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INHIBITORS OF PURINE NUCLEOSIDE PHOSPHORYLASE

C(8) AND C(5′) SUBSTITUTIONS

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Abstract—The C(8) and C(5′) positions of base and nucleoside substrates of human erythrocytic purine nucleoside phosphorylase (PNP) are promising sites for the development of an inhibitor of this enzyme. The substrate analog, 8-aminoguanine (8-AG), has the lowest dissociation constant (K_i = 0.2–1.2 μM) of any compound reported to date and V_{max} = 16 per cent (relative to guanine). Other C(8) substituents decreased the affinity for PNP and, with the exception of the methyl and sulfhydryl groups, abolished substrate activity. Halogens or a thiomethyl group at C(5′) of inosine resulted in unchanged or improved affinities (K_i = 10–30 μM) and greatly decreased substrate activity (V_{max} < 1 per cent relative to inosine). The K_i of formycin B was reduced from 100 μM to 10 μM or less by substitution of a halogen at C(5′). Phosphorolysis of purine nucleosides was inhibited significantly by 8-AG in intact human erythrocytes and murine Sarcoma 180, L1210 and L5178Y cells. Although a K_i value of 17 μM was determined for 8-aminoguanosine, it was equally effective in inhibiting PNP activity in intact cells. The nucleoside was cleaved to 8-AG which was not a substrate for guanase or hypoxanthine-guanine phosphoribosyltransferase. Despite its low substrate activity (V_{max} < 0.2%), 5′-deoxy-5′-iodoinosine was cleaved by intact L1210 and L5178Y cells.

Although the degradative enzyme, purine nucleoside phosphorylase (PNP; purine nucleoside:orthophosphate ribosyltransferase; EC 2.4.2.1), has long been of secondary chemotherapeutic interest, it is now viewed as a primary target for drug development. Several considerations have led to this reappraisal. First of all, the genetic disease, identified in 1975 [1], of deficiency in PNP specifically associated with defective cellular but not humoral immunity and the high concentrations of PNP as well as of deoxyguanosine kinase in T lymphocytes and thymus [2] indicate that a potent inhibitor of PNP could have specific immunosuppressive activity. It has been postulated that, in the absence of PNP, 2′-deoxyguanosine is converted to 2′-deoxyguanosine nucleotides rather than being degraded to guanine and deoxyribose-1-phosphate [2, 3]. The accumulated dGTP causes feedback inhibition of ribonucleotide diphosphate reductase, an enzyme that is essential for the conversion of ribonuleotides to deoxyribonucleotides [4]. Such inhibition might be lethal to cells undergoing DNA synthesis. It is likely that administration of 2′-deoxyguanosine in combination with a PNP inhibitor would have synergistic effects. Another attractive approach is the design of nucleoside analogs that are poor substrates for PNP but that have high reactivity with deoxyguanosine kinase and other enzymes of dGTP synthesis, thus enabling intracellular accumulation of analog dGTPs. These concepts may be appreciated by examination of Fig. 1.

Also, in the past a number of purine nucleoside analogs that showed promise in laboratory trials did not offer clear-cut clinical advantages over the respective purine base analogs, e.g. β-2′-deoxythio-guanosine versus 6-thioguanine. This is readily explained by the high PNP activity of human erythrocytes (10–15 E.U./ml of cells). There is sufficient PNP in the whole-body erythrocytes of an adult human to phosphorolyze about 5–6 g of inosine per min [5]. Since many potentially chemotherapeutic purine nucleoside analogs are good substrates for PNP and are also permeants of the erythrocyte [5], it is probable that these nucleosides are converted rapidly to free bases in the vascular system before reaching peripheral targets, e.g. a tumor. Therefore, co-adminstration of a potent PNP inhibitor might permit certain purine nucleoside analogs to survive transit through the bloodstream to the desired site of action.

The unusual metabolic abnormalities seen in PNP deficiency suggest that potent PNP inhibitors might have therapeutic activity in hyperuricemic states, e.g. secondary gout. PNP-deficient children display hypouricosuria, hypouricemia, increased purine biosynthesis *de novo* and marked elevations of nucleosides of guanine and hypoxanthine in plasma and urine [6]. Because these nucleosides are much more soluble than xanthine or uric acid, the excretion of nucleic acid degradation products might be facilitated and the danger of crystalluria prevented by the administration of a potent PNP inhibitor.

This laboratory has undertaken a systematic examination of the structure–activity relationships of substrates for human erythrocytic PNP with the intent of identifying promising sites for chemical modification that might accomplish certain of the goals outlined above. Studies completed to date sug-

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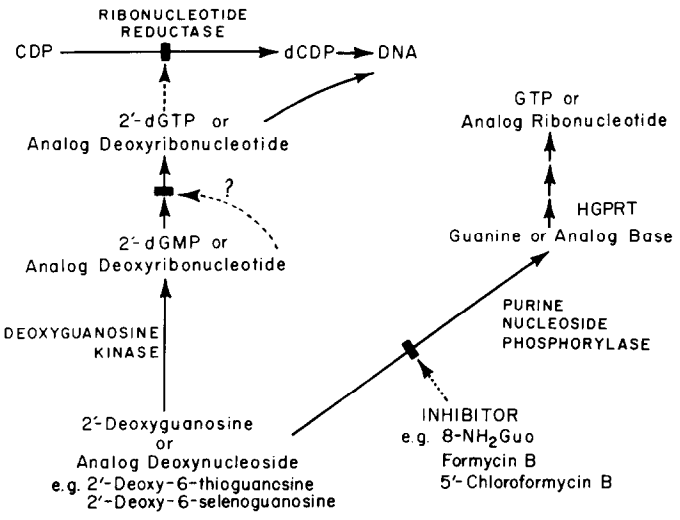

Fig. 1. Rationale for use of PNP inhibitors.

gest that various substitutions on C(8) of the purine ring and C(5′) of the pentose can cause increases in substrate affinity and decreases in reactivity, both desired objectives for inhibitor design. As "lead compounds", 8-aminoguanine (8-AG, Fig. 2) and its ribonucleoside have been selected for pilot studies to test some of these concepts. These analogs have much lower dissociation constants than the natural nucleoside substrates. Hopefully, still more potent inhibitors can be developed. Preliminary reports of portions of these studies have been presented elsewhere [7–9].

