Linear Free Energy Relationships for N(7)-Substituted Guanosines as Substrates of Calf Spleen Purine Nucleoside Phosphorylase. Possible Role of N(7)-Protonation as an Intermediary in Phosphorolysis

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Quantitative structure-activity relationships (QSAR) for a series of N(7)-substituted guanosines as substrates for calf spleen purine nucleoside phosphorylase (PNP) were developed, and compared with those for acid hydrolysis of these analogues. There is no correlation between the rates for enzymatic phosphorolysis and acid hydrolysis, indicating that for the enzymatic reaction labilization of the glycosidic bond is not the only, nor the predominant, effect of N(7)-substitution. Multiple regression analysis of the enzymatic process revealed that optimal substrate properties (minimal Michaelis constant) are associated with the Taft electronic constant equal zero and a substituent size, parametrized by the Taft steric constant, smaller than that for a methyl group. These results support the hypothesis of protonation of the N(7)-position of the base by the enzyme as a catalytic mechanism for calf spleen PNP.

Attention is drawn to the postulated similar mechanism of action of other purine N-glycosidases, including plant antiviral proteins which function as RNA N-glycosidases, and possibly some DNA N-glycosidases which function as repair enzymes.

Purine nucleoside phosphorylase (PNP, purine-nucleoside: orthophosphate ribosyltransferase, EC 2.4.2.1.) catalyzes reversible phosphorolytic reaction:

purine nucleoside + $P_i \rightleftharpoons$
purine + ribose-1-phosphate.

The mechanism for enzymatic cleavage of the glycosidic bond remains to be clarified. Kinetic studies on the acid-catalyzed hydrolysis of purine nucleosides [1], known to proceed *via* intermediary protonation of the purine ring N(7), led to the proposal that the rate-limiting step for enzymatic phosphorolysis is protonation of the ring N(7) of the purine base [2]. This derives support from the observation that, with bovine thyroid PNP, the rate of phosphorolysis of m^7Ino, which necessarily carries a positive charge at N(7), is even more rapid than for the parent Ino [2].

Other N(7)-methyl purine nucleosides, such as m^7Guo and m^7Ado, were subsequently found to be good substrates of PNP from various sources [3–5]. With the enzyme from mammalian sources (human erythrocyte and calf spleen), m^7Guo is cleaved more rapidly than the parent Guo [5]. With the enzyme from *E. coli*, the rate constants for m^7Ino, m^7Guo and m^7Ado are somewhat lower than for the parent nucleosides, but all three remain good substrates [5]. Furthermore, in contrast to the natural nucleosides, phosphorolysis of N(7)-substituted congeners is apparently non-reversible [3].

In general N(7)-methylation of Ino, Guo and Ado leads to labilization of the glycosidic bond by several orders of magnitude at neutral pH [1, 6, 7], but only moderately affects rate constants for phosphorolysis [2–5]. It also follows that the N(7) of a purine nucleoside is not a binding site for PNP, albeit necessary for catalysis [5], a conclusion reinforced by the fact that 7-deaza-Ino is an inhibitor, but not a substrate; the same applies to 7-deaza-Ado with the *E. coli* enzyme [5, 8, 9].

Abbreviations: Guo, guanosine; Ino, inosine; iso-Ado, 3-β-D-ribofuranosyladenine; m^7Guo, 7-methylguanosine, with similar connotations for other analogues: et, ethyl; pr, propyl; ipr, isopropyl; bu, butyl; ibu, isobutyl; bn, benzyl; 2phet, 2-phenylethyl; 1phet, 1-phenylethyl; al, allyl; cm, carboxymethyl; ohet, hydroxyethyl.

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We have elsewhere described the substrate properties of several N(7)-alkylguanosines [4, 10]; and have now extended this to a series of 12 analogues, involving an examination of quantitative structure-activity relationships (QSAR), with a view to establishing the possible role of N(7) protonation in cleavage of the glycosidic bond by calf spleen PNP.

