SPECIFICITY OF PURINE NUCLEOSIDE PHOSPHORYLASE FROM *Escherichia coli*

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Substrate specificity of electrophoretically homogeneous purine nucleoside phosphorylase from *E. coli* was determined; purine nucleoside analogs were tested as substrates and inhibitors of phosphorolysis and phosphateless ribosyl transfer; some purine base analogs were tested for ribosyl-acceptor activity. 9-(α-L-Lyxofuranosyl)adenine was the only sugar-modified nucleoside analog capable of phosphorolytic and arsenolytic cleavage. Chemical substitution at positions 2, 7 and 8 of purine ring was found to alter substrate activity, while position 6 was of minor importance. The introduction of thio or methylthio group into position 6 or 2 enhanced the affinity of the nucleoside for the enzyme about 20-times; cumulation of primary amino groups in 8-aminoadenosine or 2-aminoadenosine increased the affinity, while a similar cumulation of hydroxyl groups in xanthosine or 8-hydroxyguanosine caused a loss of activity. Some analogs such as 7-deazaadenosine or 7-deazainosine were inhibitory non-substrates. Some purine bases were found to inhibit the phosphorolysis of nucleosides, while several nucleoside analogs inhibited the ribosylation of guanine or adenine with ribose 1-phosphate.

Purine nucleoside phosphorylase (purine nucleoside : orthophosphate (deoxy)ribosyltransferase, E.C. 2.4.2.1) of *E. coli* catalyses two types of reactions:

$$\text{Rib-base} + P_i \rightleftarrows \text{Rib-1-P} + \text{base}\,, \qquad (A)$$

$$\text{Rib-base}^1 + \text{base}^2 \rightleftarrows \text{Rib-base}^2 + \text{base}^1\,. \qquad (B)$$

Reaction (*B*) takes place even in the absence of phosphate or arsenate. The enzyme has recently been purified to homogeneity and reaction (*A*) was shown to proceed according to sequential mechanism, with free enzyme binding primarily the nucleoside or ribose 1-phosphate[1]. Therefore it seemed of interest to investigate the specificity of substrate-binding sites of this enzyme. In the experiments described here purine nucleoside analogs, modified either in the sugar or base moieties, were assayed as substrates and inhibitors of phosphorolysis (Eq. (*A*)) or in the function of ribosyl donors in (*B*); purine bases were tested for ribosyl-acceptor ability with either ribose 1-phosphate (*A*) or a purine nucleoside (*B*) as ribosyl donors.

EXPERIMENTAL

Sugar-modified adenosine analogs were either prepared by previously described procedures (see Table I for reference) or synthesized anew. The α- and β-anomers of 9-(D/L-arabinofuranosyl)-adenine (*II*–*V*) and 9-(β-D-xylofuranosyl)adenine (*VI*) were prepared from adenine and D- or L-arabinose, or D-xylose, respectively, using polyphosphoric acid in triphenyl phosphate as described by Schramm[2] and purified by Dekker chromatography on Dowex I in 50% methanol[3]. The structural assignment of anomers was performed by means of CD spectra. 9-(β-D-Ribopyranosyl)adenine (*X*) was prepared by chemical synthesis using a known procedure[4] with slight modifications. (+)-Aristeromycin and (±)-aristeromycin were synthesized chemically[5,6].

Base-modified nucleoside analogs were either purchased from Calbiochem, Los Angeles, USA, or Waldhof, Mannheim, GFR, or synthesized chemically by previously described procedures (see Table II for reference).

Heterocyclic bases. Hypoxanthine, adenine, 6-mercaptopurine and 8-azaguanine were purchased from Calbiochem, Los Angeles, USA. 2-Methylthioadenine was prepared by methylation of 2-thioadenine (Lachema, Czechoslovakia) with methyl iodide; 6-chloropurine was prepared from hypoxanthine by a reported procedure[7] and transformed into 6-methylamino- or 6-dimethylaminopurine by treatment with 30% methanolic solution of the appropriate amine (20–25 molar excess) at 100°C for 3 hours. The bases were crystallized from methanol. 8-Aminoadenine was prepared from 8-bromoadenine *via* the 8-azido intermediate in the same way as described for 8-aminoadenosine[8]. 2,6-Diaminopurine was purchased from Lachema, Czechoslovakia.