MATERIALS AND METHODS

Materials. Partially purified human erythrocytic PNP (sp. act. = 1.5) and hypoxanthine-guanine phosphoribosyltransferase (HGPRT, sp. act. = 1.8) were isolated from outdated blood by the New England Enzyme Center, Boston, MA, by a large-scale preparative method [10]. Purified PNP (sp. act. = 70) and crystalline PNP were prepared as described elsewhere [11]. Milk xanthine oxidase (types III and IV), adenosine, inosine, hypoxanthine, 6-mercaptopurine ribonucleoside (6-MPR) and α-D-ribose-1-phosphate (R-1-P) were purchased from the Sigma Chemical Co., St. Louis, MO. Calf intestinal adenosine deaminase and guanine were supplied by Boehringer Mannheim Biochemicals, Indianapolis, IN, and 2′-deoxyguanosine (dGuo) by CalBiochem, Los Angeles, CA. Guanosine (Guo) was a product of P. L. Biochemicals, Inc., Milwaukee, WI. The synthesis of 8-aminoguanosine (8-AGuo) is described below, and 8-aminoxanthine was a gift of Dr. L. B. Townsend. All other C(8)-substituted purines were obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. The concentration of 8-AG was calculated by means of the published extinction coefficients [12]; all other C(8)-substituted compounds were assumed to be pure and concentrations were based on weight. Formycin B was purchased from CalBiochem, San Diego, CA. Formycin and adenosine analogs modified at C(5′) were synthesized from formycin and adenosine by modifications of published procedures [13–15]. The C(5′)-modified inosine compounds were prepared by deamination of the respective adenosine analogs with nitrous acid, followed by purification with activated charcoal. Calf adenosine deaminase was used to deaminate 5′-chloro-5′-deoxyformycin (K_m, 300 μM; V_{max}, < 1 per cent), 5′-deoxy-5′-iodoformycin (V_{max}, < 1 per cent) and 5′-deoxy-5′-fluoroadenosine (K_m, 470 μM; V_{max}, < 1 per cent) by a previously described procedure [16]. Guanase was obtained from homogenized rat liver. A crude extract was centrifuged at 27,000 *g* and the supernatant fluid was dialyzed against 50 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), pH 7.5. Sarcoma 180 ascites tumor cells were grown intraperitoneally in CD_1 mice; L1210 and L5178Y cells were grown intraperitoneally in B6D2f1 mice. Fresh human blood was obtained from volunteers and treated with heparin or citrate.

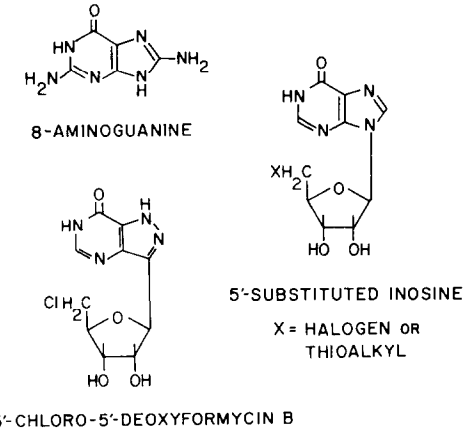

Fig. 2. C(8) and C(5′)-modified analogs.

Spectrophotometric assays for inosine and guanosine phosphorolysis. The standard assay for PNP employed the coupled xanthine oxidase procedure for inosine phosphorolysis [17]. One unit of PNP catalyzes the reaction of 1.0 μmole inosine per min

under standard conditions. Inhibition of inosine phosphorolysis was monitored by observing uric acid formation at 293 nm with 50 mM potassium phosphate (pH 7.5) 0.02 unit of xanthine oxidase, and various concentrations of inosine and the inhibitor in a volume of 1.0 ml at 30° in a Gilford model 240 spectrophotometer. Guanosine phosphorolysis or synthesis was measured directly by observing the change in absorbance at 258 nm and employing $\Delta\varepsilon = 5.3 \times 10^3\ M^{-1}\,cm^{-1}$ for the molar absorbancy change.* The reactions were performed at 30° in 50 mM potassium phosphate (pH 7.5), with various concentrations of guanosine and inhibitor in a volume of 1.0 ml such that the initial absorbance did not exceed 1.1 units in order to avoid possible spectrophotometric artifacts [18]. Kinetics at $> 80\ \mu M$ concentrations were performed in 1 mm pathlength cuvettes.

The kinetic constants were estimated from the data by employing linear regression analysis to obtain K_i values from Dixon plots [19] of 1/reaction velocity ($1/v$) versus inhibitor concentration and a weighted linear regression analysis program adapted from Cleland [20] to a Wang computer and extended to compute both K_m and K_i values from plots of $1/v$ versus $1/S$ at multiple inhibitor concentrations by Dr. S. Cha.

Assay for the inhibition of 6-MPR phosphorolysis by 8-AG in intact human erythrocytes and hemolysates. Blood was washed three times with a solution containing 50 mM potassium phosphate (pH 7.4), 75 mM NaCl, 2 mM $MgSO_4$, 10 mM glucose, 100 units/ml penicillin and 100 μg/ml streptomycin (referred to hereafter as standard medium) to remove the plasma and buffy coat.

Phosphorolysis of 6-MPR was determined by a minor modification of a method described earlier [21]. The incubation mixtures contained 500 μM 6-MPR, 1.0 mM dithiothreitol, ~2 units of xanthine oxidase and various 8-AG concentrations in 4.0 ml of standard medium. The reactions were initiated by the addition of erythrocytes to give a 2% suspension (v/v) and incubated at 37° in a shaking water bath. At 0, 10 and 20 min, 1.0-ml aliquots were removed and extracted at 4° with 4% (final concentration) perchloric acid. After removal of the KOH-neutralized perchlorate by centrifugation, 0.05 unit of xanthine oxidase was added to the supernatant fluid, which was then incubated for 15–30 min to ensure complete oxidation of 6-MP to 6-thiouric acid. The 6-thiouric acid concentration was measured by applying a molar extinction coefficient of 24.5×10^3 to the increase in absorbance at 348 nm. At this wavelength there was no interference due to the presence of 8-AG or 8-AGuo. As shown in Fig. 4, 8-AG has an absorption maximum at 291 nm, very close to the 293 nm peak of uric acid.

Hemolysates were prepared by diluting washed erythrocytes with distilled water, freezing, thawing and centrifuging at 31,000 *g* for 20 min at 4°. Incubations with 6-MPR were carried out as above except that 8-AG concentrations were 9–250 μM and the reactions were initiated by the addition of hemolysate to give a final concentration equivalent to a 1.5% suspension of intact cells. Aliquots were removed at 0 and 7 min. In control studies, the formation of 6-thiouric acid was linear with time for at least 25 min with both the intact cells and hemolysates.