As will be shown below (see Discussion), PNP is only one of a class of enzymes which catalyze the cleavage of the N-glycosidic bond of purine nucleosides and nucleotides. Its mechanism of action is consequently of relevance to that of other related enzymes.

Materials and Methods

Calf spleen PNP (25 U/mg), Ino and m^7Guo were products of Sigma (St. Louis, MO, U.S.A.). Compounds **2–7** have been previously described [4]. Compounds **8–11** were obtained by enzymatic dephosphorylation of the corresponding nucleotides [4, 11–13], kindly supplied by Dr. E. Darzynkiewicz. We are indebted to Dr. G. Eisenbrand (University of Kaiserslautern, F.R.G.) for a sample of compound **12** [14].

UV absorption spectrophotometry was performed with a Zeiss (Jena, F.R.G.) Specord M40 UV-VIS recording instrument or a VSU-2P, both fitted with thermostatic cell compartments. Measurements and control of pH utilized a Mera-Elwro (Wroclaw, Poland) instrument and a combination semi-micro electrode.

Enzyme assays

Kinetic parameters for phosphorolysis of analogues **1–7** were taken from a previous report [4, 10]. For the five new substrates **8–12,** the standard spectrophotometric method [4] was employed, and the kinetic constant K_m and V_{max} obtained at pH 7 as previously described [4], from both continuous monitoring of the reaction, and initial velocity measurements [4, 15]. The parameters from both methods gave similar results in the correlation procedures described below.

pK_a values

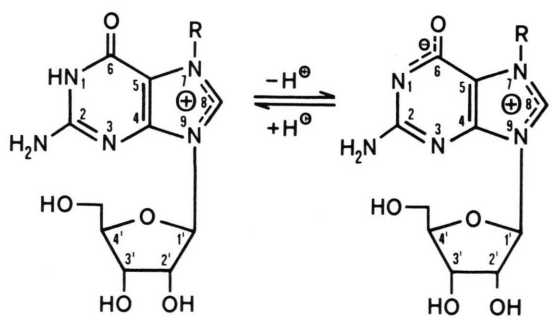
For the N(7)-substituted guanosines for which the Taft electronic constants σ for the N(7)-substituents are known, the pK_a values were calculated from the linear dependence of pK_a on σ, according to Muller and Eisenbrand [14]. For analogue **11,** for which the Taft constant for the N(7)-substituent is not known, the pK_a was determined spectrophotometrically, and the Taft constant for QSARs calculated from the σ *vs.* pK_a regression line [14].

QSAR calculations

Development of the QSAR equations, and statistical analyses, were performed on an IBM PC computer with the "Statgraphic" program commercially available from Statistical Graphics Corporation.

Results

For the N(7)-substituted guanosines embraced in this study, the N(1) proton is appreciably more mobile than that of the parent Guo, *e.g.* pK_a for m^7Guo is 7.3, as compared to 9.2 for Guo [3, 16]. Hence, when phosphorolysis is followed at pH 7, account must be taken of the existence of the N(7)-substituted guanosines as a mixture of cationic and zwitterionic species (see Scheme 1). For the mammalian enzyme employed in this study, it has been shown that only the cationic form is a substrate [3]. The zwitterionic form of m^7Guo is not bound by the enzyme, and we assume that the same holds for the zwitterionic species of the other N(7)-substituted analogues. Hence the K_m values measured at pH 7 are apparent values, which are dependent on the pK_a of a given analogue. The Michaelis constants for the cationic forms, K_m^+,



Scheme 1. Cationic and zwitterionic forms of N(7)-substituted guanosines: R = methyl (**1**), ethyl (**2**), propyl (**3**), isopropyl (**4**), butyl (**5**), isobutyl (**6**), benzyl (**7**), 2-phenylethyl (**8**), 1-phenylethyl (**9**), allyl (**10**), carboxymethyl (**11**), and hydroxyethyl (**12**).

were calculated according to the following equation:

$$K_m^+ = K_m/[1 + 10^{(pH-pK_a)}], \quad \text{where pH} = 7.0,$$

and these values employed in the QSAR calculations.

