these compounds.[10] Just as **1** and **4** exhibit essentially identical activity toward phosphorolysis, **13** and **14** appear to have nearly equal inhibitory activities. All methyl substitution (**15–17**) leads to decreased binding abilities, suggesting that N^3, N^7, and N^9 are all involved in binding or that the steric bulk at these positions cannot be tolerated in the active site. For N^7 the ratio of inhibition by **13** to **16** (fourfold) is roughly of the same order of magnitude as the ratio of K_M values (2.5 more favorable for **1** than **7**). Very likely the same factor is responsible for both observations. Finally, the substitution of N for C–H in the C^8 position appears to have a striking effect, very greatly reducing the binding ability of the base to the active site (see **18** and **19**).

Conclusions

The nucleoside binding site of purine nucleoside phosphorylase appears to have significant specificity. This specificity is reflected by the fact that the N^1 base position must not be blocked; the C^8 substituent must not be larger than $-NH_2$ (or Br); the free 5′-OH must be available for catalysis to take place but is not needed in binding. Additional observations include that on the base the N^3, N^7, and N^9 positions should not have a bulky group such as methyl and that a syn conformer (**12**) is an acceptable substrate of the enzyme. A possible model suggested for the active site in accord with current results is the following.



Cysteine and histidine have been implicated at the active site of the bovine brain enzyme,[16] cysteine in the erythrocyte one,[17,18] and histidine and arginine in both the calf-spleen and human erythrocyte enzymes.[25] Elsewhere it was also shown by isotope-exchange studies by Kalckar and more recently in this laboratory[25] that the mechanism probably involves two steps. While the role of the Arg is fairly clear from our studies,[25] the role of Cys is surmised from the studies on compound **7**, and the role of His represents one of many possibilities that require a His imidazole for catalytic activity. The results and the explanations which we put forth, which are consistent with all available evidence, provide us with a satisfactory working hypothesis for future research on this enzyme system.

Experimental Section

Chemicals. All inorganic chemicals and buffers were from Fisher Scientific. The following compounds were synthesized in this laboratory according to procedures described in the literature (and shown to be identical with those reported according to melting points and ^1H NMR and UV spectral data): 8-bromoguanosine from guanosine;[26] 8-aminoguanosine,[27] 8-*N*-methylaminoguanosine,[28] and 8-*N*,*N*-dimethylaminoguanosine[29] from 8-bromoguanosine; N^1-methylguanosine from guanosine.[30] Guanosine, 8-azaguanine, 5′-GMP, and 8-azahypoxanthine were purchased from Sigma; inosine, adenosine, guanine, and hypoxanthine from Aldrich; 2′-deoxyguanosine from Pfaltz and Bauer; N^3-methylguanine, N^7-methylguanine, N^9-methylguanine, and N^7-methylguanosine from Vega-Fox Biochemicals. Monomethylamine and dimethylamine were from Matheson Gas Products.

Spectroscopy. Ultraviolet spectra were determined on a Cary Model 14 or Beckman Acta III spectrophotometer equipped with thermostated cell compartments. ^1H NMR spectra were taken on a JEOL PS-FT-100 instrument.

pH measurements were performed on a Radiometer pH M26 meter equipped with a pH-stat assembly.

Enzyme Purification. Purine nucleoside phosphorylase from human erythrocytes was purified according to the method of Tsuboi and Hudson,[17] taken through a calcium phosphate chromatographic step and resulting in enzyme with a specific activity of 10.9 units/mg of protein. Prior to use the enzyme was diluted with 0.001 M EDTA and 0.01% Triton X-100 which reportedly stabilize the enzyme.[17] The protein content was determined by the method of Lowry et al.[31]

The enzyme activity was monitored by the orcinol reaction.[32] Employing arsenate instead of phosphate drives the reaction toward nucleoside breakdown as ribose 1-arsenate is hydrolytically labile. The enzyme activity was also measured by monitoring the amount of guanine produced while deoxyguanosine was the substrate. A unit of activity is defined as the amount liberating 1 μmol of guanine (or ribose) per minute under the specified assay

conditions. Finally, in some cases the xanthine oxidase coupled assay was employed.[3b]

Determination of Steady-State Kinetic Parameters. In determining the relative activity of various nucleosides as substrates the orcinol reaction[32] was employed (analyzing the amount of ribose formed).