High pressure liquid chromatography (h.p.l.c.). Nucleosides and bases were analyzed on a Varian 4200 high pressure liquid chromatograph equipped with a Waters μBondapak C_{18} analytical column (4.5 mm × 30 cm); nucleotide analysis was performed on a Varian LCS-1000 liquid chromatograph by previously described methods [16, 22]. For the chromatography of 5'-deoxy-5'-iodoinosine the procedure was modified by extending the isocratic elution at 20% methanol for an additional 6 min [23]. The absorbances of the eluates were monitored at 254 nm. Samples were prepared for h.p.l.c. by extraction with 4% perchloric acid at 4° for 15 min. After centrifugation, the supernatant fractions were neutralized with KOH to pH 6.5–7.5. The perchlorate salts were precipitated at 4° for 15 min and removed by centrifugation. The extracts were stored at −20°.

High pressure liquid chromatographic assay for the inhibition of inosine synthesis by 8-AGuo. The reaction mixtures containing 1.0 mM R-1-P, either 40 or 60 μM hypoxanthine, various concentrations of 8-AGuo (0.6–53 μM), and 0.0013 unit of crystalline PNP in 1.0 ml of 50 mM Tris–HCl, (pH 7.5) were incubated at 37° in a shaking water bath. The exclusion of phosphate from the incubation medium precluded phosphorolysis of the 8-AGuo. The reactions were terminated after 2.5 min by immersing the mixtures in a boiling water bath for 2 min. Inosine was measured in unextracted 150- or 200-μl aliquots by reversed-phase h.p.l.c. The retention times for hypoxanthine and inosine were 6.9 and 9.6 min respectively. The synthesis of inosine was linear with time for at least 5 min in control studies.

Inhibition of guanosine metabolism in human erythrocytes and murine Sarcoma 180, L1210 and L5178Y cells. Incubation mixtures of 100 μM guanosine with and without 100 μM 8-AG in 3.0 ml of standard medium were maintained at 37° in a shaking water bath. Reactions were initiated by the addition of erythrocytes to give 5% suspensions and terminated by extractions of 0.5-ml aliquots with perchloric acid at 0, 15, 30 and 60 min. Aliquots of 50 and 100 μl were analyzed by h.p.l.c. for guanosine and for guanine nucleotides respectively.

Sarcoma 180 cells were washed three times with standard medium and cell concentrations were determined with an Adams Autocrit centrifuge as with the erythrocytes. The incubations and h.p.l.c. were carried out as above except that the 5% cell suspensions were preincubated for 15 min and the reactions were initiated by the addition of 100 μM guanosine or 2'-deoxyguanosine. The 100 μM 8-AG was added either to the preincubation mixture or together with the substrate. L1210 and L5178Y cells were prepared similarly and counted on a model B Coulter Counter. Incubation mixtures contained 5×10^7 cells per ml and 100 μM 2'-deoxyguanosine in the presence and absence of 100 μM 8-AG or 8-AGuo.

* Unpublished observation, E. Chu of this laboratory.

Determination of the substrate activities of analogs. The C(5′)-modified inosine analogs were tested for substrate activity with partially purified PNP by means of the coupled xanthine oxidase reaction. The C(8)-substituted compounds were tested in a spectral shift assay and by h.p.l.c. For the spectral assay, solutions containing 50–75 μM compounds, 300 μM R-1-P, 25–75 mM Tris–acetate (pH 7.5) and 0.1–0.6 unit of PNP in a volume of 3.0 ml were incubated at ambient temperature in a 1 cm pathlength quartz cuvette. The u.v. spectrum was scanned in a Perkin Elmer 402 spectrophotometer at varying time intervals. The reference cuvette contained all the components except the analog. In the case of compounds having a sulfhydryl group, 10 mM mercaptoethanol was added to the solutions. The incubations were extended until no further change was observed at 30-min intervals or for 16–24 hr in cases where no spectral change occurred after addition of PNP. Values of $\Delta\varepsilon$ were calculated at the wavelengths at which the greatest absorbancy change occurred during the reaction. These $\Delta\varepsilon$ values were then employed to determine the kinetic parameters for the substrate activities. The spectral changes for the synthesis of 8-AGuo from 8-AG at pH 7.5 are illustrated in Fig. 4. The $\Delta\varepsilon$ for this reaction is $+7.5 \times 10^3\ M^{-1}\ cm^{-1}$.

Substrate kinetics were studied by observing the change in absorbance at the selected optimal wavelength. The reaction mixtures contained various concentrations of analogs (the initial absorbances did not exceed 1.1), 1.0 mM R-1-P, 25 or 100 mM Tris–acetate (pH 7.5) and an appropriate concentration of PNP in 1.0 ml at 30°.

Substrate activity was also tested by h.p.l.c. assay in some cases. The 24-hr incubation mixtures employed in the spectral shift assays above were extracted with perchloric acid and 100-μl aliquots were subjected to reversed-phase h.p.l.c.

Determination of the activity of 8-AG with human erythrocytic HGPRT. The assay for HGPRT activity with 8-AG is based on the method of Kong and Parks [24]. The reaction mixture contained 100 mM Tris–HCl (pH 7.5), 2 mM $MgSO_4$, 1.0 mM 5-phosphoribosyl-1-pyrophosphate, 5 mM mercaptoethanol, 250 μM 8-AG and 0.2 unit HGPRT. Perchloric acid extracts were prepared after a 2-hr incubation in a shaking water bath at 37° and analyzed for the formation of the 5′-monophosphate nucleotide by h.p.l.c.

Determination of the activity of 8-AG with rat liver guanase. The activity of crude guanase preparations was assayed by slight modification of the procedure of Giusti [25], using guanine as the substrate in a coupled xanthine oxidase reaction in which the xanthine product is converted to uric acid and quantitated by monitoring increasing absorbance at 293 nm, with $\Delta\varepsilon = 7 \times 10^3\ M^{-1}\ cm^{-1}$. An activity of $0.023\ \mu mole \cdot min^{-1} \cdot (mg\ protein)^{-1}$ was calculated by determining the V_{max} under the following conditions: 50 mM phosphate (pH 7.5) 0.04 unit xanthine oxidase and 4–60 μM guanine in a total volume of 1.0 ml at 37°. Negligible uricase activity was detected under these conditions. To determine the activity of 8-AG as a substrate for rat liver guanase, an incubation mixture containing 500 μM 8-AG, 50 mM phosphate (pH 7.5), and 0.25 ml of the rat liver extract in a total volume of 2.5 ml was shaken for 24 hr in a water bath at 37°. Control incubation mixtures contained either 500 μM guanine or 8-aminoxanthine in place of the 8-AG. Analysis for the bases in perchloric acid extracts was performed by reversed-phase h.p.l.c. and elution times of 2.4, 3.9, 7.5 and 7.5 min were determined for 8-aminoxanthine, xanthine, 8-aminoguanine and guanine, respectively, under the standard conditions described above. Added 8-aminoxanthine did not appear to undergo any reactions during incubation.

