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Human Erythrocytic Hypoxanthine-Guanine Phosphoribosyltransferase: Effect of pH on the Enzymatic Reaction

CHONG M. KONG AND R. E. PARKS, JR.

Section of Biochemical Pharmacology, Division of Biological and Medical Sciences, Brown University, Providence, Rhode Island 02912

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SUMMARY

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The effect of pH on human erythrocytic hypoxanthine-guanine phosphoribosyltransferase (EC 2.4.2.8) was studied, and the enzyme was found to be unstable at pH values below 5.5 and above 9.0. The enzyme exhibited marked differences in pH optima when the various purines or purine analogues were used as substrates: 8-azaguanine, 6.5; 6-mercaptopurine, 7.5–8.0; 6-selenoguanine, 7.5–8.0; 6-thioguanine, 8.0–9.0; guanine, 8.5–9.0. The enzyme was subjected to a Dixon analysis, in which the Michaelis constants and maximal velocities were determined for these substrates at various pH values between 5.5 and 9.5. Plots of the negative logarithm of the Michaelis constants (pK_m) against pH resulted in a family of graphs with downward bends. With the exception of guanine, the bending points of the curves occurred at pH values which closely approximated the pK_a values of the respective compounds. The V_{max} values increased with increasing pH within the range examined. This effect of pH on K_m values suggests that the ionization state of purines or their analogues is an important factor for binding of the substrate to the enzyme. It also indicates that the undissociated form of the molecule is the effective substrate for the enzymatic reaction. When K_m values are expressed in terms of the concentration of the un-ionized form, the pK_m plots result in straight lines in all cases.

INTRODUCTION

Hypoxanthine-guanine phosphoribosyltransferase (EC 2.4.2.8) is one of several enzymes that salvage previously formed purines or purine nucleosides and therefore are of special importance in the metabolism of cells in which purine biosynthesis *de novo* is deficient or inhibited (1–3). The enzyme is of key importance in some aspects of chemotherapy and immunosuppression, since most analogues of guanine or hypoxanthine (e.g., 6-mercaptopurine, 6-thioguanine, 8-azaguanine) must be converted to the respective nucleotides in order to exert their cytotoxic effects. Cells lacking hypoxanthine-guanine phosphoribosyltransferase are usually resistant to analogues of hypoxanthine or guanine (4).

In spite of extensive studies of many prop-

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erties of the enzyme (5–11), only limited information is available on how it is affected by pH. In early studies with the enzyme from hog liver Way and Parks (5) found striking differences in the substrate activities of guanine and 8-azaguanine at different pH values. Although the maximal reaction velocity with guanine occurred at about pH 9.3, 8-azaguanine had no detectable substrate activity above pH 7.5 and displayed a pH optimum at about pH 7.0 (5). Krenitsky *et al.* (9) observed no differences in the rate of IMP synthesis between pH 7.1 and 9.1 by the human erythrocytic enzyme. Miller and Bieber (6) found that yeast enzyme was unstable at pH values below 7.0 and above 10.0 and that it exhibited different pH optima with hypoxanthine and guanine as substrates.

The purpose of the present work is to examine and define the effect of pH on various kinetic parameters of human hypoxanthine-guanine phosphoribosyltransferase.

MATERIALS AND METHODS

Materials. [8-^{14}C]Guanine (28 mCi/mmole) and [8-^{14}C]hypoxanthine (50 mCi/mmole) were obtained from Schwarz/Mann. Guanine was purchased from P-L Biochemicals. Hypoxanthine was obtained from Boehringer/Mannheim. 5-Phosphoribosyl 1-pyrophosphate, 6-mercaptopurine, and diethylaminoethyl cellulose were Sigma products. 6-Thioguanine, 8-azaguanine, dithiothreitol, and 2-(*N*-morpholino)ethanesulfonic acid were obtained from Calbiochem. 6-Selenoguanine was synthesized and supplied by Dr. S.-H. Chu of this Division. Human erythrocytes were obtained from the Department of Hematological Research, Memorial Hospital, Pawtucket, R. I. All other reagents were of the highest purity available.

Purification of hypoxanthine-guanine phosphoribosyltranferase. Human erythrocytes stored at 4° for 5–7 days were used. After centrifugation at 10,000 × *g* for 30 min, the plasma and white cells were removed by aspiration. The erythrocytes were washed twice with equal volumes of cold 0.9 % NaCl. To 150 ml of erythrocytes, 600 ml of water were added with stirring. The mixture was then frozen at −20° and thawed. The stroma was removed by centrifugation at 13,000 × *g* for 30 min.

