Adenosine Triphosphate-Guanosine 5′-Phosphate Phosphotransferase

II. Inhibition by 6-Thioguanosine 5′-Phosphate of the Enzyme Isolated from Hog Brain and Sarcoma 180 Ascites Cells

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SUMMARY

Sarcoma 180 cells contain a highly specific guanylate kinase (ATP-GMP phosphotransferase, EC 2.7.4.8) that converts GMP to GDP. This tumor enzyme closely resembles in its kinetic parameters and molecular size the guanylate kinase of hog brain. With both enzymes the analogue nucleotide 6-thioguanosine 5′-phosphate behaves as a potent inhibitor, competitive with GMP, with an inhibition constant (K_i) of about 6×10^{-5} M. When examined with large amounts of hog brain enzyme, 6-thioguanosine 5′-phosphate behaves as an alternative substrate with a very low maximum velocity that is less than 0.04% of the reaction rate attainable with GMP as the substrate. The possible role of these findings in the cytolytic action of 6-thioguanine is discussed.

INTRODUCTION

Earlier work in this laboratory (1) demonstrated the occurrence in brain tissue of a specific guanylate kinase (ATP-GMP phosphotransferase, EC 2.7.4.8) which catalyzes the following reaction:

$$GMP + ATP \rightleftharpoons GDP + ADP.$$

This enzyme is highly specific for ATP, and among the nucleotide monophosphates tested, only GMP, dGMP, and 8-azaGMP[1] serve as substrates. On the other hand, such compounds as AMP, IMP, XMP, 6-mercaptopurine ribonucleotide, and several pyrimidine ribonucleoside monophosphates were inert, either as substrates or as inhibitors.

A finding of potential importance for the understanding of the chemotherapeutic effects of the antitumor agent 6-thioguanine is the observation that 6-thioguanosine 5′-phosphate is a potent competitive inhibitor of this enzyme. This raised the question of the possible occurrence of a similar enzyme in tumors and other tissues. The present report describes the inhibition of guanylate kinase from brain by 6-thioGMP and the isolation and characterization from Sarcoma 180 ascites cells of a similar enzyme which is also inhibited by 6-thioGMP. Preliminary reports of these findings have been presented (2–4).

MATERIALS AND METHODS

The sources of the various nucleotides, NADH, phosphoenolpyruvate, pyruvate kinase, and lactate dehydrogenase have been described elsewhere (1). 6-Thioguanine was purchased from Sigma Chemi-

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[1] The abbreviations used are: 8-azaGMP, 8-azaguanosine 5′-phosphate; 6-thioGMP, 6-thioguanosine 5′-phosphate; 6-thioGTP, 6-thioguanosine 5′-triphosphate.

cal Company, and Bio-Gel P-20 was obtained from the California Corporation for Biochemical Research. Male mice (CD-1 strain) were purchased from Charles River Breeding Laboratories. The initial inoculum of 6-thioguanine-sensitive Sarcoma 180 cells was generously supplied by Dr. A. C. Sartorelli, Yale University.

Isolation of hog brain ATP-GMP phosphotransferase (guanylate kinase). Guanylate kinase from frozen hog brain was prepared through fraction V as described previously (1). The ammonium sulfate precipitate from Fraction V was dissolved in about 3 ml of 0.01 M Tris-chloride buffer (pH 7.5) and filtered through a Sephadex G-75 column (50 × 2.5 cm). The 2-ml fractions containing the greatest enzymatic activity were pooled and were found to have a specific activity of 28 μM units/mg of protein. Sufficient solid ammonium sulfate was added to the pooled active fractions to produce 100% saturation, and the resulting suspension was stored at 4°. When required for study, aliquots of the suspension of guanylate kinase were centrifuged and the precipitates were dissolved in an appropriate amount of buffer.