Determination of the effect of 8-AG on xanthine oxidase activity. The assay mixtures contained 50 μM hypoxanthine and 0.005 unit xanthine oxidase in 1.0 ml of 50 mM potassium phosphate (pH 7.5) at 30°. Reaction rates were determined spectrophotometrically at 293 nm in the presence and absence of 50 μM 8-AG.

Synthesis of 8-AGuo with immobilized PNP. Partially purified PNP was immobilized on CNBr-activated Sepharose as described elsewhere [16]. Synthesis of 8-AGuo was conducted at 37° in a shaking water bath with 0.62 mM 8-AG, 1.0 mM R-1-P and 20 units of PNP-agarose in 40 ml of 50 mM triethylammonium bicarbonate (pH 7.0). The reaction was monitored by measuring the absorbance of aliquots at 260 nm, the wavelength at which the greatest absorbance increase occurs during synthesis, and at 291 nm, the λ_{max} of 8-AG. When no further changes in absorbance were detected, the solution was filtered, lyophilized and redissolved in a minimal volume of water. Analysis by reversed-phase h.p.l.c. showed 94 per cent conversion to nucleoside and several small contaminating peaks. The product solution was then applied to a 2.5 × 15 cm column of CF 1 cellulose powder (Whatman, Clifton, NJ) and eluted with distilled water. The fractions with the highest 260/291 nm absorbance ratios were pooled and found by h.p.l.c. to contain < 1 per cent contaminants. The absorbance maxima of the product coincided with published values for 8-AGuo [26].

RESULTS

Inhibition of PNP by C(8)-substituted purine bases. The per cent inhibition of guanosine phosphorolysis by equimolar C(8)-substituted base analogs is shown in Table 1. Most of the C(8)-substituted purines tested were inhibitors of PNP. These compounds were evaluated in the direct spectrophotometric assay for guanosine phosphorolysis to avoid possible inhibition of or reaction with xanthine oxidase in the coupled assay, since some of the analogs, e.g. 8-aminohypoxanthine, were readily oxidized to the C(8)-substituted xanthines. In the present assays, guanosine and the inhibitors were both at a concentration of 30 μM (the approximate K_m value for guanosine [16]) so that > 50 per cent inhibition indicates a K_i value < 30 μM. Only 8-AG and 8-aminohypoxanthine are potent inhibitors by this criterion. With the compounds studied in Table 1, the presence of an amino group on C(8) results in the highest affinity for PNP.

Table 2 presents kinetic parameters for the most effective inhibitors of PNP and compares them to those of the parent compounds, guanine and hypo-

Table 1. Inhibition of guanosine phosphorolysis by C(8)-substituted purine analogs*

	Purine ring substituent			% Inhibition	Substrate activity
	C(2)	C(6)	C(8)		
1	NH_2	OH	NH_2	95	+
2	H	OH	NH_2	57	+
3	H	SH	NH_2	26	+
4	NH_2	OH	SH	25	+
5	NH_2	OH	I	22	−
6	H	OH	*S*-(*o*-nitrobenzyl thio)	22	−
7	H	OH	*S*-(*m*-nitrobenzyl thio)	20	−
8	H	OH	$SONH_2$	18	−
9	NH_2	SH	CH_3	18	−
10	NH_2	OH	CH_3	17	+
11	H	OH	*S*-(*m*-aminobenzyl thio)	17	−
12	H	OH	CHOH-phenyl	13	−
13	H	OH	*m*-nitrophenyl	12	−
14	NH_2	OH	SCH_3	12	−
15	CH_3	OH	OH	11	−
16	NH_2	SH	$CH_2CH_2CH_3$	10	−
17	H	SH	OH	9	−
18	H	SH	*S*-(*p*-nitrobenzyl thio)	8	−

* The percent inhibition was determined by spectrophotometric assays with substrate and inhibitor both present at 30 μM concentration. Substrate activity was detected by the spectral shift assay. The results are the averages of two experiments. The 8-Br, -SH and -SO_2F analogs of hypoxanthine caused 6 percent inhibition and showed no substrate activity; no significant inhibition was detected with 8-phenyl- or 8-hydroxyguanine.

xanthine. K_i values of 50 μM were estimated from Dixon plots for the 8-thio and 8-iodo analogs of guanine which had shown only 22–26 per cent inhibition of phosphorolysis in Table 1. Double reciprocal plots with 8-iodoguanine showed competitive inhibition with a K_i value of 51 μM.

In contrast, 8-AG has a K_i value substantially lower than that of guanine. The affinity of 8-AG for PNP was determined by studying the inhibition of inosine phosphorolysis since 8-AG did not inhibit the xanthine oxidase reaction. Concentrations of the substrate were held below 150 μM to avoid the concentration range at which substrate activation is normally observed. The double-reciprocal plots (Fig. 3A) indicated competitive inhibition of PNP by 8-AG. However, the replot of the slopes (Fig. 3B) was concave downward: at concentrations of 8-AG < 0.5 μM, a line could be extrapolated indicating a K_i value of 0.2 μM; at concentrations of 8-AG > 1.0 μM, the line could be extrapolated to a K_i value of 1.2 μM. A Dixon plot of reciprocal velocity versus varying inhibitor concentrations at a constant substrate concentration of 27 μM inosine also revealed a concave downward curve with the break occurring at concentrations of 8-AG < 2 μM (Fig. 3C). The K_i values of 0.7 and 4.1 μM which were determined for 8-AG at concentrations < 2 μM and > 2 μM, respectively, by extrapolations from the V_{max} value for inosine, were in general agreement with the values determined from the replot of slopes.