Hemoglobin was removed by the method of Hennessey *et al.* (12) as modified by Rubin *et al.* (13). The stroma-free lysate was added to 600 ml of DEAE-cellulose (40 g, 0.9 mEq/g) suspension that had been equilibrated in 0.01 M potassium phosphate buffer (pH 7.0), and the mixture was stirred for 2 hr. The cellulose was collected by suction filtration on a Buchner funnel, and the remaining hemoglobin was removed by washing with 2 liters of 0.01 M potassium phosphate, pH 7.0. The filtrate and washes contained negligible hypoxanthine-guanine phosphoribosyltransferase activity and were discarded.

The DEAE-cellulose was suspended in 500 ml of a solution composed of potassium phosphate (pH 7.4), 0.01 M; potassium chloride, 0.2 M; and dithiothreitol, 0.005 M, and stirred for 2 hr. The suspension was filtered and washed with an additional 250 ml of the same eluting solution. The filtrates were combined and concentrated about 50-fold with an Amicon ultrafiltration cell, using a PM-30 membrane, to a volume of about 14.0 ml. The concentrated solution was frozen at −20° and thawed, and the precipitated proteins were removed by centrifugation at 10,000 × *g* for 15 min. The supernatant fluid contained hypoxanthine-guanine phosphoribosyltransferase at a specific activity of about 0.16 enzyme unit/mg of protein, which represented approximately 95-fold purification from the hemolysate. The recovery of enzymatic activity was usually about 60 %. Subsequent freezing did not cause precipitation of protein. The enzyme solution could be stored at −20° without loss of activity for a least 6 weeks.

This partially purified transferase proved satisfactory for the kinetic studies described below. When the various purine or analogue substrates were incubated with enzyme, Tris-HCl (pH 7.5), 100 mM, and $MgSO_4$, 2 mM, but in the absence of PRPP,[1] no spectral changes were observed over a 30-min period. Therefore there was no apparent interference from competing enzymatic reactions. When K_m and V_{max} values of 6-

[1] The abbrevation used is: PRPP, 5-phosphoribosyl 1-pyrophosphate.

mercaptopurine and 6-thioguanine were determined with this preparation, they were essentially identical with the kinetic values obtained with a more highly purified preparation (specific activity, 0.64 unit/mg of protein) that was available only in small quantity. Recent studies by Arnold and Kelley (11) as well as in this laboratory suggest the possible occurrence of multiple forms of this enzyme. When the partially purified enzyme preparation was subjected to isoelectric focusing, two peaks of activity were observed: a large peak at pI 5.5, which contained about 95% of the activity, and the remainder in a smaller peak, at pI 6.0. The quantity of enzyme isolated in the smaller peak was insufficient to permit further study.

Enzymatic assay. Hypoxanthine-guanine phosphoribosyltransferase activity was assayed spectrophotometrically by measuring the rate of 5′-monophosphate nucleotide synthesis as indicated by the increase in absorbance at the most favorable wavelength for the substrate selected.

The standard assay was carried out at room temperature in a 1-ml reaction mixture containing Tris-HCl (pH 7.5), 100 mM; PRPP, 1.0 mM; $MgSO_4$, 2.0 mM; 6-thioguanine, 0.05 mM; and enzyme. The reaction was initiated by addition of the enzyme, and the rate of increase in absorbance at 345 nm was measured for several minutes with a Gilford model 240 spectrophotometer equipped with a model 6040 recorder. The enzymatic activity observed by this method closely parallels that measured by an isotopic assay that employs [8-^{14}C]guanine or [8-^{14}C]hypoxanthine as described by Henderson *et al.* (8) and modified by Parks *et al.* (14). One micromolar unit of hypoxanthine-guanine phosphoribosyltransferase is defined as the amount that catalyzes the formation of 1 μmole of 5′-monophosphate nucleotide per minute under the conditions of the standard assay.