All compounds tested were analytically pure, chromatographically homogeneous and their UV absorption spectra agreed with those published in the literature. The homogeneity of ribonucleosides was also checked by paper electrophoresis in borate buffer.

[8 ^{3}H]Guanosine (9·1 Ci/mmol), [8 ^{3}H]guanine (9·1 Ci/mmol) and [8 ^{3}H]adenine (11·6 Ci/mmol) were obtained from ÚVVVR, Prague. 9-(α-L-Lyxofuranosyl)-[8 ^{3}H]-adenine (22 mCi/mmol) was prepared as described earlier.[9]

The enzyme, purine nucleoside phosphorylase, was purified[1] from *E. coli* B induced with inosine. Phosphorolytic activity of the electrophoretically purified preparation with guanosine as substrate was 39 μmol/min/mg protein.

Substrate activity of the nucleoside analogs was tested as follows. The reaction mixture contained 1·5 mM nucleoside, 50 mM phosphate or arsenate and 7 μg enzyme protein, in a total volume 0·4 ml. Samples (0·1 ml) were taken in 20 min intervals and applied onto paper Whatman No 3; the sheets were chromatographed in 50% Na_2HPO_4 saturated with isoamyl alcohol. The chromatograms were scanned in UV light, using a Mineralite lamp, and gradual appearance of a spot with an R_F lower than that of the nucleoside was taken as indicating phosphorolysis. Some nucleosides which gave negative or ambiguous results in this test were assayed using paper electrophoresis in 0·1M triethylammonium borate buffer, pH 7·5 (20 V/cm, 2 hours).

Inhibition of phosphorolysis of guanosine, or ribosylation of guanine, by nucleoside and base analogs was tested using [^{3}H] labeled substrates. Phosphorolysis or arsenolysis were tested in 50 mM phosphate or arsenate buffers, pH 7·5, with guanosine in the conc. range of 10 μM up to 1 mM, and the inhibitors in conc. up to 4 mM. The reaction was started by adding the enzyme (usually 7 μg per 0·4 ml of reaction mixture). Samples (0·1 ml) were taken in intervals and immediately applied onto spots on paper Whatman No 3, where a mixture of nonlabeled guanosine and guanine (50 μg of each, dissolved in 50 μl water) had been applied before the start of the experiment, and left to dry. The sheets were then chromatographed as above. Spots of guanosine

and guanine, visible in UV light, were cut out and their radioactivity determined with a liquid-scintillation counter. Ribosylation of guanine, either with ribose 1-phosphate or a nucleoside, was tested in a similar manner, using 50 mM Tris buffer, pH 7·5. Ribosyl-donor activity of nucleoside analogs in phosphate-free medium was determined using [8 ^{3}H]adenine as ribosyl acceptor; the amount of adenosine formed was assayed chromatographically as above, using nonradioactive adenine and adenosine as carriers. The acceptor function of bases was estimated by measuring the rate of conversion of guanosine to guanine in Tris-medium, lacking phosphate or arsenate.

The uptake of guanosine by whole cells of *E. coli* B was measured with the [^{3}H] labelled substance, using rapid-filtration technique[10]; the rates were normalized for 10^{12} bacteria/l.

RESULTS

Specificity of the Sugar Moiety of Nucleoside Analogs

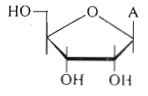
Among the various analogs of adenosine modified in the sugar moiety, 9-(α-L-lyxofuranosyl)adenine (*VII*) was the only substrate for phosphorolysis or arsenolysis by purine nucleoside phosphorylase (Table I). The identity of enzymatic activities

TABLE I

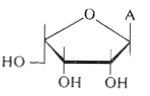
Substrate and Inhibitory Activity of Purine Nucleoside Analogs Modified in the Sugar Component