Table 2. Kinetic parameters of some C(8)-substituted purines with PNP

Purine ring substituent			K_i (μM)	K_m (μM)	Relative V_{max}
C(2)	C(6)	C(8)			
NH_2 (Guanine)	OH	H	5*	20	100
NH_2	OH	NH_2	0.2–1.2*, 4	11	16
NH_2	OH	SH	53		
NH_2	OH	I	51		
H (Hypoxanthine)	OH	H	17*	19†	
H	OH	NH_2	10*	6	6
H	SH	NH_2	79		

* K_i values were estimated from replots of double-reciprocal plots. All others were determined from Dixon plots.

† Published value [27].

Both the replot (Fig. 3B) and the Dixon plot (Fig. 3C) indicated that the reaction rate observed at the higher 8-AG levels is faster than would be expected by extrapolation of the inhibition line obtained from the low 8-AG range. This suggested that at concentrations > 1.0 μM, although it functions as an inhibitor, 8-AG activates the enzyme through the postulated negative cooperativity phenomenon termed substrate activation. A downward curvature in double-reciprocal plots at high substrate concentrations has been shown with the nucleosides inosine and 2′-deoxyinosine [17, 28] and was found in the present study with guanosine as well as with its base, guanine. To test whether 8-AG simulates substrate activation, inosine phosphorolysis was studied over a wide concentration range (30–500 μM) in the presence of 0.16 and 3.0 μM 8-AG. Substrate activation was seen in the presence of 0.16 μM 8-AG, with the apparent break in the curve occurring at ~100 μM inosine as opposed to ~200 μM inosine in the absence of inhibitor. No break in the double-reciprocal plot was found when 3.0 μM 8-AG was added (figures not shown). In the latter case only the high K_m region for inosine was observed.

Hypoxanthine showed weaker product inhibition

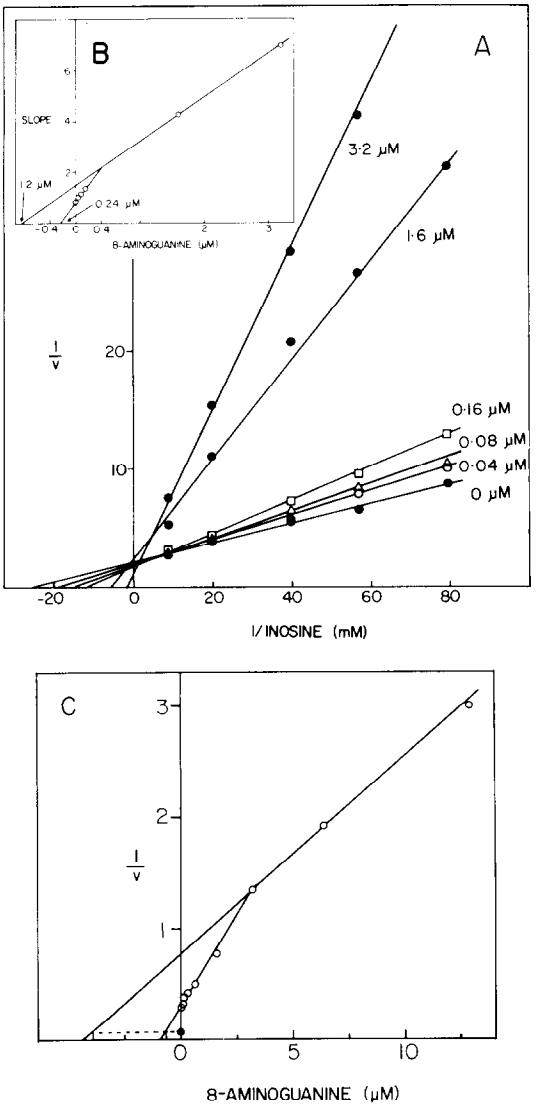

Fig. 3. Inhibition of PNP by 8-AG. Assay conditions are described in Materials and Methods. Panel A: Double-reciprocal plot of the inhibition of inosine phosphorolysis by different fixed concentrations of 8-AG. Panel B (inset): Replot of slopes from panel A versus 8-AG concentration. Panel C: Dixon plot showing inhibition of the phosphorolysis of 27 μM inosine by 8-AG.

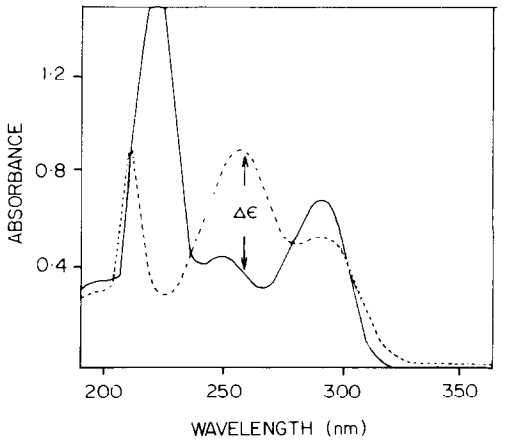

Fig. 4. Spectral changes for the enzymatic conversion of 8-AG to 8-AGuo in the presence of excess R-1-P. Key: (——) 8-AG spectrum; and (----) 8-AGuo spectrum. The reaction mixture contained 68 μM 8-AG, 330 μM R-1-P and 0.2 unit PNP in 3.0 ml of 25 mM Tris–HCl, pH 7.8. The arrows indicate the maximum absorbance change which occurred at 260 nm.

than guanine, the K_i values being 17 and 5 μM respectively. Similarly, 8-aminohypoxanthine displayed lower affinity for PNP (K_i = 10 μM) than 8-AG although it bound almost twice as tightly as hypoxanthine. The double-reciprocal plot also showed a pattern of competitive inhibition for 8-aminohypoxanthine. The 8-amino-6-thiopurine analog which had displayed only 26 per cent inhibition of guanosine phosphorolysis (Table 1) showed weaker inhibition, K_i = 79 μM. The 8-nitrobenzylthio derivatives of hypoxanthine were not subjected to kinetic studies because of their poor solubilities and high absorbance values.

Substrate activities of 8-AG, 8-AGuo and other C(8)-substituted bases. The 8-amino derivatives of guanine and hypoxanthine that displayed competitive inhibition of PNP are alternate substrates for this enzyme. Figure 4 shows the spectral shift for the enzymatic synthesis of 8-aminoguanosine (8-AGuo) from 8-AG. A direct spectrophotometric assay was used to determine the kinetic parameters of the synthetic reaction. The V_{max} of 8-AG was only 16 per cent of the rate for guanine, but the K_m was also lower, 11 μM. Since 8-AG simulated the substrate activation phenomenon when used as an inhibitor (see above and Fig. 3), it is likely that this value represents the high K_m of the substrate activation range, and a lower value might be determined if it were possible to measure the reaction at concentrations below 1 μM.