Determination of changes in molar extinction coefficients (Δε) for conversion of guanine and various analogues to respective 5′-monophosphate nucleotides. When guanine and the various analogues are converted to their respective 5′-monophosphate nucleotides, specific spectral changes occur that form the bases of the spectrophotometric assays of the hypoxanthine-guanine phosphoribosyltransferase employed in this study. The difference in molar absorbance (Δε) at the wavelengths and pH values indicated in Table 1 were determined for guanine and the analogues by the following procedure. A reaction mixture (1 ml) which contained 100 mM buffer at the desired pH; PRPP, 1 mM; $MgSO_4$, 2 mM; and 0.001–0.002 micromolar unit of enzyme was incubated at room temperature for 3–4 min. Purine or purine ana-

TABLE 1

Changes in molar absorbance (Δε) for hypoxanthine-guanine phosphoribosyltransferase reaction with guanine and several analogues at various pH values

The procedure used to determine the Δε values is described in MATERIALS AND METHODS. Guanine and 8-azaguanine were dissolved in a minimal volume of 0.1 M NaOH and then brought to volume for the desired concentration with water; 6-mercaptopurine, 6-thioguanine, and 6-selenoguanine, were dissolved by heating the suspended compounds in a boiling water bath for 1–2 min. For 6-selenoguanine an equal concentration of dithiothreitol was added, and the solution was protected from light to avoid photodegradation.

Substrate	Wavelength	Δε							
		pH 6.2	pH 6.5	pH 7.0	pH 7.5	pH 8.0	pH 8.5	pH 8.8	pH 9.1
	nm								
Guanine	255			5000	4800	5000	4950	4950	5000
8-Azaguanine	260	6100	6200	6200	6150	6000			
6-Mercaptopurine	312		5750	5750	4300	3600	3300	3350	
6-Thioguanine	345		3350	3650	3600	3800	3150	2250	
6-Selenoguanine[a]	360		3350	3450	3600	3350	1800	650	

[a] Assayed in the presence of 0.1 M dithiothreitol.

logue substrate was added to the reaction mixture to a known final concentration within the range of 10–40 μM to start the reaction. An initial absorbance reading was obtained immediately after mixing, and the change in absorbance was monitored every 3–4 min until no further changes occurred. The total increase in absorbance during the reaction and the amount of substrate added were used to calculate $\Delta\epsilon$. When the changes in absorbance observed were plotted against the concentration of the purine substrate, linear graphs were obtained that extrapolated approximately through zero concentration. When [8-^{14}C]guanine was the substrate, over 95% of the radioactivity was converted to GMP under the conditions described here.

The change in molar extinction coefficient ($\Delta\epsilon$) for the conversion of hypoxanthine to inosinate, less than 1500 at ultraviolet wavelengths, is unfavorable for the development of a sensitive spectrophotometric assay. Therefore hypoxanthine was not included in the present study.

Determination of kinetic constants. Initial reaction velocities were measured spectrophotometrically as described for the standard assay. All buffers were added to a final concentration of 100 mM. The buffer systems used are described in the legend to Fig. 1. In order to rule out effects on the enzymatic activity due to differences in the buffer system, reaction rates were measured in Tris buffer at pH 7.5 in the presence and absence of 2-(*N*-morpholino)ethanesulfonic acid and glycine. Identical velocities were observed. The reaction mixtures were incubated for 3–4 min at room temperature, and reactions were initiated by addition of the enzyme. All velocities were measured in duplicate or triplicate. The pH values of the reaction mixtures were measured before and immediately after each reaction by a Beckman Zeromatic II pH meter. The pH values did not differ by more than 0.05 pH unit.

All Lineweaver-Burk plots were linear. To obtain the best estimation of Michaelis constants, maximal velocities, and standard errors, a computer program described by Cleland (15) and adapted to a Wang model 700 computer was used.

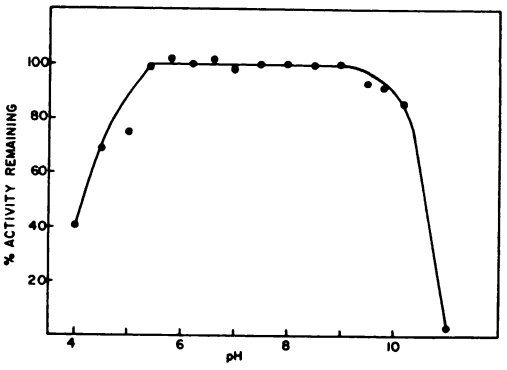

FIG. 1. *Effect of pH on enzymatic stability*
The following buffers were used throughout all pH studies: below pH 7.0, 2-(*N*-morpholino)-ethanesulfonic acid–KOH; pH 7.0–9.5, Tris-HCl; above pH 9.5, glycine–KOH. After a 20-min incubation in 0.1 M buffer at the indicated pH values and at room temperature, aliquots were removed and assayed at pH 7.5. The 100% value represents the synthesis of 11.7 nmoles of GMP per minute.