Formula	Compound	Substrate activity[a]	K_I[b]
I	adenosine	+	350
	guanosine	+	350
	2′-deoxyadenosine	+	890
	2′-deoxyguanosine	+	1 100
II	9-(α-L-arabinofuranosyl)adenine[3]	–	>2 300
III	9-(β-L-arabinofuranosyl)adenine[3]	–	>1 800
IV	9-(α-D-arabinofuranosyl)adenine[3]	–	>1 800
V	9-(β-D-arabinofuranosyl)adenine[3]	–	>1 800
VI	9-(β-D-xylofuranosyl)adenine[3]	–	>1 800
VII	9-(α-L-lyxofuranosyl)adenine[16]	+	>3 500
VIII	2′-O-methyladenosine[17]	–	>3 500
IX	3′-O-methyladenosine[17]	–	>3 500
X	9-(β-D-ribopyranosyl)adenine[4]	–	>3 500
XI	L-adenosine[18]	–	> 3500
XII	aristeromycin[6]	–	>3 500

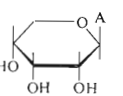
[a] Formation of base by phosphorolysis and arsenolysis as detected by paper chromatography. [b] Inhibition constant of phosphorolysis of guanosine.



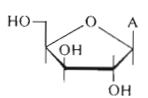
I



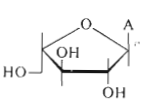
VII



X



VI



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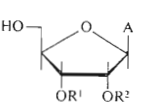
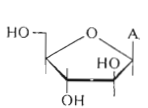
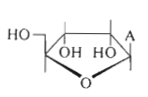


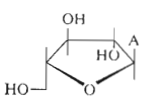
VIII, $R^1 = H$, $R^2 = CH_3$
IX, $R^1 = CH_3$, $R^2 = H$



V



XI



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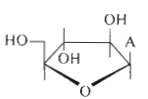
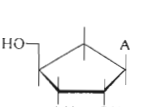


III



(+)-*XII*

responsible for the phosphorolysis of *VII* and inosine was verified by acrylamide gel electrophoresis (Fig. 1). The lack of measurable competition of *VII* with guanosine,

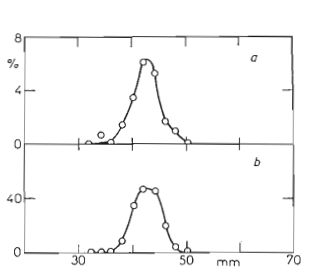


FIG. 1

Coincidence of Phosphorolytic Activities for 9-(α-L-Lyxofuranosyl)adenine and Inosine on Electrophorograms of Purine Nucleoside Phosphorylase

Abscissa: Distance of migration of proteins on 8% acrylamide gel; ordinate: % phosphorolysis with eluates from single slices of the gel, measured after 30 min incubation. *a*, phosphorolysis of 9-(α-L-lyxofuranosyl)-[8 ^{3}H]adenine determined by measuring the radioactivity of chromatographically separated adenine; *b*, phosphorolysis of inosine determined spectrophotometrically after chromatographic separation of inosine and hypoxanthine.

TABLE II

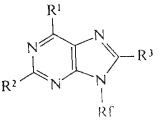
Substrate and Inhibitory Activity of Purine Nucleoside Analogs