QSAR for acid-catalyzed depurination of N(7)-substituted guanosines and comparison with kinetic parameters for enzymatic phosphorolysis

For a series of N(7)-substituted guanosines, it has been shown that there is a direct correlation between the electron-withdrawing effect, expressed by the Taft constant σ for the various substituents, and the rate-constant for acid-catalyzed depurination [11, 14]. Table I presents the published rate-constants for the acid-catalyzed cleavage of the glycosidic bond of a number of N(7)-substituted guanosines [11]; and Table II the kinetic parameters for phosphorolysis of the same compounds by calf spleen PNP. The correlation matrix for interrelationship of these variables is exhibited in Table III.

Table I. First order rate constants, k, for acid-catalyzed depurination for N(7)-substituted guanosines, and Taft electronic constants, σ, for the substituents.

No.	Compound	N(7)-substituent	σ[a]	k[b] (10^{-1} s^{-1})	log$k$ (rel to m^{7}Guo)
1	m^{7}Guo	CH_3	0.000	1.37	0.000
2	et^{7}Guo	CH_2CH_3	−0.115	1.09	−0.099
5	bu^{7}Guo	$(CH_2)_3CH_3$	−0.130	1.04	−0.120
6	ibu^{7}Guo	$CH_2CH(CH_3)_2$	−0.125	1.46	0.028
7	bn^{7}Guo	$CH_2C_6H_6$	+0.215	2.62	0.283
8	2 phet^{7}Guo	$(CH_2)_2C_6H_6$	+0.080	1.72	0.099
9	1 phet^{7}Guo	$CH(CH_3)(C_6H_5)$	+0.110	1.88	0.137
10	al^{7}Guo	CH_2CHCH_2	+0.152[c]	1.89	0.140

[a] Taft electronic constants from ref. [17] p. 619.
[b] From ref. [11].
[c] For the substituent $CH_2CHCHCH_2$.

Table II. Kinetic parameters for phosphorolysis of N(7)-substituted guanosines.

Compound	N(7)-substituent	K_m [μM]	V_{max} (% rel. to Ino)	pK$_a$ N(1)H	$(K_m^+)^{exp}$ [μM]	$(K_m^+)^{calc}$ [μM]	$\frac{\Delta K_m^+}{(K_m^+)^{exp}}$	log$(K_m^+)^{exp}$	log(V_{max}/K_m^+) (V_{max}/K_m^+ % rel. to Ino)	σ[a]	E_s[b]	E_s'[c]
Ino		13	100	8.90								
Guo		11	220	9.20								
m^{7}Guo	CH_3	15	317	7.30	9.99	8.57	+0.142	1.000	2.615	0.000	0.00	0.00
et^{7}Guo	CH_2CH_3	13	298	7.44	9.54	11.80	−0.237	0.980	2.609	−0.100	−0.07	−0.08
pr^{7}Guo	$(CH_2)_2CH_3$	25	413	7.47	18.67	20.09	−0.076	1.271	2.459	−1.115	−0.36	−0.31
ipr^{7}Guo	$CH_2(CH_3)_2$	69	173	7.57	54.37	42.07	+0.226	1.735	1.617	−0.190	−0.47	−0.48
bu^{7}Guo	$(CH_2)_3CH_3$	34	682	7.49	25.69	23.12	+0.100	1.410	2.538	−0.130	−0.39	−0.31
ibu^{7}Guo	$CH_2CH(CH_3)_2$	90	538	7.48	67.61	78.70	−0.164	1.830	2.015	−0.125	−0.93	−0.93
bn^{7}Guo	$CH_2C_6H_6$	78	596	6.99	38.55	44.67	−0.159	1.586	2.303	+0.215	−0.38	−0.39
2 phet^{7}Guo	$(CH_2)_2C_6H_6$	30	347	7.18	18.06	17.82	+0.013	1.257	2.398	+0.080	−0.38	−0.35
1 phet^{7}Guo	$CH(CH_3)(C_6H_5)$	290	7.2	7.14	168.17	157.76	+0.062	2.226	−0.254	+0.110	−1.19	−0.90
al^{7}Guo	$CH_2CH{=}CH_2$	29	890	7.31	19.47	20.04	−0.029	1.289	2.774	+0.152[d]	−0.31[e]	−0.31
cm^{7}Guo	CH_2COO^-	750	15.5	7.65	612.81	612.35	+0.001	2.787	−0.483	−0.250[f]	−1.25[g]	−1.25[g]
ohet^{7}Guo	$(CH_2)_2OH$	63	693	7.00	31.50	31.50	+0.021	1.498	2.456	+0.200[f]	−0.25[h]	−0.25[h]