Guanylate kinase assay. Guanylate kinase activity was measured at 30° by following the decrease in absorbance at 340 mμ as a function of time in a 1-ml reaction volume that contained 100 mM Tris-chloride buffer (pH 7.5), 100 mM KCl, 10 mM $MgCl_2$, 1.5 mM phosphoenolpyruvate, 0.15 mM NADH, 8 mM ATP, 2.5–7.5 μM units of pyruvate kinase, 3.3–9.9 μM units of lactate dehydrogenase, an appropriate amount of guanylate kinase, and 0.1 mM GMP. Guanylate kinase activity is defined in terms of micromolar units; the details of the assay have been described elsewhere (1). A Zeiss spectrophotometer, model M4QII, a Gilford absorbance converter, model 200, and a Barber-Coleman recorder, model 8000, were used to record changes of absorbance as a function of time. Protein concentrations were determined by the method of Warburg and Christian (5).

Preparation of guanylate kinase from Sarcoma 180 cells. Sarcoma 180 cells, harvested from mice 6 days after intraperitoneal tumor inoculation, were washed with isotonic sodium chloride. The packed cells were suspended in an equal volume of isotonic sodium chloride and stored at —20°. Between 200 and 300 ml of the frozen cell suspension were thawed and homogenized with an equal volume of distilled H_2O in a Waring Blendor. The homogenate was centrifuged at 13,000 × *g* for 20 min. The resulting supernatant fluid contained guanylate kinase activity within the range of 0.32–0.40 μM unit/ml with a specific activity of approximately 0.03 μM unit/mg of protein and a ratio of guanylate kinase to adenylate kinase activities ranging from 0.15 to 0.17. This extract was treated with solid ammonium sulfate, and the 40–60% ammonium sulfate precipitate (guanylate kinase; specific activity, 0.08–0.10; guanylate kinase to adenylate kinase ratio, 0.36–0.48) was dialyzed by continuous flow overnight against 15 liters of 0.01 M Tris-chloride (pH 7.5) containing 0.001 M EDTA. The dialyzed preparation was treated for 30 min with calcium phosphate gel (6) in a gel to protein ratio of 1:1 and then centrifuged to remove the gel. The supernatant fluids from several different preparations contained guanylate kinase activities that ranged in specific activities from 0.22 to 0.60, and guanylate kinase to adenylate kinase ratios of 0.6 to 6.8. Solid ammonium sulfate was added to this supernatant fluid to a final concentration of 100%. The resulting suspension was allowed to stand at 4° for at least 48 hr; then the precipitate was collected by centrifugation and dissolved in 4 ml or less of 0.01 M Tris-chloride (pH 7.5) containing 0.001 M EDTA and filtered through a Sephadex G-75 column (2.5 × 50 cm) with 0.01 M Tris-chloride (pH 7.5) containing 0.01 M EDTA. Two-milliliter fractions were collected, and the peak of guanylate kinase activity in fractions 44–48 was pooled and stored at —20°. The guanylate kinase specific activity of these pooled fractions ranged from 1.1 to 1.2, with a guanylate kinase to adenylate kinase ratio of 38 to 70. It should be noted that the behavior of guanylate kinase from Sarcoma 180 cells during the isolation pro-

cedure followed closely that of the brain enzyme, which has been studied extensively in this laboratory.

Enzymatic synthesis of 6-thioGMP. The chemical synthesis of 6-thioGMP has been described earlier (7), and the enzymatic synthesis of this nucleotide has been reported (8). The synthesis employed here is a modification of the latter method.