Formula	Nucleoside	Substrate activity			K_I[d] μM
		phosphorolysis[a]	ribosyl-transfer donor[b] %	ribosyl-transfer acceptor[c] %	
I	adenosine[e]	+	n.d.	100	350
XIII	inosine[e]	+	91	164	380
XIV	purine riboside[f,g]	+	14	n.d.	490
XV	6-chloropurine riboside[22]	+	2·4	44	17
XVI	6-methoxypurine riboside[21]	+	57	n.d.	150
XVII	6-thioinosine[f]	+	125	54	22
XVIII	6-methylthiopurine riboside[f]	+	82	n.d.	17
XIX	6-hydroxylaminopurine riboside[f]	+	n.d.	n.d.	690
XX	N^6-methyladenosine[23]	+	63	133	490
XXI	N^6-dimethyladenosine[23]	+	11	30	220
XXII	2-aminopurine riboside[19]	+	n.d.	n.d.	120
XXIII	2-aminoadenosine[27]	+	38	74	48
XXIV	2-methylthioadenosine[24]	+	33	56	98
XXV	xanthosine[e]	—	n.d.	n.d.	>3 500
XXVI	isoguanosine[25,26]	—	n.d.	n.d.	>2 300
XXVII	7-deazainosine[e]	—	0·5	n.d.	250
XXVIII	7-deazaadenosine[c]	—	6·0	n.d.	120
XXIX	8-azaguanosine[28]	—	0·0	38	>1 700
XXX	inosine N^1-oxide[20]	weak	n.d.	n.d.	>2 300
XXXI	adenosine N^1-oxide[20]	+	18	n.d.	380
XXXII	1-methylinosine[21]	+	120	n.d.	380
XXXIII	1-methyladenosine[24]	very weak	10·5	n.d.	130
XXXIV	8-bromoadenosine[8]	—	n.d.	n.d.	>1 700
XXXV	8-methoxyadenosine[8]	—	n.d.	n.d.	260
XXXVI	8-thioadenosine[8]	—	n.d.	n.d.	>1 700
XXXVII	8-aminoadenosine[8]	+	21	137	5·5
XXXVIII	8-hydroxyguanosine[8]	—	n.d.	n.d.	>3 500
XXXIX	8-dimethylamino-guanosine[29]	—	n.d.	n.d.	>3 500
XL	8-methylguanosine[30]	—	n.d.	n.d.	860
XLI	8-aminoguanosine[8]	+	35	n.d.	690
XLII	isoadenosine[31]	+	6·9	n.d.	1 150

[a] Formation of base by phosphorolysis and arsenolysis as detected by paper chromatography. [b] Relative rate of ribosylation of adenine in Tris buffer (no phosphate) with nucleoside analogs (rate with Guo = 100%). [c] Relative rate of conversion of guanosine to guanine in Tris buffer (no phosphate) in the presence of purine base analogs (rate with adenine = 100%). [d] Inhibition constant of phosphorolysis of guanosine with nucleoside analogs. [e] Purchased from Calbiochem,

however, indicated that the binding of the lyxofuranosyl group is weak. In the presence of the enzyme and *VII* a slight portion of labelled adenine was converted to a form with chromatographic mobility similar to that of adenosine, indicating that the enzyme is capable of catalyzing the exchange of the lyxofuranosyl group.

All other isomeric adenine 9-aldopentafuranosides, including L-adenosine (*XI*) were completely inactive (Table I). Neither substrate nor inhibitory activity could have been detected with the carbocyclic adenosine analog aristeromycin (*XII*) or with the isomeric 9-(β-D-ribopyranosyl)adenine (*X*). On the other hand isoadenosine, *i.e.* 3-(β-D-ribofuranosyl)adenine (*XLII*) was substrate of the phosphorolytic reaction, inhibited the phosphorolysis of guanosine and was capable of functioning as ribosyl donor for the ribosylation of adenine (Table II).



I, $R^1 = NH_2$, $R^2 = R^3 = H$
XIV, $R^1 = R^2 = R^3 = H$
XV, $R^1 = Cl$, $R^2 = R^3 = H$
XVI, $R^1 = OCH_3$, $R^2 = R^3 = H$
XVII, $R^1 = SH$, $R^2 = R^3 = H$
XVIII, $R^1 = SCH_3$, $R^2 = R^3 = H$
XIX, $R^1 = NHOH$, $R^2 = R^3 = H$
XX, $R^1 = NHCH_3$, $R^2 = R^3 = H$
XXI, $R^1 = N(CH_3)_2$, $R^2 = R^3 = H$
XXII, $R^2 = NH_2$, $R^1 = R^3 = H$
XXIII, $R^1 = R^2 = NH_2$, $R^3 = H$
XXIV, $R^1 = NH_2$, $R^2 = SCH_3$, $R^3 = H$
XXV, $R^1 = R^2 = OH$, $R^3 = H$
XXVI, $R^1 = NH_2$, $R^2 = OH$, $R^3 = H$
XXXIV, $R^1 = NH_2$, $R^2 = H$, $R^3 = Br$
XXXV, $R^1 = NH_2$, $R^2 = H$, $R^3 = OCH_3$
XXXVI, $R^1 = NH_2$, $R^2 = H$, $R^3 = SH$
XXXVII, $R^1 = R^3 = NH_2$, $R^2 = H$
XXXVIII, $R^1 = R^3 = OH$, $R^2 = NH_2$
XXXIX, $R^1 = OH$, $R^2 = NH_2$, $R^3 = N(CH_3)_2$
XL, $R^1 = OH$, $R^2 = NH_2$, $R^3 = CH_3$
XLI, $R^1 = OH$, $R^2 = R^3 = NH_2$