RESULTS

Effect of pH on enzymatic stability and determination of pH optima. Identical quantities of the enzyme were incubated in buffer, 100 mM, at the indicated pH values for 20 min at room temperature. At the end of the incubation period, 25-μl samples were removed and assayed at pH 7.5. As shown in Fig. 1, the enzyme was stable under these conditions between pH 5.5 and 9.0. Below pH 5.5 and above pH 9.0, there was marked loss of activity.

The rates of synthesis of 5′-monophosphate nucleotides from guanine and the analogues added in 0.05 mM concentration were determined at various pH values. The enzyme exhibits distinctly different pH optima with the different substrates (Fig. 2): guanine, pH 8.5–9.0; 8-azaguanine, pH 6.5; 6-mercaptopurine, pH 7.5–8.0; 6-selenoguanine, pH 7.5–8.0; 6-thioguanine, pH 8.0–9.0. In each case the rate of nucleotide synthesis reached a plateau at approximately the pK_a value of the respective substrate (Table 2) and then decreased when pH was increased above the pK_a value of the compound (16–20).

The determination of pH optima in this manner, i.e., at constant, relatively low substrate concentrations, reflects a number of

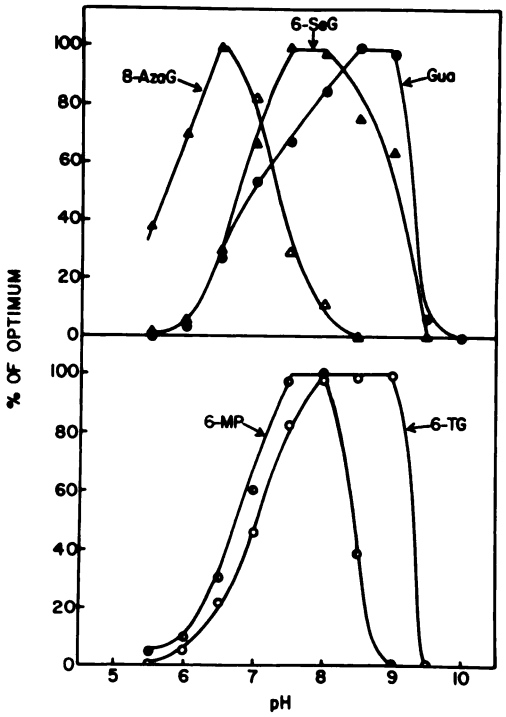

FIG. 2. *Effect of pH on reaction rates with different substrates*

Rates of 5′-monophosphate ribonucleotide synthesis from the corresponding substrates were measured at the pH indicated. Reaction mixtures contained 50 μM purine or analogue substrate, 1 mM PRPP, 2 mM $MgSO_4$, and 100 mM buffer. The 100% values represent the synthesis of 9.7 nmoles of GMP per minute (●——●). ○——○, 9.75 nmoles of 6-thioGMP per minute; △——△, 3.56 nmoles of 8-azaGMP per minute; ◐——◐, 6.25 nmoles of 6-thioIMP per minute; ▲——▲, 14.8 nmoles of 6-selenoGMP per minute. 8-AzaG, 8-azaguanine; 6-SeG, 6-selenoguanine; 6-MP, 6-mercaptopurine; 6-TG, 6-thioguanine; Gua, guanine.

factors such as the effects of pH on the enzymatic conformation, substrate binding, and catalytic activity. Therefore the effects of pH on the individual kinetic parameters were examined by the method of Dixon (21).

Effect of pH on Michaelis constants. The apparent Michaelis constants were determined for PRPP, guanine, and the analogues over a range of pH values between 5.9 and 9.35 (Table 3). Although the K_m values for PRPP are relatively constant, the apparent K_m values for guanine and the analogues are affected markedly by pH. In all cases the apparent K_m values increase with increasing pH. From pH 7.5 to 8.8 the approximate increases are: guanine, 3-fold; 6-mercaptopurine, 10-fold; 6-thioguanine, 5-fold; 6-selenoguanine, 10-fold; from pH 6.5 to 8.0 the K_m for 8-azaguanine increases about 48-fold.

TABLE 2

pK_a values of guanine and various analogues

Compound	pK_a	Reference
Guanine	9.2	16
6-Thioguanine	8.2	17
6-Selenoguanine	7.7	18
6-Mercaptopurine	7.7	19
8-Azaguanine	6.5	20

pK_m vs. pH relationship. The negative logarithms of the apparent Michaelis constants (pK_m) were plotted against pH (Fig. 3). All curves have a downward curvature. With the exception of guanine, the curves bend at a pH near the pK_a of the respective compounds (Table 2).