[a] Taft electronic constant from ref. [17] p. 619.
[b] Taft steric constant from ref. [17] p. 598.
[c] Revised Taft steric constant from ref. [12].
[d] For substituent $CH_2CHCHCH_2$.
[e] Revised Taft constant.
[f] Calculated from experimental pK$_a$ values for dissociation of N(1)H according to ref. [14].
[g] For substituent $CH_2SO_3^-$ from ref. [18].
[h] Calculated as $E_s'\{(CH_2)_2CH_3\} - E_s'\{CH_2CH_3\} + E_s'\{CH_2OH\}$, E_s' values from ref. [19].

Table III. Correlation matrix for interrelationships of first-order rate constant (k) for acid-catalyzed cleavage of glycosidic bond and kinetic parameters for phosphorolysis catalyzed by calf spleen PNP for a series of eight N(7)-substituted guanosines.

	σ	$\log(k)$	$\log(K_m^+)$	$\log(V_{max})$	$\log(V_{max}/K_m^+)$
σ	1	0.922	0.217	−0.176	−0.212
$\log(k)$		1	0.420	−0.145	−0.277
$\log(K_m^+)$			1	−0.622	−0.849
$\log(V_{max})$				1	+0.942
$\log(V_{max}/K_m^+)$					1

It will be seen that there is no correlation between the first-order rate constants (k) for acid-catalyzed hydrolysis and the rate constants (V_{max}/K_m^+) for enzymatic phosphorolysis ($R = -0.277$) or the corrected Michaelis constants (K_m^+) ($R = 0.420$). Consequently there is also no correlation between V_{max}/K_m^+ (or K_m^+) and the Taft electronic constants σ, whereas the correlation coefficient between $\log(k)$ and σ is 0.922, indicating that the electronic effect accounts for 85% of the variance in $\log(k)$. It follows that, for the enzyme-catalyzed reaction, labilization of the glycosidic bond is not the predominant effect of N(7)-substitution.

QSAR for phosphorolysis of N(7)-substituted guanosines

Attention was then directed to multiple regression analysis of the kinetic parameters for phosphorolysis of a series of N(7)-substituted guanosines. As a measure of the substrate properties of the nucleosides, we have employed the Michaelis constants for the active cationic forms, K_m^+, since, for a reaction involving two substrates and two products (as is the case for PNP), this constant better characterizes the overall course of the reaction (further discussed below).

From the Michaelis constants for the cationic forms (K_m^+) of the 12 compounds listed in Table II, equation (1) was derived. The Taft steric (E_s) and electronic (σ) constants served as criteria for determining the influence of steric and electronic effects [17, 18], respectively (Table II). The more recently revised Taft steric constants [19] were also tested, with essentially similar results (hence not shown).

