of decamethonium in the medium, indicating that decamethonium is taken up by a concentrating mechanism that can be saturated. Table 1 further shows that the S/M concentration ratio of decamethonium decreases when nonlabelled hexamethonium chloride (May & Baker Ltd.) is added to the medium.

The results suggest that decamethonium is taken up by liver slices of mice by a process showing several of the characteristics of active transport. There is an uptake against a concentration gradient, the process is saturable, it is inhibited by low temperature and under anaerobic conditions. Further, hexamethonium acts as an inhibitor of decamethonium uptake, suggesting that the two methonium compounds share a common process involved in the transport.

The fate of decamethonium taken up in the liver of mice *in vivo* is not known. As mentioned we found no evidence of biotransformation in the liver within the first 20 min after i.v. injection. It is possible that decamethonium is either redistributed to plasma or excreted into bile. Biliary excretion does not represent a major pathway of elimination in rat, rabbit and cat[3–5]. In these species, however, the hepatic uptake of decamethonium is significantly lower than in the mouse.

Further studies of the hepatic uptake of decamethonium in various species are in progress.

Department of Pharmacology,
University of Copenhagen,
Denmark.

C. BROEN CHRISTENSEN

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An hypothesis on the mechanism of action of 6-thioguanine

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THE GUANINE analog, 6-thioguanine (TG), is one of the purine antimetabolites that possess potent growth-inhibitory activity against both transplanted tumors in animals and neoplasms in man. For this reason, the biochemical mode of action of this compound has been the subject of many investigations, and a considerable number of metabolic alterations have been ascribed to this agent; these findings have been adequately summarized in recent reviews.[1–6] Several hypotheses have been suggested to account for the carcinolytic potency of TG, and these include: (a) incorporation of the analog into the nucleic acids; (b) feedback inhibition of the *de novo* biosynthetic pathway for purine nucleotides; and (c) interference with the interconversion of purine nucleotides. Recent findings in our two laboratories now make it possible to propose a unifying concept. Inhibitions of the enzymes, phosphoribosylpyrophosphate (PRPP) amidotransferase, inosine 5′-phosphate dehydrogenase, and ATP:GMP phosphotransferase by 6-thioguanosine 5′-phosphate (6-thioGMP) have been shown. These demonstrated inhibitions are proposed to act in concert with competitive substrate interaction between guanine and TG with guanylic pyrophosphorylase as well as with product inhibition of this enzyme by 6-thioGMP to diminish the rate of biosynthesis of guanine nucleotides. Such metabolic blockade would be expected to limit markedly the availability of guanine nucleotides for both coenzyme function and nucleic acid synthesis, thereby resulting in inhibition of growth and death of neoplastic cells.

RESULTS

ATP:GMP phosphotransferase

In earlier reports from one of our laboratories,[7, 8] it was demonstrated that there occurs in brain tissue a highly specific enzyme which catalyzes the following reaction:

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

Among the natural nucleoside 5′-phosphates, only GMP and d-GMP were active as substrates, whereas IMP, AMP, XMP, CMP, UMP, and several other nucleotides were inactive as substrates and inhibitors of this enzyme (Table 1). Of the analogs of purine nucleotides tested, only 8-azaguanosine 5′-phosphate (8-azaGMP) was active as a substrate, whereas 6-mercaptopurine ribonucleoside 5′-phosphate (6-thioIMP) was inactive both as a substrate and an inhibitor. Of considerable interest was the finding that 6-thioGMP behaved as a competitive inhibitor of the enzyme.[9] The inhibition constant ($5–8 \times 10^{-5}$ M) was relatively low and was of the same order of magnitude as

TABLE 1. SUBSTRATE SPECIFICITY OF ATP:GMP PHOSPHOTRANSFERASE FROM HOG BRAIN*

Fixed substrate†	Variable substrate†	Brain	
		K_m	% V_{max}
ATP	GMP	$2·0 \times 10^{-5}$M	100
ATP	*d*-GMP	$1·0 \times 10^{-4}$M	~50
ATP	8-azaGMP	$1·6 \times 10^{-4}$M	~50
GMP	ATP	$1·0 \times 10^{-4}$M	100
GMP	*d*-ATP	$3·0 \times 10^{-4}$M	100
GMP	CTP		<5
GMP	UTP		<2
GMP	ITP		<1
GMP	GTP		0

* Data summarized from reference 8.