K_M, V_{max}, and K_I were determined, assuming Michaelis–Menten behavior, from Lineweaver–Burk plots.[33] Relative activities were based on the amount of ribose formed in the course of a given incubation time compared to the amount produced under the same conditions by guanosine, the standard of comparison. In a typical reaction mixture 100 μmol of pH 6.0 acetate buffer, 5 μmol of nucleoside, 50 μmol of arsenate, and a given amount of enzyme in a total volume of 1.0 mL were incubated at 37 °C for 30 min. An aliquot of this mixture was removed and the reaction was quenched by the addition of 0.5 mL of 7% $HClO_4$ containing 60 mg of activated charcoal as a suspension to remove unutilized nucleoside. Next the reaction was left at room temperature for about 10 min and shaken occasionally. The mixture was centrifuged and an aliquot of the supernatant solution was used for assay of the ribose employing the orcinol reaction.[32] In a control reaction 0.5 mL of 7% $HClO_4$ and 60 mg of activated charcoal were added to the enzyme before the addition of substrate and the usual protocol was followed from there on.

The inhibition vis-à-vis guanosine phosphorolysis was determined by analyzing for the guanine formed according to the phenol reaction.[34]

$$\text{guanosine} + HAsO_4^{2-} \xrightarrow[\text{phosphorylase}]{\text{purine nucleoside}} \text{guanine} + \alpha\text{-D-ribose 1-arsenate}$$

$$\text{guanine} + \text{phenol reagent} \rightarrow \text{blue color}$$

The protocol was the same as when determining alternate substrate activity (vide supra) except that quenching was done by the addition of 2.0 mL of 20% Na_2CO_3. Guanine then was determined directly in the reaction mixture by (1) addition of 2.5 mL of H_2O and 0.5 mL of phenol reagent, (2) allowing the blue color to develop for 30 min at 25 °C (no absorbance change was observed after this time), and (3) reading the absorbance at 660 nm. Appropriate substrate blanks were also measured.

On guanosine as substrate the orcinol and phenol reagent reactions were shown to lead to nearly identical results.

Experiments on 5′-Deoxyinosine. 5′-Deoxyadenosine (10 mg) (U.S. Biochemical Corp.) was incubated with 0.1 mL (40 units) of adenosine deaminase [Boehringer-Manheim; 2 mg/mL suspended in 3.2 M $(NH_4)_2SO_4$] in 3.75 mL of 0.1 M pH 7.0 Tris buffer for 3.5 h at 37 °C or 15 h at 25 °C. The UV spectral differences of inosine and adenosine suggested that the conversion of 5′-deoxyadenosine to 5′-deoxyinosine could be ascertained by monitoring the decrease in absorbance at 265 nm. At the end of the incubation periods at least 80% of the 5′-deoxyadenosine had been deaminated. The resulting solution was employed directly in the subsequent studies on purine nucleoside phosphorylase. The residual 5′-deoxyadenosine would give rise to insignificant substrate or inhibitory activity (see Results and Discussion). The activity of purine nucleoside phosphorylase toward 5′-deoxyinosine as substrate was measured spectrophotometrically by the coupled xanthine oxidase method.[3b] The reaction mixture contained 0.268 mM 5′-deoxyinosine, 95.8 mM pH 7.5 phosphate buffer, 0.05 unit of xanthine oxidase, and 0.002 unit of purine nucleoside phosphorylase in a total volume of 3.12 mL.

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