Equation (1) was developed by the sequential addition and deletion of parameters considered important in the enzymatic reaction, with concomitant use of the F-test to determine the significance of each modification. Table IV shows the final sequence arrived at

$$\log(K_m^+) = -0.53\,(\pm 0.22)\,E_s + 10.00\,(\pm 1.22)\,(\sigma)^2 + 0.36\,(\pm 0.16)\,(E_s)^2 + 0.93\,(\pm 0.06) \quad (1)$$

$$N = 12 \qquad R = 0.994 \qquad s = 0.069 \qquad F_{3,8} = 206.56.$$

The parenthesized values in the equation are the 95% confidence intervals; N represents the number of data points employed; R is the correlation coefficient; s the standard deviation for the regression; and $F_{n,m}$ the significance F-test for n parameters and m degrees of freedom. To reduce the possibility of a chance correlation [20], the number of data points ($N = 12$) permits the use of only three parameters in the equation.

For the N(7)-substituted guanosines embraced in this study, Fig. 1 displays a plot of the experi-

Table IV. Development of equation (1).

Eq. no.	Coeff. E_s	Coeff. $(\sigma)^2$	Coeff. $(E_s)^2$	Constant	s	R	n	m	$F_{n,m}$	$F_{n,m}$; $\alpha = 0.95$
(1a)	−1.20 (±0.15)[a]			0.98 (±0.10)	0.193	0.936	1	10	70.66	10.04
(1b)	−1.02 (±0.07)	9.68 (±1.49)		0.84 (±0.05)	0.085	0.989	2	9	202.49	8.02
(1)	−0.53 (±0.22)	10.00 (±1.22)	0.36 (±0.16)	0.93 (±0.06)	0.069	0.994	3	8	206.56	7.59

[a] Numbers in parantheses are standard deviations.

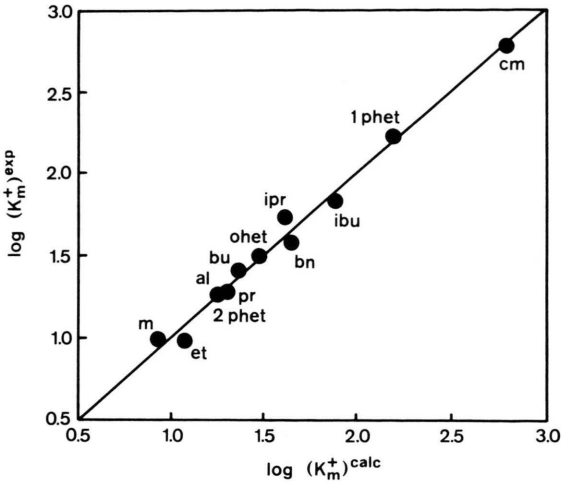


Fig. 1. Relationship of experimental $\log(K_m^+)^{exp}$ values for twelve N(7)-substituted guanosines to $\log(K_m^+)^{calc}$ values calculated according to equation (1).

mentally measured values of $\log(K_m^+)^{exp}$ *vs.* the calculated values, $\log(K_m^+)^{calc}$, obtained from equation (1).

Amongst the individual parameters considered, the Taft steric constant E_s contributed most to the measured Michaelis constants, K_m^+, for the cationic forms of the N(7)-substituted guanosines, and accounted for 88% of the variance in the data (Eqn. (1a) in Table IV). Addition of the term $(\sigma)^2$ led to Eqn. (1b) (Table IV) with a highly significant improvement, accounting for 98% of the variance. It should be noted that the influence of the electronic parameter $(\sigma)^2$ is an order of magnitude greater than that for the steric parameter E_s. Addition of the term $(E_s)^2$ to Eqn. (1b) to give Eqn. (1) led to small increases in the correlation coefficient R and the F parameter. Overall, Eqn. (1) accounted for 99% of the variance in the data. Actually, the difference between Eqn. (1b) and (1) is not particularly significant, albeit addition of the term $(E_s)^2$ led to small increases in both the correlation coefficient and F. It should, nonetheless, be noted that both equations give the same global dependence of $\log(K_m^+)$ on the steric parameter, *i.e.* an increase in $\log(K_m^+)$ with increasing bulk of the N(7)-substituent expressed by the Taft steric constant.