The apparent K_m values at the various pH values were corrected and expressed in terms of the molar concentrations of the undissociated form by the formula

$$\text{Corrected } K_m = \frac{\text{apparent } K_m}{1 + 10^{(\text{pH}-\text{p}K_a)}}$$

where pK_a is the negative logarithm of the dissociation constant of the substrate. When the corrected pK_m values are plotted against pH, one obtains a family of relatively straight lines, as shown in Fig. 4.

Effect of pH on V_{max}. The V_{max} values for each purine or analogue substrate at various pH values between 5.9 and 9.35 are presented in Table 3 and are illustrated in Fig. 5. Since identical amounts of enzyme were used in all assays, the V_{max} values for different substrates can be compared. The V_{max} values increase in an approximately linear relationship with increasing pH. The correlation coefficient of the straight line in Fig. 5 is 0.895. It is of interest that at any selected pH the V_{max} values with the different substrates are relatively similar.

TABLE 3

Michaelis constants and maximal velocities for reaction of guanine, various analogues and PRPP with hypoxanthine-guanine phosphoribosyltransferase

K_m and V_{max} values are expressed in terms of micromolarity and nanomoles of 5'-monophosphate synthesized per minute, respectively. The assay procedures are described in MATERIALS AND METHODS. The kinetic parameters and their standard errors were determined by a computer program described by Cleland (15).

pH	8-Azaguanine		6-Mercaptopurine		6-Selenoguanine		6-Thioguanine		Guanine		PRPP K_m
	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}	$M \times 10^4$
5.9	23.3 ±2.37	1.50 ±0.08									
6.2	17.1 ±1.95	2.11 ±0.08									
6.5	25.2 ±1.82	2.73 ±0.07	8.59 ±0.89	4.85 ±0.17	9.85 ±0.65	4.08 ±0.07	8.13 ±1.20	4.42 ±0.14			3.83 ±0.37
7.0	111.8 ±8.82	6.90 ±0.33	14.0 ±2.05	6.15 ±0.40	14.4 ±1.11	8.40 ±0.27	12.8 ±1.33	4.18 ±0.10	5.24 ±0.41	5.93 ±0.14	5.05 ±0.33
7.5	504.8 ±87.0	12.5 ±1.79	14.8 ±1.03	6.35 ±0.20	16.2 ±1.49	6.30 ±0.20	12.9 ±1.49	4.73 ±0.15	4.91 ±0.49	6.89 ±0.20	3.01 ±0.285
8.0	1208 ±472	11.0 ±3.33	23.6 ±2.11	9.25 ±0.47	26.0 ±1.34	8.05 ±0.19	17.7 ±2.46	7.45 ±0.37	6.06 ±0.40	7.40 ±0.16	1.42 ±0.12
8.5			30.5 ±3.41	9.80 ±0.68	59.3 ±4.07	11.4 ±0.38	38.6 ±5.41	11.48 ±0.66	10.6 ±0.70	12.2 ±0.35	1.34 ±0.12
8.8			155.5 ±93.3	13.1 ±3.13	177.4 ±24.0	40.7 ±3.30	68.8 ±6.77	13.0 ±0.54	14.5 ±1.74	15.4 ±0.94	2.36 ±0.15
9.1									20.5 ±0.35	17.3 ±1.55	
9.35									34.2 ±6.20	24.4 ±2.80	

DISCUSSION

This investigation presents the results of a Dixon pH analysis of human erythrocytic hypoxanthine-guanine phosphoribosyltransferase. As noted by Dixon (21), a downward curvature in the plot of pK_m vs. pH indicates that the ionizing group is either in the substrate or in the free enzyme, and that the bend occurs at the pH corresponding to the pK_a of the ionizing group. An upward curvature suggests that the ionizing group is in the enzyme-substrate complex. The fact that the downward bends in Fig. 3 occurred at about pH 8.0, 7.7, 7.6, and 6.3 when 6-thioguanine, 6-selenoguanine, 6-mercaptopurine, and 8-azaguanine, respectively, were used as substrates indicates that the ionizations take place in the substrates rather than in the enzyme. If the downward curvatures were due to ionization in the free enzyme, they would occur at the same pH, regardless of the substrate used. With the exception of guanine, the bending points approximate the pK_a values of the corresponding compounds (Table 2). Since both the enzyme and PRPP are unstable above pH 9.0, estimation of kinetic parameters above this pH may be subject to error, which may account for the deviation from expected values in the case of guanine (pK_a = 9.2). Since the proportion of undissociated purine or purine analogue molecules decreases with increasing pH, the apparent increase in K_m with increasing pH indicates that the undissociated species is the effective form of the substrate. Indeed, when apparent K_m values are recalculated in terms of the concentrations of the undissociated (protonated) forms, the downward curvatures in plots of pK_m vs. pH are eliminated (Fig. 4). Thus decreases in the rates of