By similar spectrophotometric assays a V_{max} of only 6 per cent (relative to guanine) and a K_m of 6 μM were determined for 8-aminohypoxanthine. Of the twenty-three compounds tested, only three others showed substrate activity as determined from spectral shift assays: 8-amino-6-thiopurine, and 8-thio- and 8-methylguanine. No substrate activity was found with substitution of the bulkier 8-iodine atom, although the resultant analog had the same affinity for PNP as 8-thioguanine.

Enzymatically synthesized 8-AGuo (see Materials and Methods) was tested as a substrate for PNP in the presence of 50 mM potassium phosphate (pH 7.5) and was found to have a V_{max} of 12 per cent (relative to inosine) and a K_m value of 7 μM. When 8-AGuo was tested as an inhibitor of the formation of inosine from hypoxanthine, a K_i of 17 μM was determined, a value about 10-fold greater than the K_i estimated for 8-AG.

Since 8-AG was found to be a substrate for PNP it was also tested with other enzymes to determine whether it could be incorporated into nucleotide pools or catabolized. No nucleotide synthesis was detected by anion exchange h.p.l.c. when 8-AG was incubated with HGPRT under conditions that normally result in rapid nucleotide synthesis [24]. Also,

Table 3. Kinetic parameters of C(5')-substituted nucleosides with PNP

Nucleoside	C(5')-substituent	K_m (μM)	Relative V_{max}	K_i (μM)
Inosine	OH	33	100	
	H	31	45	
	S-CH_3	15	0.7	22*
	S-isobutyl	42	0.2	105
	S-*n*-butyl	32	0.6	63
	S-benzyl		ND†	300*
	S-COOH		ND†	
	F	13	4	
	Cl	10	0.8	26*
	Br	20	0.4	50
	I	12	0.1	18*
Formycin B	OH			100‡
	Cl			10*
	I			7*

* Values were estimated from replots of double-reciprocal plots; others were determined from Dixon plots.
† Not detectable with 1.5 units of PNP.
‡ Published value [21].

8-AG showed only very weak inhibition of HGPRT, $K_i > 200$ μM, compared with $K_m = 5$ μM for guanine [24]. When 8-AG was incubated for 24 hr with 0.016 unit of guanase from rat liver extracts, there was no detectable conversion to 8-aminoxanthine as judged by reversed-phase h.p.l.c. Also, 70 μM 8-AG did not inhibit the deamination of 7.8 μM guanine in the spectrophotometric assay.

Reactivity of C(5')-substituted nucleosides with PNP. Replacement of the C(5')-hydroxyl of inosine by a halogen (other than F) or thioalkyl resulted in a reduction of the V_{max} values to < 1 per cent of that of the parent compound (Table 3). Despite their extremely low substrate activities, the halogenated analogs and those with smaller thioalkyl groups bound as well or more tightly than the natural nucleoside substrates. An anomalous relationship of the K_i to the K_m value is seen with these compounds since, by definition, K_m may be $> K_i$, whereas the reverse is not true. A possible explanation for the present results is that the substrate parameters had to be determined at enzyme concentrations 100–300 times greater than those employed for the inhibition studies. Within the 5'-halogenated series and the 5'-*S*-alkylated series, the V_{max} decreased with increasing substituent bulk. No activity was detected with the thiobenzyl derivative, which was also a poor inhibitor. Its low solubility prevented testing of this analog at high concentration. The charged carboxylate derivative was also not a substrate. The most dramatic increase in affinity was seen when the relatively weak PNP inhibitor, formycin B (a C-C nucleoside), was halogenated at C(5').

Studies with intact cells. The inhibition of intracellular PNP by 8-AG and 8-AGuo was studied in several ways. The phosphorolysis of the purine nucleoside analog, 6-MPR, was inhibited by 8-AG in intact human erythrocytes. Figure 5 shows the effects of increasing 8-AG concentrations on the phosphorolysis of 500 μM 6-MPR by red cells and hemolysates. Since V_{max} values were not determined

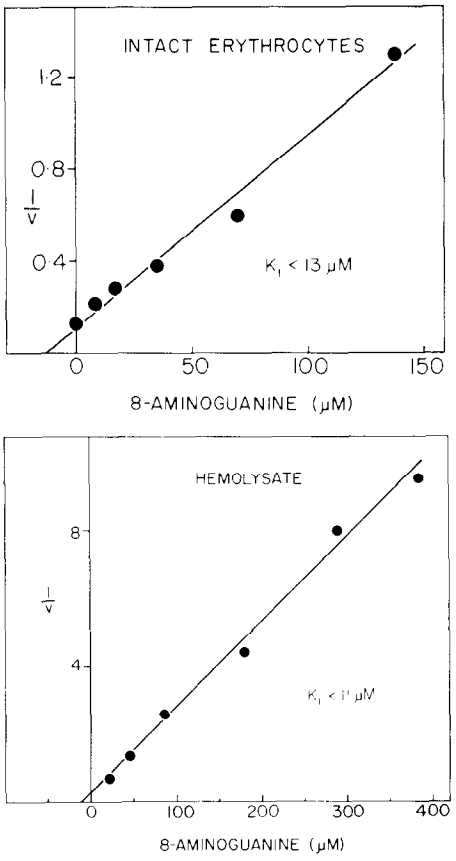

Fig. 5. Inhibition of the phosphorolysis of 6-mercaptopurine ribonucleoside by 8-AG in intact and hemolyzed human erythrocytes. Assay conditions are described in Materials and Methods. The x-intercepts show the upper limits of the K_i values.

for 6-MPR, exact K_i values could not be determined (as in Fig. 3A). However, the extrapolated lines intersect at 11 and 13 μM, respectively, and therefore the K_i values must be lower than these and similar for intact and hemolyzed cells. The similarity of these values also indicates that membrane transport of the nucleosides and bases is not rate-limiting.

The phosphorolysis of Guo and its incorporation into guanine nucleotide pools in intact human erythrocytes was also inhibited by 8-AG (Fig. 6). In 5% suspensions of red cells incubated with 100 μM Guo, only 15 per cent of the nucleoside remained after 15 min and none after 30 min. When 100 μM 8-AG was co-incubated, 38 per cent of the Guo remained after 15 min and 8 per cent after 30 min. Thus, the lifetime of the nucleoside was prolonged although these cells have very high PNP activity (10–15 units per ml packed cells [5]). Only 88 per cent of the Guo was converted to GTP after 60 min in the uninhibited cells, possibly a reflection of the relatively low HGPRT activity in erythrocytes (0.3 unit per ml packed cells [5]), and 62 per cent was converted in the 8-AG-treated cells.