The parabolic relation between $\log(K_m^+)$ and the Taft steric constant E_s, and the signs associated with the linear and quadratic terms, are indicative of an N(7)-substituent size smaller than that for a methyl group for optimal substrate properties, *i.e.* a minimal K_m^+ value. Eqn. (1b) demonstrates the linear dependence of $\log(K_m^+)$ on E_s, with the minimal value corresponding to that for a methyl group.

The fact that a quadratic function of σ is significantly correlated with $\log(K_m^+)$, as well as the positive sign associated with $(\sigma)^2$ in Eqn. (1), implies that the influence of σ is at minimum value with σ equal zero. This requires that both large positive and large negative values of σ lead to a high value of K_m^+; *i.e.* poor substrate activity.

Several additional parameters, not listed in Table II, were considered during the course of development of Eqn. (1). These included the Hansch parameter π as a measure of hydrophilicity [21, 22], the molar refraction MR [21], the molecular weight MW as a measure of "steric" bulk [21], and the F and R parameters of Swain and Lupton [21, 23], representing field-inductive and resonance effects of the substituents.

The colinearity amongst significant variables is presented in Table V. Ideally the squared correlation coefficients should be less than 0.30 [24], equivalent to $-0.548 < R < 0.548$. Amongst the

Table V. Correlation between parameters used to derive equation (1).

	σ	E_s	E_s'	$(\sigma)^2$	$(E_s)^2$	$(E_s')^2$	$\log(V_{max}/K_m^+)$
σ	1	0.279	0.343	−0.097	−0.292	−0.418	0.277
E_s		1	0.980	−0.402	−0.969	−0.930	0.892
E_s'			1	−0.503	−0.944	−0.963	0.854
$(\sigma)^2$				1	0.365	0.500	−0.457
$(E_s)^2$					1	0.954	−0.940
$(E_s')^2$						1	−0.872
$\log(V_{max}/K_m^+)$							1

parameters employed in Eqn. (1), good correlations were observed only for E_s and $(E_s)^2$. But, as pointed out above, addition of the term $(E_s)^2$ to Eqn. (1 b) did not affect the global conclusions.

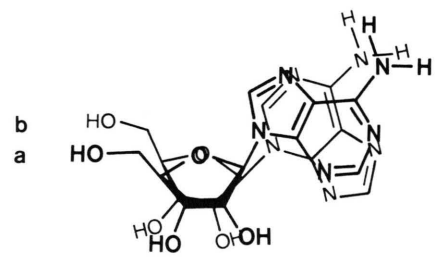
A similar analysis was conducted for the pseudo first-order rate constants for phosphorolysis, V_{max}/K_m^+. As with the Michaelis constants, K_m^+, the Taft steric constants were major contributors, amongst the various parameters taken into consideration, and accounted for 80% of the variance in the data, with a correlation coefficient $R = -0.872$ (see Table V). But, in marked contrast to the situation with the values K_m^+, addition of other terms to this simple equation did not significantly improve the F parameter. Since, for a reaction involving two substrates and two products, the rate constant corresponds to the rate of formation of the complex of the enzyme with the first substrate bound, the observed correlation of V_{max}/K_m^+ with the parameter E_s, with a negative correlation coefficient, implies that, with increasing bulk of the N(7)-substituent, access of the nucleoside to the active site is rendered more difficult, *i.e.* the enzyme-nucleoside complex is formed more slowly.