† Inactive as substrates or inhibitors: guanosine, 3′-GMP, CMP, UMP, XMP, IMP, AMP, and 6-thioIMP.

the K_m values for GMP (2×10^{-5} M) and d-GMP (1×10^{-4} M) (Table 2). 6-ThioGMP can also serve as a substrate for ATP:GMP phosphotransferase, but the reaction rate is less than 1/100 of the rate obtained with GMP. Thus, in experiments in which 6-thioGMP was incubated for 12 hr with

TABLE 2. COMPARISON OF KINETIC PARAMETERS FOR ATP:GMP PHOSPHOTRANSFERASE ISOLATED FROM HOG BRAIN AND SARCOMA 180 CELLS*

Nucleotide	Brain		Sarcoma 180	
	K_m	K_i	K_m	K_i
ATP	$1·2 \times 10^{-4}$M		4×10^{-5}M	
GMP	$2·0 \times 10^{-5}$M		1×10^{-5}M	
d-GMP	$1·0 \times 10^{-4}$M		3×10^{-5}M	
8-azaGMP	$1·6 \times 10^{-4}$M		7×10^{-5}M	
6-thioGMP		$5–8 \times 10^{-5}$M		5×10^{-5}M

* R. H. York, R. P. Miech and R. E. Parks, Jr., unpublished observations.

ATP:GMP phosphotransferase, ATP, phosphoenolpyruvate, pyruvic kinase, and the required metal ions, 6-thioGTP was formed in quantitative yields. The 6-thioGTP was isolated by ion-exchange chromatography on DEAE cellulose bicarbonate and was shown to serve as a substrate for heart muscle succinic thiokinase.[10] The inhibition by 6-thioGMP of ATP:GMP phosphotransferase is an

example of inhibition of an enzymatic reaction by an alternative substrate. The effect *in vivo* of an alternative substrate that has both a low K_m and a low V_{max} should be one of profound inhibition of that enzymatic activity. The incorporation of TG into DNA and RNA, reported by LePage and colleagues,[11–14] indicates, however, that 6-thioGDP and 6-thioGTP and their deoxyribonucleotide counterparts are indeed formed in intact neoplastic cells. It is possible that the conversion of 6-thioGMP to 6-thioGDP and 6-thioGTP may be catalyzed by as yet unidentified isozymes of ATP:GMP phosphotransferase or by some other unknown mechanism(s). Since 6-thioGMP does serve as a substrate, however, even at a very slow rate, it is possible that ATP:GMP phosphotransferase is principally responsible for the conversion of 6-thioGMP to higher nucleotide forms in tumor cells.

Recent studies from one of our laboratories[9] have shown that a similar enzyme is present in Sarcoma 180 cells. Thus, an ATP:GMP phosphotransferase was purified about 100-fold from the neoplastic cells.* The brain and tumor enzymes are notably similar in substrate specificity, the magnitude of various kinetic constants, and in their molecular size (mol. wt. $\simeq$ 19,000; Table 2).

Accumulation of 6-thioGMP in Sarcoma 180 ascites cells

It has been amply demonstrated that 6-thioGMP can be synthesized enzymatically by the interaction of TG with PRPP and GMP and IMP pyrophosphorylase.[2] Furthermore, Moore and LePage[15] have shown that ion-exchange chromatography of cold perchloric acid extracts of Ehrlich ascites cells isolated from animals treated with TG revealed a large peak of material absorbing at 345 mμ that was identified as 6-thioGMP. This major component was followed by several smaller fractions that were believed to be polyphosphate ribonucleotides of 6-thioguanosine. The injection of TG in doses of 0·25–4 mg/animal (about 8–130 mg/kg) leads to the formation of 6-thioGMP in concentrations of 0·05–0·3 μmole/ml packed tumor cells in 45 min.[15] Studies in one of our laboratories[16, 17] have shown that the i.p. injection of TG in low doses into mice bearing 6-day implants of Sarcoma 180 cells also results in the accumulation of 6-thioGMP. Thus, the injection of 2·5 mg of TG/kg resulted in a concentration of 6-thioGMP, at 1 hr after treatment, of 0·016 μmole/ml tumor cells. If it is assumed that the analog nucleotide distributes uniformly throughout the cell and that water comprises 75 per cent of the cell volume, the concentration of 6-thioGMP would be about 2×10^{-5} M. Similarly, treatment with TG in doses of 5 and 10 mg/kg yielded 6-thioGMP in concentrations of 9×10^{-5} M and 1×10^{-4} M, respectively.