As shown in Fig. 6, in uninhibited Sarcoma 180 cells rapid phosphorolysis of Guo (100 μM) occurred with only 4 per cent remaining after 15 min. The accumulation of GTP kept pace with virtually com-

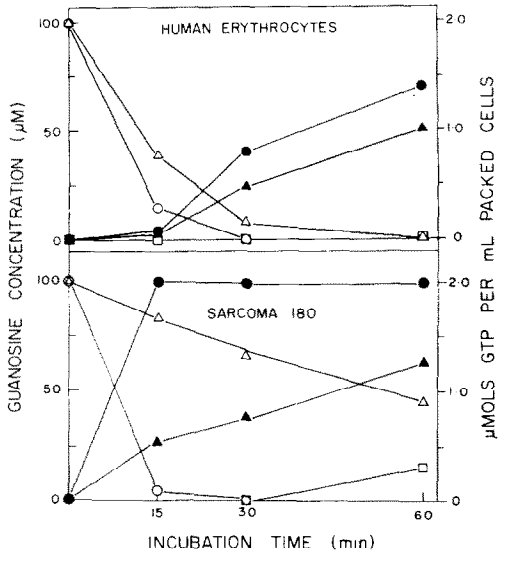

Fig. 6. Inhibition of Guo phosphorolysis and GTP synthesis by 8-AG in human erythrocytes and Sarcoma 180 cells. The conditions used are described in Materials and Methods. Key: (○) Guo concentration and (●) GTP concentration when 8-AG was omitted from the incubation; (△) Guo concentration and (▲) GTP concentration when 8-AG was added at 0 time; (□) GTP concentration in the absence of added Guo or 8-AG. The results are averages of duplicate samples.

plete conversion of added Guo to intracellular GTP. When 8-AG was co-incubated, however, 80 per cent of the Guo remained after 15 min and almost 50 per cent after 60 min. Synthesis of GTP was correspondingly decreased. When 8-AG was preincubated with the cells for 15 min before Guo addition, essentially the same degree of inhibition was seen.

Both 8-AG and 8-AGuo were tested as inhibitors of dGuo metabolism in L1210 and L5178Y cells as shown in Fig. 7. Phosphorolysis of 100 μM dGuo was complete within 5 min upon incubation with uninhibited L1210 cells, whereas in the presence of 100 μM 8-AG, complete phosphorolysis required 15 min. GTP synthesis lagged by about 25 per cent at 15 min in the 8-AG-treated cells but was complete at 60 min both with and without inhibitor. In contrast, L5178Y cells were affected more profoundly. In uninhibited cells the phosphorolysis of 100 μM dGuo was complete within 10 min, but 40 per cent remained after 60 min when 8-AG (100 μM) was pre- or co-incubated. GTP synthesis was decreased markedly in the presence of 8-AG. Essentially similar results were obtained with L1210 and L5178Y cells when 8-AGuo was used as the inhibitor. L5178Y cells had much lower PNP activity (0.009–0.031 unit/mg protein) than L1210 cells (0.073–0.164 unit/mg protein). PNP activities were determined on homogenates after centrifugation for 60 min at 100,000 *g*.

To determine whether C(5′)-substituted nucleosides that have relative V_{max} values of <1 per cent can function as PNP substrates intracellularly, L1210 and L5178Y cells were incubated at 37° in standard medium with 200 μM 5′-iodoinosine. Reversed-phase h.p.l.c. profiles showed that 70 and 20 per cent, respectively, of the analog was cleaved after 60 min, in accord with the relative PNP activities in these cell lines. These results will be described elsewhere.

DISCUSSION

For the reasons outlined above, the identification of a potent PNP inhibitor has become an important goal. Earlier studies showed that modification of the purine ring or ribose moiety of PNP substrates could lower the reaction rate, but there was always a concomitant loss in affinity for the enzyme [16, 28–31]. The present report documents the first cases of substrate analogs with decreased V_{max} values but the same or improved binding affinities.

An amino group at C(8) of guanine or hypoxanthine interacts with PNP in a unique manner since it significantly lowers the dissociation constants, whereas all other modifications at this position reduce affinity. There appears to be less specificity at the C(4′) and C(5′) positions of inosine. PNP can accommodate inversion of the 5′-hydroxymethyl group, as in α-L-lyxosylhypoxanthine [16], and a variety of uncharged substitutions at C(5′), i.e. halogen or thioalkyl. These C(5′)-substituted inosines bind reasonably well but are such poor alternate substrates that they almost approach the status of true inhibitors. An analog that combines the C(8) and C(5′) modifications, e.g. 8-amino-5′-deoxy-5′-iodoguanosine, might have excellent inhibitory properties. Attempts at such a synthesis are currently under way.

Although 8-AG has the highest affinity for PNP of any compound reported to date, it may not bind tightly enough to simulate the situation found in the

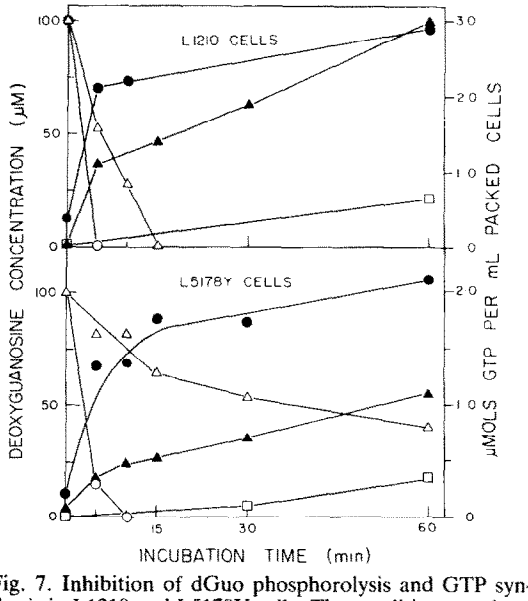

Fig. 7. Inhibition of dGuo phosphorolysis and GTP synthesis in L1210 and L5178Y cells. The conditions used are described in Materials and Methods. Key: (○) dGuo concentration and (●) GTP concentration when 8-AG was omitted from the incubation; (△) dGuo concentration and (▲) GTP concentration when 8-AG was added at 0 time; (□) GTP concentration in the absence of added dGuo or 8-AG. The results are averages of duplicate samples.

immunodeficient state. For this purpose it may be necessary to identify specific PNP inhibitors with K_i values $<10^{-9}$ M, as has occurred with adenosine deaminase and inhibitors such as deoxycoformycin [32]. However, 8-AG can significantly prolong the lifetime of co-administered analog nucleosides that are substrates for PNP. The more soluble nucleoside, 8-AGuo, was equally effective in inhibiting PNP intracellularly since it is rapidly converted to the more potent base analog which undergoes no further degradation. The important question of whether 8-AGuo and 8-AG enter cells via specific transport mechanisms is a topic for further study.