Discussion

It has been amply demonstrated that non-enzymatic hydrolysis of purine nucleosides proceeds by a mechanism involving preequilibrium protonation of the purine base, followed by rate-limiting glycosyl bond cleavage [1]. The neutral nucleoside may undergo protonation at N(1), N(3) and/or N(7). But, in the case of guanosine, the thermodynamically favoured site of protonation is N(7), as observed experimentally both in solution and in the solid state [25–27].

In the PNP-catalyzed cleavage of the glycosyl bond of guanosine, the two substrates (nucleoside and phosphate) are precisely oriented at the active site, and it is not inconceivable that N(3) is protonated by the enzyme; but this may be ruled out on the basis of the substrate properties of 3-deazaguanosine [28] and 3-deazainosine [5], showing than N(3) is not involved in the enzyme catalysis.

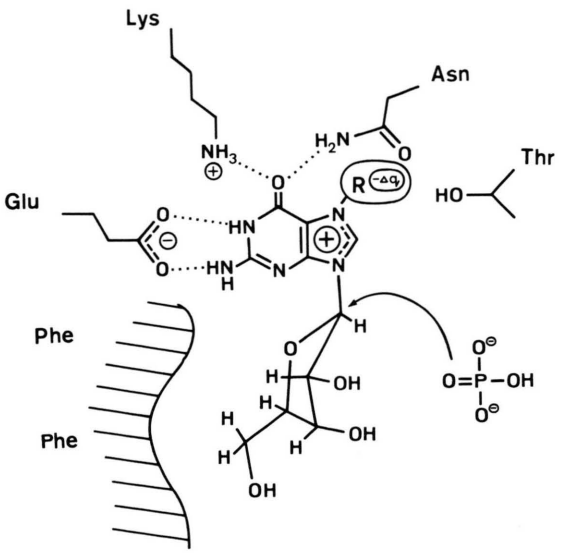
With the *E. coli* enzyme, 3-β-D-ribofuranosyladenine (iso-Ado) was reported to be a substrate [8], but no quantitative data were presented. By contrast, 3-β-D-ribofuranosylhypoxanthine was found to exhibit substrate properties comparable to those for Ino [29]. The latter authors concluded that the enzyme operates without the expected protonation of the base, on the grounds that it is difficult to visualize how some protein residue, defined relative to ribofuranosyl ring, could protonate both N(9)-linked and N(3)-linked purine rings. However, in the case of iso-Ado, which behaves like Ado in several enzyme systems, Leonard and coworkers [30] showed that iso-Ado in the *syn* conformation about the glycosidic bond is virtually superimposable with Ado in the *anti* conformation (see Scheme 2). And, at least in the solid state, iso-Ado is in the *syn* conformation, and in the amino form [31], consistent with the fact that 3-methyladenine is in the amino form in solution [32]. Furthermore, the pK_a (presumably for protonation) of iso-Ado is 5.6, in agreement with a value of 5.7 for 3-methyladenine [33], but considerably higher than that for Ado, 3.6 [33]. It now would obviously be desirable to establish the site of protonation of iso-Ado and/or 3-methyladenine. Finally, bearing in mind the marked differences in structural requirements for substrates between the *E. coli* and mammalian enzymes, it would be of interest to determine whether the 3-β-D-nucleosides are substrates for the latter.



Scheme 2. Iso-Ado in the *syn* conformation (b) superimposable with Ado in *anti* conformation (a).

In contrast to the N(3), the N(7) nitrogen appears to be involved in enzymatic catalysis, but is not necessary for binding to the enzyme, since 7-deaza analogues have no substrate activity, but are relatively good inhibitors [5, 8, 9]. This supports the hypothesis of N(7) protonation as a catalytic mechanism for phosphorolysis, as do results of our structure-activity correlation. The fact that a quadratic function of σ is significantly correlated with $\log(K_m^+)$, and the positive sign associated