Methylation of the 2′ and 3′ hydroxyl groups of the ribosyl moiety of adenosine produced completely inactive compounds *VIII* and *IX*, respectively.

Effect of Substitution in the Purine Ring

Many analogs substituted in the purine ring were substrates of the enzyme and most of them inhibited the phosphorolysis of guanosine. The competitive character of inhibition was demonstrated by Lineweaver–Burk plots with 6-chloropurine ribofuranoside (*XV*), 8-aminoadenosine (*XXXVII*), 2-methylthioadenosine (*XXIV*),

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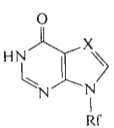
Los Angeles, USA. [f] Purchased from Waldhof, Mannheim, GFR. [g] In all compounds listed, riboside represents a 9-(β-D-ribofuranosyl) derivative.

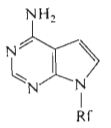
7-deazaadenosine (*XXVIII*), and 8-aminoguanosine (*XLI*). For other co-substrates the competitive character of inhibition was presumed and the K_I values (Table II) were calculated from the extent of inhibition at two different substrate/inhibitor ratios.

Positions 6 and 1. Most 6-substituted purine nucleosides, including the parent compound, purine nucleoside (*XIV*) were substrates of purine nucleoside phosphorylase; many of these derivatives had very high affinity for the enzyme as evident from extremely low values of the K_I (Table II). Although the affinity for the enzyme appeared to increase with the electronegativity of the substituent (—Cl > —SCH_3 > > —SH > —OCH_3), no linear correlation of the K_I-values with either the σ_{meta} or σ_M and σ_I could be demonstrated.

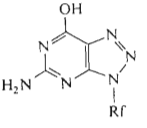
Since purine ribonucleoside, (*XIV*), adenosine (*I*) and inosine (*XIII*) were good substrates of the enzyme, it appeared that the lactim-lactam tautomerism of the purine ring does not affect the affinity of the nucleoside for the enzyme. 1-Methylinosine (*XXXII*) with forced oxo form had about the same K_I and slightly higher ribosyl-donor activity in comparison with inosine. In 1-methyladenosine (*XXXIII*), however, the ribosyl-donor activity was strongly reduced. This behavior may be understood in the following manner: the methylation in position 1, or the occurrence of imino form in position 6 forced by methylation, do not affect much the aromaticity of the purine ring system of the nucleoside and therefore do not interfere with the binding of the nucleosides to the enzyme. The corresponding free bases, however, may be expected to assume different structures: 1-methyladenine occurs predominantly in the 6-amino form with unprotonated N^9 atom; in this form the base cannot be



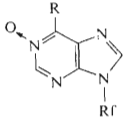
XIII, X = —N=
XXVII, X = —CH=



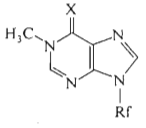
XXVIII



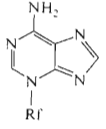
XXIX



XXX, R = OH
XXXI, R = NH



XXXII, X = O
XXXIII, X = NH



XLII

Rf = β-D-ribofuranosyl residue

ribosylated and may cause a dead-end inhibition of the enzyme; with 1-methylhypoxanthine, however, such situation does not arise.

The inactivity of inosine N^1-oxide (*XXX*) is probably due to electron withdrawal by the strongly polarized N→O-group; in adenosine N^1-oxide (*XXXI*) this effect is compensated by electron-donor ability of the 6-amino group.