Although C(5')-halogenated compounds, e.g. 5'-deoxy-5'-iodoinosine, have very low V_{max} values, the high activity of PNP in certain tissues, i.e. ~10 units per ml in human erythrocytes, may be sufficient for significant rates of phosphorolysis. This could result in the intracellular formation of potentially toxic analogs of ribose-1-phosphate, e.g. 5-iodoribose-1-phosphate. In accord with this suggestion, cultured L1210 cells, with PNP activity about fifteen times higher than in cultured L5178Y cells, are inhibited by 5'-deoxy-5'-iodoinosine ($ID_{50} = 7.6 \times 10^{-6}$ M), whereas L5178Y cells are relatively insensitive ($ID_{50} > 10^{-4}$ M).*

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REFERENCES

1. E. R. Giblett, A. J. Ammann, R. Sandman, D. W. Wara and L. K. Diamond, *Lancet* **i**, 1010 (1975).
2. D. A. Carson, J. Kaye and J. E. Seegmiller, *Proc. natn. Acad. Sci. U.S.A.* **74**, 5677 (1977).
3. A. Cohen, L. J. Gudas, A. J. Ammann, G. E. J. Staal and D. W. Martin, Jr., *J. clin. Invest.* **61**, 1405 (1978).
4. E. C. Moore and R. B. Hurlbert, *J. biol. Chem.* **241**, 4802 (1966).
5. R. E. Parks, Jr., G. W. Crabtree, C. M. Kong, R. P. Agarwal, K. C. Agarwal and E. M. Scholar, *Ann. N.Y. Acad. Sci.* **255**, 412 (1975).
6. A. Cohen, D. Doyle, D. W. Martin, Jr. and A. J. Ammann, *New Engl. J. Med.* **295**, 1449 (1976).
7. R. E. Parks, Jr., K. C. Agarwal, R. P. Agarwal, G. W. Crabtree, T. Rogler-Brown, T. M. Savarese and J. D. Stoeckler, in *Nucleosides, Nucleotides and Their Biological Applications* (Eds. J-L. Barascut and J-L. Imbach), Vol. 81, p. 209. INSERM, Paris (1978).
8. R. E. Parks, Jr., J. D. Stoeckler, C. Cambor, T. Savarese, G. W. Crabtree and S. H. Chu, in *Molecular Actions and Targets for Cancer Chemotherapeutic Agents* (Eds. A. Sartorelli, J. S. Lazo and J. R. Bertino), Academic Press, New York p. 229 (1981).
9. J. D. Stoeckler, C. Cambor, F. W. Burgess, S. B. Erban and R. E. Parks, Jr., *Pharmacologist* **22**, 99 (1980).
10. R. P. Agarwal, K. C. Agarwal and R. E. Parks, Jr., *Meth. Enzym.* **51**, 581 (1978).
11. J. D. Stoeckler, R. P. Agarwal and R. E. Parks, Jr., *Meth. Enzym.* **51**, 530 (1978).
12. L. F. Cavalieri and A. Bendich, *J. Am. chem. Soc.* **72**, 2587 (1950).
13. S. D. Dimitrijevich, J. P. H. Verheyden and J. G. Moffatt, *J. org. Chem.* **44**, 400 (1979).
14. K. Kikugawa and M. Ichino, *Tetrahedron Lett.* p. 87 (1971).
15. K. Kikugawa, K. Iizuka, Y. Higuchi, H. Hirayma and M. Ichino, *J. med. Chem.* **15**, 387 (1972).
16. J. D. Stoeckler, C. Cambor and R. E. Parks, Jr., *Biochemistry* **19**, 102 (1980).
17. B. K. Kim, S. Cha and R. E. Parks, Jr., *J. biol. Chem.* **243**, 1763 (1968).
18. R. L. Cavalieri and H. Z. Sable, *Analyt. Biochem.* **59**, 122 (1974).
19. M. Dixon and E. C. Webb, *Enzymes*, p. 329. Academic Press, New York (1964).
20. W. W. Cleland, *Biochim. biophys. Acta* **67**, 104 (1963).
21. M. R. Sheen, B. K. Kim and R. E. Parks, Jr., *Molec. Pharmac.* **4**, 293 (1968).
22. G. W. Crabtree, R. P. Agarwal, R. E. Parks, Jr., A. F. Lewis, L. L. Wotring and L. B. Townsend, *Biochem. Pharmac.* **28**, 1491 (1979).
23. T. M. Savarese, G. W. Crabtree and R. E. Parks, Jr., *Biochem. Pharmac.* **30**, 189 (1981).
24. C. M. Kong and R. E. Parks, Jr., *Molec. Pharmac.* **10**, 648 (1974).
25. G. Giusti, in *Methods in Enzymatic Analysis* (Ed. H. U. Bergmeyer), Vol. 2, p. 1086. Academic Press, New York (1974).
26. R. E. Holmes and R. K. Robins, *J. Am. chem. Soc.* **87**, 1772 (1965).
27. T. P. Zimmerman, N. Gersten, A. F. Ross and R. P. Miech, *Can. J. Biochem.* **49**, 1050 (1971).
28. R. P. Agarwal and R. E. Parks, Jr., *J. biol. Chem.* **244**, 644 (1969).
29. T. A. Krenitsky, G. B. Elion, A. M. Henderson and G. H. Hitchings, *J. biol. Chem.* **243**, 2876 (1968).
30. R. E. Parks, Jr. and R. P. Agarwal, in *The Enzymes* (Ed. P. E. Boyer), Vol. 7, p. 483. Academic Press, New York (1972).
31. L. B. Townsend, B. L. Cline, R. P. Panzica, P. E. Fagerness, L. W. Roti-Roti, J. D. Stoeckler, G. W. Crabtree and R. E. Parks, Jr., *Heterocyclic Chemistry Series* **3**, S-79 (1978).
32. R. P. Agarwal, T. Spector and R. E. Parks, Jr., *Biochem. Pharmac.* **26**, 359 (1977).

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