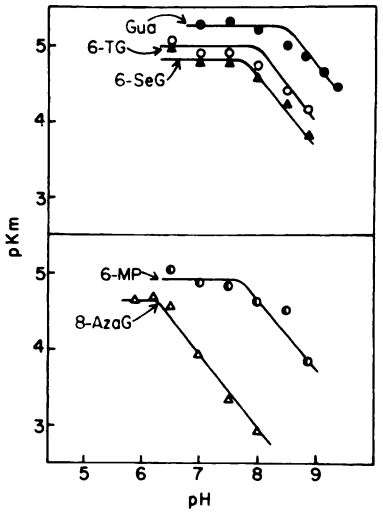

FIG. 3. *Effect of pH on apparent Michaelis constants*

The negative logarithms of the apparent Michaelis constants for guanine (Gua, ●——●), 6-thioguanine (6-TG, ○——○), 6-selenoguanine (6-SeG, ▲——▲), 6-mercaptopurine (6-MP, ◐——◐), and 8-azaguanine (8-AzaG, △——△) from Table 3 are plotted against pH according to the method of Dixon (21).

5′-monophosphate nucleotide synthesis at pH values above the corresponding pK_a values of these compounds (Fig. 2) seem principally due to the decreased percentage of the effective species of the substrates.

It is not yet unequivocally established which ionizing groups of purine or purine analogues are responsible for each pK_a value observed. Considerable evidence suggests that guanine and hypoxanthine, as well as many of their analogues, exist predominantly in the 6-keto form (22–26). For guanine, a pK_a value of 3.3 was assigned to the amino group at position 2 (27), and a pK_a of 12.3 for the proton on N-9 of the imidazole ring (28). The pK_a value found at 9.2 is generally believed to be due to the dissociation of the lactam structure (28). This ionization may be represented as shown in Fig. 6. The negative charge which is probably distributed throughout the lactam group may make the molecule incapable of binding to the active site of the enzyme.

The logarithms of the maximal velocities increase linearly as the pH increases from

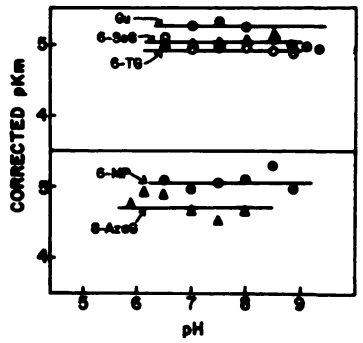

FIG. 4. *Effect of pH on corrected Michaelis constants*

The apparent Michaelis constants from Table 3 are corrected and expressed as the molar concentrations of the undissociated forms of the substrates at each pH value. The negative logarithms of these are plotted against pH. ●——●, guanine (Gu); ○——○, 6-thioguanine (6-TG); ▲——▲, 6-selenoguanine (6-SeG); ◐——◐, 6-mercaptopurine (6-MP); △——△, 8-azaguanine (8-AzaG).

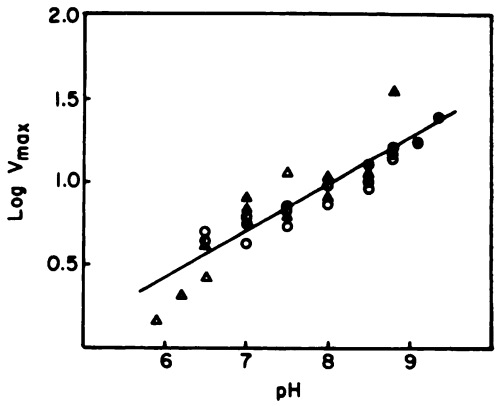

FIG. 5. *Effect of pH on maximal velocities*

The maximal velocities of the reactions of guanine (●——●), 6-thioguanine (○——○), 6-selenoguanine (▲——▲), 6-mercaptopurine (◐——◐), and 8-azaguanine (△——△) with hypoxanthine-guanine phosphoribosyltransferase are plotted against pH. The correlation coefficient (r) of the linear regression line is 0.895.