Furthermore, in this neoplasm it was not possible to demonstrate the occurrence of more than trace amounts of either the di- or triphosphates of 6-thioguanosine. Hence, the finding of relatively high concentrations of 6-thioGMP and negligible amounts of 6-thioGDP and 6-thioGTP is consistent with an extremely slow rate of conversion of 6-thioGMP to 6-thioGDP *in vivo*.

Inhibition of inosine 5′-phosphate dehydrogenase (IMP dehydrogenase) from Sarcoma 180 cells by 6-thioGMP

IMP dehydrogenase isolated from *Aerobacter aerogenes* has been shown by Hampton[18] to be inhibited by 6-thioGMP; inhibition was partially reversed by addition of reduced glutathione (GSH) to the reaction mixture. It was proposed that 6-thioGMP formed a disulfide bond with the enzyme and that the GSH reduced the inhibition by restoration of free sulfhydryl groups on the enzyme.

Work in one of our laboratories[19] with IMP dehydrogenase isolated from Sarcoma 180 ascites cells gave results similar to those reported for the *A. aerogenes* enzyme. Concentrations of about 4×10^{-5} M of 6-thioGMP produced 50 per cent inhibition of the activity of this enzyme after 5–10 min of preincubation in the absence of substrates (Table 3). The following facts suggest that a covalent bond was formed between 6-thioGMP and IMP dehydrogenase of Sarcoma 180: (a) the inhibition by 6-thioGMP was progressive with time; (b) the inhibition was not reversed by dialysis; (c) GSH produced a partial reversal of the inhibition; and (d) the inhibition could be completely reversed by treatment with dithiothreitol.

DISCUSSION

There is general agreement that for TG to exert its antitumor action it must first be converted to a 5′-nucleotide.[1–6] Transplanted neoplasms carry out this conversion actively, and in the experiments of Moore and LePage[15] 6-thioGMP accumulated to much higher levels in tumor cells than in

* R. H. York, R. P. Miech and R. E. Parks, Jr., unpublished observations.

normal tissues. Competition between guanine and TG for guanylic pyrophosphorylase,[20] as well as probable product inhibition of this enzyme by GMP and 6-thioGMP, offers explanation for the decreased incorporation of guanine into nucleic acids of tumors in the presence of TG.[21]

A number of enzymes are inhibited by 6-thioGMP. The initial enzyme in the pathway of synthesis of purine nucleotides *de novo*, phosphoribosylpyrophosphate amidotransferase, has been shown by

TABLE 3. INHIBITION OF INOSINE 5′-PHOSPHATE DEHYDROGENASE OF SARCOMA 180 ASCITES CELLS BY 6-thioGMP*

Time of preincubation with 6-thioGMP (min)	Per cent inhibition
0	27
2·5	37
5	48
10	58

* The potassium salt of 6-thioGMP ($4{\cdot}0 \times 10^{-5}$M) was preincubated with the enzyme preparation, 0·12 M Tris Cl, pH 8, and 0·12 M KCl in a volume of 0·8 ml at 37°. After the indicated length of time, 0·2 ml of a mixture of substrates was added to bring the final concentration of IMP to $2{\cdot}3 \times 10^{-3}$M, NAD to $2{\cdot}8 \times 10^{-4}$M, and 6-thioGMP to $3{\cdot}2 \times 10^{-5}$ M, and the residual enzymatic activity was measured during the subsequent 15 min by determining the rate of change in absorbancy at 320 mμ that was the result of the production of NADH. The results are expressed relative to an untreated enzyme control that was preincubated in the absence of 6-thioGMP. IMP dehydrogenase was partially purified from Sarcoma 180 ascites cells by centrifugation of sonicates at 104,000 *g*, followed by ammonium sulfate fractionation of the supernatant; the precipitate that formed between 20 and 40 per cent saturation with $(NH_4)_2SO_4$ contained most of the enzymatic activity.