Three milliliters of packed, washed Sarcoma 180 cells, harvested from mice 6 days after intraperitoneal tumor inoculation, were ground in a mortar and pestle with 3 ml of 0.15 M KCl and 0.001 M EDTA. The disrupted cells were suspended in an additional 6 ml of 0.15 M KCl and 0.001 M EDTA, and the resulting suspension was centrifuged at 38,000 x *g* for 20 min. The supernatant fluid was dialyzed against three 1-liter changes of 0.15 M KCl and 0.001 M EDTA over a 3-hr period. The dialyzed preparation was adjusted to a volume of 20 ml and added to an equal volume of a reaction mixture composed of 200 mM Tris-chloride (pH 8.0), 20 mM $MgCl_2$, 1.0 mM 6-thioguanine, and 2 mM magnesium 5-phospho-α-D-ribosylpyrophosphate. The reaction mixture was allowed to stand for 12–18 hr at room temperature before being diluted 5-fold with water. The diluted reaction mixture was added to a DEAE-cellulose-bicarbonate column (2.5 × 15 cm), which then was washed with water until the absorbance at 260 mμ fell to a constant low level. A linear gradient of triethylammonium bicarbonate (0.05–0.25 M over a volume of 2 liters) was applied to the column, and 6-thioGMP was eluted between 0.11 and 0.13 M triethylammonium bicarbonate. The fractions containing 6-thioGMP, as detected by absorbance at 345 mμ, were pooled, taken to dryness by flash evaporation, and redissolved in water, and flash evaporation was repeated until the odor of triethylamine was no longer detectable. The material isolated in this manner had spectra at pH 4.7 and 8.1 similar to those reported for 6-thioguanosine (9) and was further characterized after being converted enzymatically to 6-thioGTP.

In a typical preparation 11 μmoles of the triethylammonium salt of 6-thioGMP were isolated. The concentrations of 6-thioGMP were determined by absorption at 345 mμ ($a_M = 24.9 \times 10^3 M^{-1}$ cm^{-1} at pH 4.7) (9). It should be noted that 6-thioGMP is relatively unstable even when stored frozen in solution at —20°. This was evidenced by the loss of capacity of 6-thioGMP preparations to inhibit guanylate kinase after a few weeks of storage. Therefore, the studies reported in this paper were carried out within a few days after the preparation of 6-thioGMP.

Enzymatic conversion of 6-thioGMP to 6-thioGTP. A reaction mixture (2 ml) containing 200 mM Tris-acetate (pH 7.5), 6.3 mM phosphoenolpyruvate, 1.6 mM ATP, 2.5 mM 6-thioGMP, 100 mM KCl, 10 mM $MgCl_2$, 0.8 mg of pyruvate kinase (specific activity, 254 μM units/mg), and 8.9 μM units of guanylate kinase (specific activity, 28 μM units/mg) from hog brain was incubated at 25° for 60 hr. The reaction mixture was diluted to 100 ml with water and added to a DEAE-cellulose-bicarbonate column (2.5 × 15 cm), and a linear gradient of triethylammonium bicarbonate (0.05–0.3 M over a volume of 2 liters) was applied. ATP was eluted from the column between 0.16 and 0.18 M gradient. A second ultraviolet-absorbing peak, eluted between 0.22 and 0.25 M gradient, replaced GTP in the succinate thiokinase reaction (10), and had spectra at pH 8.1 and pH 4.7 identical with those of 6-thioGMP. The yield of 6-thioGTP was approximately 90%.

RESULTS

Purification and substrate specificity. Guanylate kinase from Sarcoma 180 cells was purified to a specific activity of 1.2 μM units/mg of protein by a combination of techniques used to purify guanylate kinase from hog brain, such as 40–60% ammonium sulfate fractionation, absorption of contaminating proteins on calcium phosphate gel, and filtration through Sephadex G-75. The ratio of guanylate kinase to adenylate kinase activities tended to increase with storage at —20°, presumably because of the instability of the adenylate kinase activity.

The nucleoside 5′-monophosphate and nucleoside 5′-triphosphate substrate specificity of guanylate kinase from Sarcoma 180 cells (Table 1) is similar to that reported for the hog brain enzyme (1). Guanylate kinase from Sarcoma 180 cells is highly specific for purine nucleoside 5′-monophosphates that have an amino group on C-2 of the purine ring and a keto group on C-6. The slight reaction of other nucleoside 5′-monophosphates in the presence of a large excess of guanylate kinase from Sarcoma 180 is most likely due to contamination by traces of other enzymes in the partially purified preparations of guanylate kinase. These findings indicate that the nucleoside 5′-monophosphate-binding site on guanylate kinase from Sarcoma 180 cells is highly specific for the guanine moiety.