about 6.0 to 9.0, while at the same pH values the V_{max} values are quite similar for the various purine substrates. If the effect of pH on V_{max} were due to the ionization of a single group in the enzyme-substrate complex, one would expect a 10-fold change over 1 pH unit. Since a change of about 10-fold in V_{max} is observed over 3 pH units, an alterna-

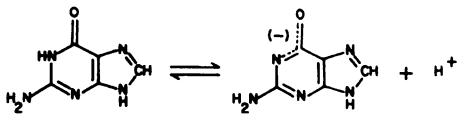

FIG. 6

tive explanation must be sought. Besides the effect on the ionization state of the substrate and on enzyme-substrate binding, pH may have additional effects on the enzyme, such as an alteration in conformation. With conformational changes, there may be changes in energy of activation for the reaction catalyzed. Miller and Bieber (7) have reported that with yeast hypoxanthine-guanine phosphoribosyltransferase the energy of activation (E_a) decreased as the pH increased from 7.4 to 8.5 when reactions were carried out above 19° and guanine was used as the substrate. At pH 7.4 the activation energy was 11,600 cal/mole; at pH 8.5 it decreased to 8800 cal/mole. This decrease in activation energy may account for the increase in reaction rate observed when the pH increased from 7.4 to 8.5. Preliminary studies at various pH values of the effect of temperature on the V_{max} values of human erythrocytic hypoxanthine-guanine phosphoribosyltransferase with 6-thioguanine as the substrate indicate that similar findings occur with this enzyme. This interesting, and as yet unexplained, observation will be the subject of further study.

The type of pH effect described here for guanine and several purine analogues is essentially similar to those observed with uridine diphosphate glucose and its 5-fluoro and 6-aza analogues and the enzyme uridine diphosphate glucose dehydrogenase (29), and in related studies with uridine diphosphate glucuronyltransferase (30). Thus selected analogue compounds may provide useful tools for analyzing the binding of substrates to enzymes.

Considerable ambiguity exists in the literature on the nature of the interaction between hypoxanthine-guanine phosphoribosyltransferase and a number of purine analogues. For example, both 6-mercaptopurine and 6-thioguanine, which are demonstrated in the present studies to be excellent substrates, have been described as inhibitors of this enzyme. The apparent inhibitions reported in fact resulted from the nature of the assays employed in these studies, i.e., the synthesis of IMP from ^{14}C-labeled hypoxanthine. Such an assay does not distinguish between a true competitive inhibitor and a competing alternative substrate. Clearly the analogues described above must be regarded as alternative substrates rather than inhibitors.

The results reported here are of potential importance in chemotherapy and immunosuppression. The findings indicate that several important purine analogues have higher activities as substrates for hypoxanthine-guanine phosphoribosyltransferase at lower pH values. In addition, the ionization state of the purine may have a marked effect on its ability to enter the cell, since a generalization in pharmacodynamics is that a drug diffuses or is transported across cell membranes more readily if it is in an uncharged form. Another potentially crucial factor worthy of further study is the effect on the cytotoxic behavior of certain purine analogues of the ionization state of the compounds after they are incorporated into coenzymes or nucleic acids. Thus the pK_a is a factor for consideration in the design of various analogues, and it may become an important criterion for the prediction of potential effectiveness. For instance, if one assumes that all other conditions are identical, it may be predicted that 6-thioguanine (pK_a 8.2) will be more effective than 8-azaguanine (pK_a 6.5) at physiological pH (7.4). Since some tumor tissues have lower intracellular pH values than normal tissues because of more vigorous aerobic glycolysis (31), analogues with relatively low pK_a values may have greater effects on certain tumors than on normal tissues. Also, if one can produce a local decrease in pH in the tumor tissue (32, 33), the inhibitory effects of these analogues might be potentiated.

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Memorial Hospital, for supplying fresh human erythrocytes.