Position 2. The presence of an amino or methylthio group at position 2 (*XXII* to *XXIV*) enhances the affinity for the enzyme in comparison with the parent compound, while the ribosyl-donor ability is slightly decreased. A hydroxyl group at position 2 inactivates the analog completely (xanthosine *XXV*, isoguanosine *XXVI*). This may be due to a conformational change in *XXV* and *XXVI*, where the 2-hydroxyl group may interact with the sugar moiety when in *syn*-conformation; however, there are no data available on the extent of this interaction. The acidity of xanthosine (pK_a 5·7) may also prevent its binding to the enzyme.

Position 7. The replacement of the N^7 atom by a methine group in tubercidin (7-deazaadenosine *XXVIII*) and 7-deazainosine (*XXVII*) results in inhibitory non-substrates.

Position 8. Most substituents at this position (—Br, —OCH_3, =S, —OH, —CH_3, —Me_2N) inactivate the analogs by changing their conformation[11,12]. 8-Aminoadenosine (*XXXVII*), however, is the strongest known competitive inhibitor of purine nucleoside phosphorylase; the compound is a comparatively weak ribosyl donor (Table II and III) but the corresponding base, 8-aminoadenine, is a good ribosyl acceptor with guanosine as donor. On the other hand, 8-aminoguanosine (*XLI*) is a weak inhibitor, but a better ribosyl donor than 8-aminoadenosine. 8-Azaguanosine (*XXIX*) turned out to be inert, but 8-azaguanine was a good ribosyl acceptor.

TABLE III

Relative Reaction Rates (in % of Control) of Phosphorolysis and Arsenolysis of Nucleosides as Compared with the Rates of Ribosylation of Corresponding Bases

Base component of the reactant	Phosphorolysis[a]	Arsenolysis[a]	Ribosylation of base[b]
6-Chloropurine	74	134	9
8-Aminoadenine	31	53	78
2-Methylthioadenine	100	89	85

[a] Reaction rate with adenosine = 100%. [b] Rate of ribosylation of adenine with ribose 1-phosphate (10 mM) = 100%.

Inhibition of Phosphorolysis by Base Analogs

Theoretical considerations (see Discussion) indicated that the strong inhibitory but poor substrate activity of some nucleoside analogs (Tables II and III) might be due to tight binding of the base to the purine-binding site of the enzyme, forming a dead-end enzyme–base–phosphate complex[1]. In this case we would expect that the addition of the base analog would inhibit phosphorolysis. To test this assumption the inhibition of phosphorolysis and arsenolysis of guanosine with a selected group of nucleoside–base pairs was studied (Table IV). Some bases, notably 8-aminoadenine and 6-chloropurine, markedly inhibited both arsenolysis and phosphorolysis, but always less then the corresponding nucleosides. Conversely, some nucleosides inhibited ribosylation of guanine with ribose 1-phosphate, but less than the corresponding bases (Table V).

Specificity of Inhibition of Guanosine Phosphorolysis by Whole Cells of E. coli

The specificity pattern of purine nucleoside phosphorylase of *E. coli* is different from any other known enzyme of purine nucleoside metabolism[13]. Therefore the participitation of this enzyme in metabolic conversion of purine nucleosides *in vivo* may be detected by characteristic inhibition pattern. Comparing the effect of selected analogs on purified nucleoside phosphorylase with the inhibition of phosphorolysis of guanosine by whole cells and with guanosine uptake in short pulses, an almost identical pattern was observed in all three cases (Table VI). This indicates that *a*) the enzyme isolated was identical with that responsible for guanosine phosphorolysis in whole cells, and *b*) interaction with purine nucleoside phosphorylase is a major

TABLE IV

Inhibition of Phosphorolysis and Arsenolysis of Guanosine with Purine Nucleoside Analogs and Corresponding Bases

The conc. of guanosine was 20 μM, that of the analogs 200 μM. The reaction rates were expressed in % control.

Base component of the inhibitors	Rate of phosphorolysis in the presence of		Rate of arsenolysis in the presence of	
	nucleoside	base	nucleoside	base
8-Aminoadenine	1·7	16	3·8	32
6-Chloropurine	3·2	16	5·9	20
6-Mercaptopurine	2·0	10·3	n.d.	6·8
6-Dimethylaminopurine	n.d.	62	n.d.	62
2-Methylthioadenine	5·8	73	3·0	48

pathway of guanosine uptake by the cells. It appears that cellular purine nucleoside phosphorylase is accessible to guanosine from the medium directly, without the intervention of any permease[14].