The small, but detectable, substrate activity of CTP, GTP, and UTP with Sarcoma 180 guanylate kinase may be due either to trace amounts of ATP in these nucleoside triphosphates or to contaminating enzymes in the partially purified enzyme. The determination whether guanylate kinase from Sarcoma 180 cells is absolutely specific for ATP must be deferred until measurements can be made with homogeneous preparations of guanylate kinase and with highly purified nucleotides. Only in the presence of a large excess of hog brain guanylate kinase (8.9 μM units; specific activity, 28 μM units/mg) could 6-thioGMP be shown to serve as a substrate under the standard assay conditions described above. Under these conditions and at a 6-thioGMP concentration of 0.1 mM, the reaction rate was slightly less than 0.04% of the rate measured with GMP as the substrate. The poor ability of guanylate kinase to catalyze the phosphorylation of 6-thioGMP is further indicated by the large amount of guanylate kinase and prolonged reaction time necessary to convert 6-thioGMP to 6-thioGTP in the presence of an excess of pyruvic kinase and phosphoenolpyruvate (see MATERIALS AND METHODS).

Kinetic studies. A comparison of the kinetic parameters (Table 2) for guanylate kinase preparations isolated from Sarcoma 180 cells and hog brain shows that they do not vary by more than 2–3-fold. These

TABLE 1

Substrate specificity of guanylate kinase from Sarcoma 180 cells

The reaction vessels contained 100 mM Tris-acetate buffer (pH 7.5), 100 mM KCl, 10 mM $MgCl_2$, 1.5 mM phosphoenolpyruvate, 0.15 mM NADH, 8 mM ATP, 2.5 μM units of pyruvate kinase, 3.3 μM units of lactate dehydrogenase, 0.06 μM unit of guanylate kinase (specific activity, 1.1 μM units/mg), and 0.1 mM AMP, IMP, XMP, CMP, or UMP in a 1-ml reaction volume at 30°. In the cases of GMP, dGMP, and 8-azaGMP, the amount of guanylate kinase was reduced to 0.006 μM unit. The reactions with nucleoside 5′-triphosphates were made with 1.4 mM ATP, CTP, GTP, or UTP in the presence of 0.1 mM GMP and 0.12 μM unit of guanylate kinase (GTP, CTP, and UTP) or 0.0048 μM unit of guanylate kinase in the case of ATP.

Nucleoside 5′-monophosphates	Percent of velocity with GMP	Nucleoside 5′-triphosphates	Percent of velocity with ATP
GMP	100	ATP	100
dGMP	48	CTP	7
8-AzaGMP	23	GTP	2
AMP	2.6	UTP	2
IMP	2		
XMP	2		
CMP	2		
UMP	2		

TABLE 2

Comparison of kinetic parameters for guanylate kinase isolated from hog brain and Sarcoma 180 cells

The Michaelis constants for the various nucleotides with 0.006 μM unit of Sarcoma 180 guanylate kinase (specific activity, 1.1 μM units/mg) were determined under the conditions described in the text. The Michaelis constants for the various nucleotides with hog brain guanylate kinase are summarized from a previously published report (1).