with $(\sigma)^2$ in Eqn. (1), is indicative of an intimate relationship between electron density around the N(7)-substituent (and consequently the imidazole ring and the C-N glycosidic bond) and substrate properties of the analogues. The relationships require large positive or large negative values of σ to produce a high K_m^+ value. Similar relationships of σ to the activity of molecules as substrates [34, 35] or inhibitors [36] of enzymatic reactions have been explained on the basis that several steps are involved in the enzyme reaction, with one of them limiting when σ is positive, and another when σ is negative. In the case of enzymatic phosphorolysis it may be argued that the more electron-withdrawing substituent on N(7) (σ positive) labilizes the glycosidic bond and facilities the nucleophilic attack of the phosphate at C(1′) more effectively (see Scheme 3) as a result of withdrawal of a charge $-\Delta q$ from the imidazole ring and the C-N glycosidic bond. But this positive effect is partially cancelled by the impediment of the enzyme to participate in protonation of the N(7) (which is blocked with a substituent), a process hindered by both steric (E_s constant) and electronic (σ constant) interactions, the second being stronger with more electron-withdrawing substituents, *i.e.* a more positive σ (as a result of greater positive charge distributed on the imidazole ring, *i.e.* the site of protonation, see Scheme 3).



Scheme 3. Hydrogen bonding arrangement for mammalian nucleoside phosphorylase active site and orientation of reactants for the phosphorolytic reaction (from ref. [37]); and a possible role of substituent R at N(7) of guanosine. The substituent was assumed to be electron-withdrawing (positive Taft electronic constant), hence the charge $-\Delta q$ is withdrawn from imidazole ring as a result of this substitution (see Discussion).

Other nucleoside and nucleotide N-glycosidases

The foregoing proposal regarding the mechanism of action of PNP may be equally applicable to a number of other enzymes which catalyze the cleavage of the glycosidic bonds of nucleosides and nucleotides. One example is nucleoside hydrolase, which cleaves the N-glycosidic bond of ribonucleosides in the absence of phosphate to release ribose and the base. Although not found in mammalian cells, it is present in trypanosomes, where it replaces PNP as the source of purine salvage. The transition state for hydrolysis of Ino by the enzyme from *Crithidia fasciculata* has been characterized with the aid of multiple kinetic isotope effects, and bond-energy bond-order vibrational analysis, and shown to involve protonation of the ring N(7) nitrogen [38], further supported by the fact that, whereas Ado is also a substrate, 7-deaza-Ado is not.

The enzyme AMP nucleosidase cleaves 5′-AMP to release adenine and ribose-5-phosphate. Analogous transition-state analysis with the native enzymes from *E. coli* and *Azotobacter vinelandii,* and a mutant enzyme from the latter organism, by Schramm and coworkers ([39] and references cited) likewise led to the conclusion that the transition state for glycosidic bond cleavage involves protonation of the ring N(7) of the substrate.

An even more striking example is pokeweed antiviral protein. This is a member of a group of plant proteins, including the A-chain of ricin, which inactivate eucaryotic ribosomes. The mechanism of inactivation has been shown to involve the deadenylation of a single adenylate residue, A_{4324}, in the 28 S ribosomal RNA. The proteins are therefore site-specific RNA N-glycosylases. Site-directed mutagenesis studies with this enzyme have led to the proposal that one possible mechanism for cleavage of the adenine residue proceeds *via* protonation of the adenine ring N(7) ([41], for review see [40]).

The postulated mechanism of action of the foregoing RNA N-glycosylase logically directs attention to the broad class of DNA repair enzymes

which efficiently remove from damaged DNA such residues as 3-methyladenine, 7-methylguanosine, 7-methyladenosine, etc. (for review see [42]). Although the structure of some of these enzymes, as well as their specificities for various damaged residues, have been extensively investigated, virtually no attention has hitherto been devoted to their mechanism of action. Bearing in mind that most of these are N-glycosidases, the results of the present investigation, together with the postulated mechanism of action of the pokeweed antiviral protein RNA N-glycosidase, furnish a useful lead for initiation of such studies.

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