TABLE V

Inhibition of Ribosylation of Guanine with Purine Bases and Corresponding Nucleosides

The conc. of guanine was 20 μM, that of ribose 1-phosphate 100 μM. Inhibitors were at 200 μM. The reaction rates were expressed in % control.

Base component of the inhibitor	Rate of ribosylation of guanine in the presence of	
	nucleoside	base
8-Aminoadenine	21	6·1
6-Chloropurine	57	5·4
6-Dimethylaminopurine	89	43
2-Methylthioadenine	87	21
6-Mercaptopurine	67	8·3

TABLE VI

Inhibition of Guanosine Uptake and Phosphorolysis by Whole Cells and with Purified Purine Nucleoside Phosphorylase

All reactions were performed in the medium of Spizizen[32]; the conc. of guanosine was 17·7 μM, that of the inhibitors 200 μM; the numbers indicate % inhibition.

Formula	Inhibitor	Uptake[a] whole cells	Phosphorolysis[a]	
			whole cells	enzyme
XIII	inosine	87	95	90
XIV	purine riboside	39	41	60
XVII	6-thioinosine	92	95	90
XIX	6-hydroxylaminopurine-riboside	40	60	59
XX	N^6-methyladenosine	77	80	59
XXII	2-aminopurine riboside	94	96	96
XXV	xanthosine	5	6	5
XXXVII	8-aminoadenosine	99	100	99
XXXVIII	8-hydroxyguanosine	25	26	35
XXXIX	8-dimethylaminoguanosine	10	12	5
XLI	8-aminoguanosine	57	77	60

[a] The rates of guanosine conversion without inhibitors (100%) were 1·7 μM/min in uptake, 1·5 μM/min in the phosphorolysis by whole cells, and 3·1 μM/min with 20 μg/ml purified enzyme.

DISCUSSION

Purine nucleoside phosphorylase of *E. coli* consists of six identical subunits, each containing two binding sites[1]. The experiments with isomeric aldopentafuranosides indicate strict specificity of the enzyme for the configuration of 2′ and 3′ hydroxyl groups, *i.e.* for their mutual orientation and the position relative to the purine ring. These results are compatible with the presumed existence of two partial binding sites, acting in a cooperative manner, one for the ribosyl group and the other for the purine base. The essential part of the ribosyl-binding site is a region with the affinity for the 2′ and 3′ hydroxyl groups in β-D-*ribo* configuration with respect to the purine base; the isomeric β-D-ribopyranoside (*X*) has no affinity for the enzyme, since the dimensions of the hydrophilic part of the molecule and its distance and orientation with respect to the purine ring are different from the parameters of the β-D-ribofuranoside. On the other hand, the 4′-hydroxymethyl group is less critical, since the inversion of configuration at the 4′-atom in 9-(α-L-lyxofuranosyl)adenine (*VII*) does not suppress substrate activity. However, the lack of measurable competition with guanosine (Table I) indicates that *VII* is bound by the enzyme in an atypical manner, *i.e.* by the base moiety and not primarily by the pentosyl moiety as in the case of β-D-ribofuranosides.

Though stereochemically non-variant, the connection between the ribosyl- and purine-binding sites appears to be flexible enough to permit a translational displacement necessary for the binding of isoadenosine (*XLII*).

As already mentioned, some negative effects of chemical modification of nucleosides may be due to conformational changes. Most substrates of purine nucleoside phosphorylase such as adenosine occur predominantly in the *anti* conformation; although the energy required for rotating around the nucleoside bond is probably not large in purine series, the strong restriction of rotation found in β-D-arabinoside[15] and the forced *syn* conformation of some 8-substituted purine nucleosides[11,12] may account for the inactivity of these compounds. Similarly the inactive carbocyclic analog of adenosine, aristeromycin (*XII*) has been shown by one of us to possess a *syn* conformation in aqueous solution[6].