Nucleotide	Brain: K_m (K_i)	Sarcoma 180: K_m (K_i)
	M	M
ATP	1.2×10^{-4}	4×10^{-5}
GMP	2.0×10^{-5}	1×10^{-5}
dGMP	1.0×10^{-4}	3×10^{-5}
8-AzaGMP	1.6×10^{-4}	7×10^{-5}
6-ThioGMP	(5.3×10^{-5})	(6×10^{-5})

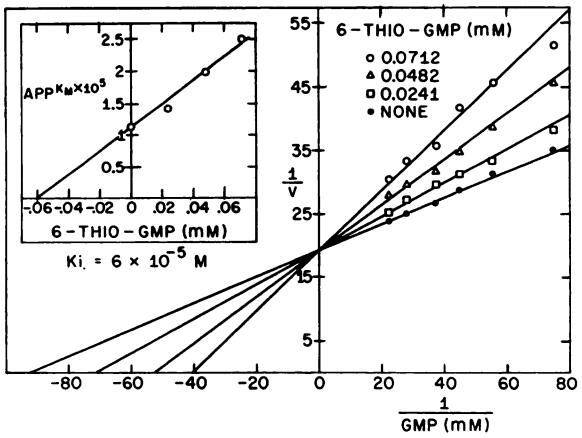

FIG. 1. *Competitive inhibition of guanylate kinase from Sarcoma 180 cells by 6-thioGMP with GMP as the variable substrate*

The reciprocal of the initial velocities ($1/A_{340}$ min^{-1}) is plotted against the reciprocal of the concentrations of GMP at four different concentrations of 6-thioGMP. Reaction conditions were identical with those described in the text, and the concentration of ATP was 8 mM. The guanylate kinase had a specific activity of 1.1 μM units/mg of protein. The inset is a plot of the apparent K_m values for GMP against the concentration of 6-thioGMP. The abscissa intercept gives an estimate of the K_i values of 6-thioGMP.

findings illustrate the similarity of these two enzymes.

In the presence of guanylate kinase from Sarcoma 180 cells, 6-thioGMP acts as a potent competitive inhibitor of GMP ($K_i = 6 \times 10^{-5}$ M, Fig. 1). This value is similar to that determined for guanylate kinase from hog brain (K_i for 6-thioGMP = 5.3×10^{-5} M), where the inhibition is also competitive with GMP (Fig. 2). Furthermore, the noncompetitive inhibition pattern (Fig. 3) obtained with 6-thioGMP and ATP as the variable substrate and GMP fixed at a nonsaturating concentration (5×10^{-5} M) is consistent with that predicted for a reversible inhibitor of a two-substrate,

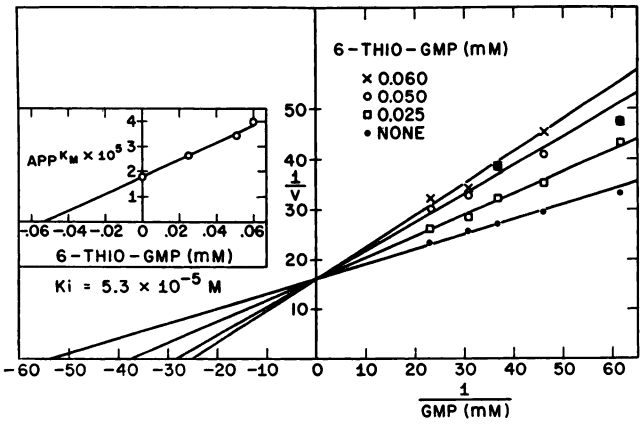

FIG. 2. *Competitive inhibition of guanylate kinase from hog brain by 6-thioGMP with GMP as the variable substrate*

The reciprocal of the initial velocities ($1/A_{340}$ min^{-1}) is plotted against the reciprocal of the concentrations of GMP at four different concentrations of 6-thioGMP. Reaction conditions were identical with those described in the text, and the concentration of ATP was 8 mM. The guanylate kinase had a specific activity of 28 μM units/mg of protein. The inset is a plot of the apparent K_m values for GMP against the concentration of 6-thioGMP. The abscissa intercept gives an estimate of the K_i value for 6-thioGMP.