REFERENCES

1. Murray, A. W. (1971) *Annu. Rev. Biochem.*, **40**, 811–826.
2. Lowy, B. A., Williams, M. K. & London, I. M. (1962) *J. Biol. Chem.*, **237**, 1622–1625.
3. Holmsen, H. & Rozenberg, M. C. (1968) *Biochim. Biophys. Acta*, **157**, 266–279.
4. Brockman, R. W., Sparks, M. C. & Simpson, M. S. (1957) *Biochim. Biophys. Acta*, **26**, 671–672.
5. Way, J. L. & Parks, R. E., Jr. (1958) *J. Biol. Chem.*, **231**, 467–480.
6. Miller, R. L. & Bieber, A. L. (1968) *Biochemistry*, **7**, 1420–1426.
7. Miller, R. L. & Bieber, A. L. (1968) *Biochemistry*, **8**, 603–608.
8. Henderson, J. F., Brox, L. W., Kelley, W. N., Rosenbloom, F. M. & Seegmiller, J. E. (1968) *J. Biol. Chem.*, **243**, 2514–2522.
9. Krenitsky, T. A., Papaioannou, R. & Elion, G. B. (1969) *J. Biol. Chem.*, **244**, 1263–1270.
10. Krenitsky, T. A. & Papaioannou, R. (1969) *J. Biol. Chem.*, **244**, 1271–1277.
11. Arnold, W. J. & Kelley, W. N. (1971) *J. Biol. Chem.*, **246**, 7398–7404.
12. Hennessey, M. A., Walterdorph, A. M., Huennekens, F. M. & Gabrio, B. W. (1962) *J. Clin. Invest.*, **41**, 1257–1262.
13. Rubin, C. S., Dancis, J., Yip, L. C., Norwinski, R. C. & Balis, M. E. (1971) *Proc. Natl. Acad. Sci. U. S. A.*, **68**, 1461–1464.
14. Parks, R. E., Jr., Brown, P. R., Cheng, Y.-C., Agarwal, K. C., Kong, C. M., Agarwal, R. P. & Parks, C. C. (1973) *Comp. Biochem. Physiol.*, **45b**, 355–364.
15. Cleland, W. W. (1967) *Adv. Enzymol.*, **29**, 1–32.
16. Taylor, H. F. W. (1948) *J. Chem. Soc. (Lond.)*, 765–766.
17. Fox, J. J., Wemper, I., Hampton, A. & Doerr, I. L. (1958) *J. Am. Chem. Soc.*, **80**, 1669–1675.
18. Ross, A. F. (1972) Ph.D. thesis, Brown University.
19. Albert, A. & Brown, D. J. (1954) *J. Chem. Soc. (Lond.)*, 2060–2071.
20. Parks, R. E., Jr. & Agarwal, K. C. (1973) in *Handbook of Experimental Pharmacology, Antineoplastic and Immunosuppressive Agents* (Sartorelli, A. C. & John, D. G., eds.), Springer-Verlag, Berlin, in press.
21. Dixon, M. (1953) *Biochem. J.*, **55**, 161–171.
22. Bugg, C. E. & Thewalt, U. (1970) *J. Am. Chem. Soc.*, **92**, 7441–7445.
23. Sletten, E., Sletten, J. & Jensen, L. H. (1969) *Acta Crystallogr., Sect. B*, **25**, 1330–1338.
24. Brown, G. M. (1969) *Acta Crystallogr., Sect. B*, **25**, 1338–1353.
25. MacIntyre, W. M., Singh, P. & Werkema, M. S. (1965) *Biophys. J.*, **5**, 697–713.
26. Chan, S. I. & Lee, G. C. Y. (1972) in *Jerusalem Symposia on Quantum Chemistry and Biochemistry*, Vol. 4, *The Purines—Theory and Experiment* (Bergmann, E. D. & Pullman, B., eds.), pp. 277–296, The Israel Academy of Sciences and Humanities, Jerusalem.
27. Bendich, A. (1955) in *The Nucleic Acids* (Chargaff, E. & Davidson, J. N., eds.), Vol. 1, pp. 81–136, Academic Press, New York.
28. Jordan, D. O. (1955) in *The Nucleic Acids* (Chargaff, E. & Davidson, J. N., eds.), Vol. 1, pp. 447–492, Academic Press, New York.
29. Goldberg, N. D., Dahl, J. L. & Parks, R. E., Jr. (1963) *J. Biol. Chem.*, **238**, 3109–3114.
30. Goldberg, N. D. (1963) Ph.D. thesis, University of Wisconsin.
31. Warburg, O., Posenor, K. & Negelein, E. (1924) *Biochem. Z.*, **152**, 309–335.
32. Eden, M., Haines, B. and Kahler, H. (1955) *J. Natl. Cancer Inst.*, **16**, 541–556.
33. Kung, S., Goldberg, N. D., Dahl, J. L., Kline, B. & Parks, R. E., Jr. (1963) *Science*, **141**, 627–628.