The data of Tables II and III show that many nucleoside analogs are very strong inhibitors of phosphorolysis, while their ribosyl-donor activities, with either P_i or adenine as acceptors, are relatively poor. To account for this behaviour, the differential effect of chemical substitution on the affinity of the nucleoside and the corresponding free base should be considered. Since the extent and even the sign of these changes in nucleosides and bases may be different, the overall effect of the substitution upon the kinetics of the reactions catalysed by purine nucleoside phosphorylase may be complex. Although the pertinent kinetic parameters could not be determined, an attempt was made to express the effects of purine substitution in a qualitative manner (Table VII).

Kinetic analysis of phosphorolysis of inosine with purine nucleoside phosphorylase indicated that the nucleoside is the first substrate to attach to, and ribose 1-phosphate the last product to leave the surface of the enzyme[1]. The same order of binding was found studying guanosine phosphorolysis[33]. Besides that, binding studies indicate the formation of enzyme–base–P_i complex[1]. If this ternary complex involving a substituted base is abnormally strong, dead-end inhibition of phosphorolysis of the corresponding nucleoside analog may be expected to occur. Good ribosyl donors such as adenosine, 2′-deoxyadenosine or inosine are relatively weak inhibitors of phosphorolysis of other nucleosides, indicating that the binding of adenine or hypoxanthine moiety in the ternary complexes is relatively weak.

Other types of behaviour of some nucleoside–base pairs may be interpreted in a similar manner, taking into account the differential effect of substitution on the affinity of the nucleoside and base (Table VII). *E.g.* 7-deazainosine (*XXVII*) appears to be bound relatively weakly in the nucleoside form, while the free base appears to bind very strongly, causing complete dead-end inhibition of the enzyme. 8-Azaguanosine (*XXIX*) apparently does not interact with the enzyme at all, while 8-azaguanine behaves like a normal purine base, serving as ribosyl acceptor.

TABLE VII

Tentative Interpretation of Interaction of Some Nucleoside-Base Pairs with Purine Nucleoside Phosphorylase

Interpretation: *A*) Nucleoside bound primarily by the ribosyl moiety; free base does not bind to free enzyme (normal substrate). *B*) Binding of the nucleoside enhanced by substitution. *C*) Binding of the base, or base moiety of the nucleoside, is strongly enhanced by substitution. *D*) Free base forms a very strong enzyme–P_i–base complex; dead-end inhibition of the enzyme. *E*) Free base, but not the nucleoside, is recognized by the enzyme. *F*) Base component of the nucleoside strongly bound; free base is not bound to the Rib 1–P–enzyme complex, but may be bound to the guanosine–enzyme complex by base exchange.

Activities of nucleoside		Activity of base (pentosyl acceptor)	Example	Interpretation
Pentosyl donor	inhibitor			
Good	weak	good	*XIII*	*A*
Good	strong	good	*XVIII*	*B*
Poor	very strong	good	*XXXVII*	*C*
None	moderate	n.d.	*XXVII*	*D*
None	none	good	*XXIX*	*E*
Poor	very strong	[a]	*XV*	*F*

[a] Good with Guo, poor with Rib 1-P.

The experiments with nucleoside analogs substituted at the purine base do not permit any simple conclusion about the structural requirements of the enzyme. The affinity and the reactivity of the nucleoside analogs is not primarily determined by the electron density at the C^9 atom immediately engaged in the reaction, but appears to be defined by the electron distribution in the whole purine ring system. This is illustrated by the effect of introducing additional primary amino groups, which enhance the affinity if the parent compound has an aromatic π-electron distribution. Thus, in terms of affinity, adenosine (*I*) > purine riboside (*XIV*); 2-aminopurine riboside (*XXII*) > purine riboside (*XIV*); 2-aminoadenosine (*XXIII*) > adenosine (*I*); 8-aminoadenosine (*XXXVII*) ⩾ adenosine. In the partially aromaticized systems such as 6-oxoderivatives, the introduction of an amino group does not enhance affinity; neither guanosine nor 8-aminoguanosine (*XLI*) possess higher affinity than inosine. The insufficient knowledge of electron distribution in substituted purine bases and nucleosides does not permit more detailed evaluation of the empirical data at present.

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