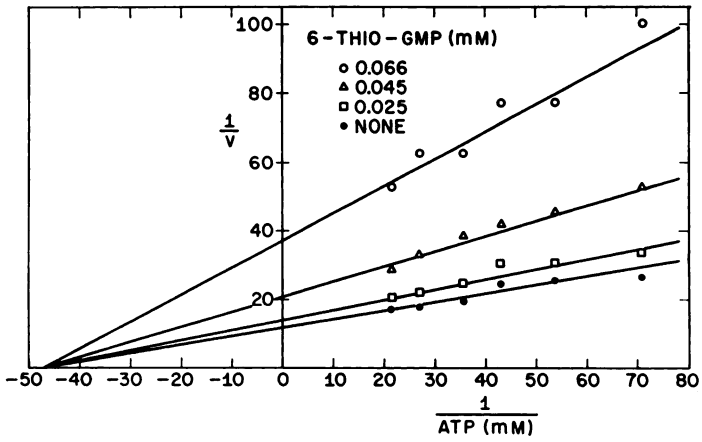

FIG. 3. *Noncompetitive inhibition of hog brain guanylate kinase by 6-thioGMP with ATP as the variable substrate*

The reciprocal of the initial velocities ($1/A_{340}$ min^{-1}) is plotted against the reciprocal of the concentration of ATP at four different concentrations of 6-thioGMP. Reaction conditions were identical with those described in the text, and the concentration of GMP was 0.05 mM. The guanylate kinase had a specific activity of 28 μM units/mg of protein.

two-product reaction (11). This finding is in accord with the concept that guanylate kinase has separate sites for the guanine and adenine moieties and that 6-thioGMP interacts with the guanine-specific site.

Prolonged incubation of brain guanylate kinase with 6-thioGMP (0.1 mM) did not result in the sort of progressive or irreversible inhibition seen with inosinate dehydrogenase (12).

Comparison of molecular weights. Almost identical peak elution volumes were obtained when guanylate kinase preparations (Fig. 4) from Sarcoma 180 cells and from hog brain were filtered individually through the same gel filtration column (exclusion limit, 20,000 mol wt). A single, relatively symmetrical peak of guanylate kinase activity was found when a mixture of guanylate kinase preparations from Sarcoma 180 cells and hog brain was filtered through the same gel filtration column. As will be reported in detail elsewhere, the estimated molecular weight of guanylate kinase from these tissues is about 19,000.

DISCUSSION

Guanylate kinase is of interest as a potential site of chemotherapeutic attack, since it appears to be the primary or perhaps the only mechanism in many cells for the conversion of GMP to GDP. In this report it is shown that 6-thioGMP is a good inhibitor of guanylate kinase, competitive with GMP ($K_i = 6 \times 10^{-5}$ M), by virtue of being an alternative substrate with a very low V_{max} (0.04% of the velocity attainable with GMP as the substrate). The inhibition of guanylate kinase by 6-thioGMP may play a key role in the cytolytic action of the purine analogue 6-thioguanine (4). It has been known for a number of years that 6-thioguanine is converted by guanylic pyrophosphorylase from tumor cells to 6-thioGMP (8). It has also been shown that 6-thioGMP accumulates to surprisingly high concentrations (as high as 0.1 mM) in susceptible tumor cells (13, 14). This concentration appears to be considerably greater than the normal tissue level of GMP, based on the reported value (0.03 mM) for rat brain (15). Because of the very low tissue levels, reliable measurements of the concentrations of GMP in other tissues are not available. The accumulation of 6-thioGMP may be explained by its continuing formation through the reaction of 6-thioguanine with guanylate pyrophosphorylase and its relatively slow removal due to its very poor

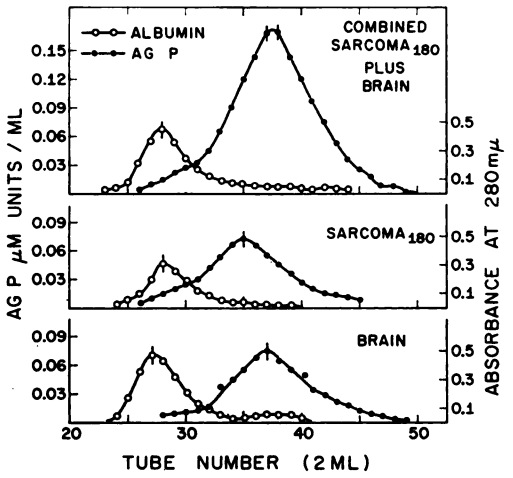

FIG. 4. *Comparison of the molecular sizes of guanylate kinase (AG P) from hog brain and Sarcoma 180 cells*

Guanylate kinase preparations from hog brain (1.7 μM units) and from Sarcoma 180 cells (1.7 μM units) were filtered individually and together through a column (40 × 2.5 cm) of polyacrylamide gel (Bio-Gel P-20; exclusion limit, 20,000 mol wt) with 0.01 M Tris-chloride (pH 7.5) containing 0.001 M EDTA. Bovine plasma albumin was added to guanylate kinase preparations to serve as a marker of the exclusion volume.

reactivity with guanylate kinase. The consequence should be an increased steady-state concentration of 6-thioGMP, resulting in a sequential blockade of guanine nucleotide biosynthesis (4).

It has been proposed that the marked accumulation of 6-thioGMP in tumor cells brings about a shutdown of the biosynthesis of GMP (4) through negative feedback inhibition of the enzyme phosphoribosylamine synthetase (16) and progressive, irreversible inhibition of inosinate dehydrogenase (12, 17). The postulated consequence of this coordinated series of enzymatic inhibitions, triggered by the inhibition and poor reactivity of 6-thioGMP with guanylate kinase, is a drastic decrease in the concentrations of GDP and GTP. Since these nucleotides are required for nucleic acid synthesis and function as coenzymes in a number of vital enzymatic reactions which are highly specific for guanine nucleotides, a marked fall in their concentration could result in cell death.

The incorporation of 6-thioguanine into the DNA of tumor cells has been observed (18), and it has been postulated that this incorporation plays an important role in the cytolytic action of the drug (19). Therefore a key question for future study is whether effects at the level of nucleotide metabolism or at the nucleic acid level are primarily responsible for the cytolytic action of 6-thioguanine. Another unsettled question of some importance is whether the slow reactivity of 6-thioGMP with guanylate kinase is sufficient to explain the incorporation of 6-thioguanine into DNA, or whether an as yet undetected isozyme or some other mechanism exists for the phosphorylation of 6-thioGMP.

It is of interest to consider the phenomenon of inhibition of guanylate kinase by 6-thioGMP. The alteration from the normal guanine moiety in this analogue nucleotide is the substitution of a sulfhydryl group for the hydroxyl group on position 6 of the purine ring. The reaction catalyzed by this enzyme is a transphosphorylation and presumably occurs at a significant distance from the purine ring. Therefore it might have been expected that structural modification of the purine substituent in the analogue would lead to effects on substrate binding, but that once the substrate was bound to the enzyme surface the chemical reaction would proceed unimpeded. Thus one might have expected to see an alteration in the Michaelis constant with little change in the maximal velocity. However, quite the reverse was observed. The K_i of 6-thioGMP is of the same order of magnitude as the K_m for GMP, suggesting that the two compounds have comparable affinities for the enzyme. On the other hand, the reaction velocity with 6-thioGMP is markedly lower than that with GMP, so much so that the analogue nucleotide behaves as a competitive inhibitor. It is possible that this phenomenon can be explained by the induced-fit hypothesis of Koshland (20). Presumably the increased bulk caused by the substitution of a sulfhydryl group for a hydroxyl group does not sig-

nificantly affect the ability of the nucleotide to bind with the active site but does interfere with the conformational change in the structure of the protein which is postulated to occur during the catalytic event.

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