Wyngaarden and Ashton[22] to be notably sensitive to allosteric regulation by the purine nucleotides present in the cell. The enzyme is inhibited by AMP, GMP, IMP, and, in addition, by a number of mononucleotide analogs. McCollister *et al.*[23] demonstrated that 6-thioGMP has a K_i of 2×10^{-4} M for this enzyme; furthermore, cooperative inhibition of this enzyme by AMP and 6-thioGMP might well be expected to occur in a manner analogous to that produced by mixtures of GMP and AMP, thereby reducing the concentration of each nucleotide required for effective blockade of enzymatic activity. The accumulation of 6-thioGMP that occurs in neoplastic cells caused a significant inhibition of this enzyme *in vivo*; this phenomenon has been monitored by measurement of the TG-induced inhibition of both the accumulation of FGAR in azaserine (*O*-diazoacetyl-L-serine)-treated tumors, and the incorporation of glycine-^{14}C into the purine bases of the nucleic acids.[21] That the inhibition by 6-thioGMP of IMP dehydrogenase from Sarcoma 180 cells is intense is shown by the finding that inhibition of this enzyme is progressive and increases with time (Table 3). Thus, one might expect that the intracellular accumulation of 6-thioGMP would cause inhibition of both phosphoribosylpyrophosphate amidotransferase and IMP dehydrogenase, two enzymes in the pathway for synthesis of guanine nucleotides *de novo*, as well as blockade of ATP:GMP phosphotransferase. Such multistep inhibition should result in a profound lowering of the intracellular concentration of not only GMP, but also of GDP, GTP, d-GDP and d-GTP. Since GTP is a specific coenzyme in a number of vital metabolic reactions, depletion of this nucleotide would result in a marked depression

in cellular metabolism that presumably would lead to cell death. Fig. 1 summarizes the sites of inhibition by 6-thioGMP and the biochemical systems affected by such blockade. The multisite inhibition of guanine nucleotide formation induced by 6-thioGMP is an example of sequential blockade as described by Potter.[24]

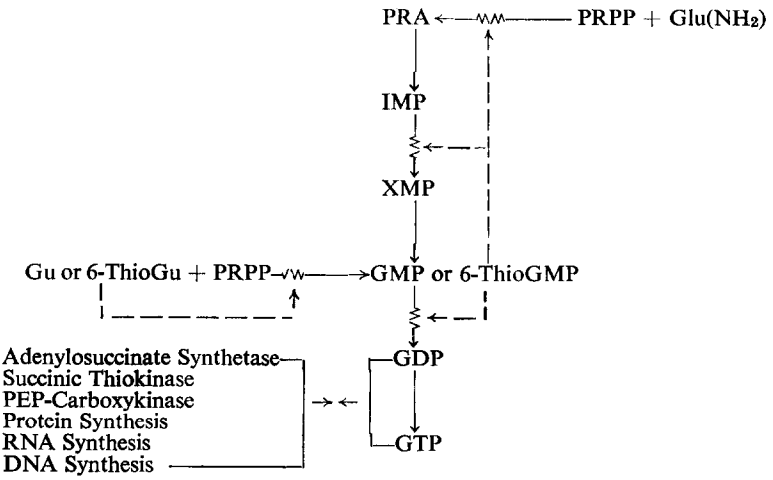

FIG 1. Sites of inhibition of 6-thioGMP and the biochemical systems affected.

If inhibition of ATP:GMP phosphotransferase activity does indeed exist *in vivo*, as is expected by the concentrations of 6-thioGMP that accumulate within the cell, one would expect to find initially a rise in the GMP pool size with a concomitant decrease in the combined pools of GDP and GTP, followed by a progressive lowering of the GMP concentration as the consequences of the inhibition of IMP dehydrogenase and purine synthesis *de novo* become manifest. Measurement of changes in guanine nucleotide levels in cells is required to test this hypothesis; analytical procedures to accomplish this have recently become available.[25] Therefore, it should soon be possible to measure accurately the guanine nucleotide pool sizes in tumor cells exposed to TG.

In studies with ^{14}C-labeled TG, LePage[12] has shown the incorporation of small amounts of 6-thioGMP into the DNA and RNA of tumors and stressed an apparent correlation between the susceptibility of the tumors to the growth inhibitory activity of TG and the degree of incorporation of the analog into DNA. That inhibition of the growth of neoplastic cells is not directly related to the accumulation of 6-thioGMP was shown by the findings of LePage *et al.*[26] with the Mecca lymphosarcoma; these neoplastic cells, which are completely resistant to the growth-inhibitory properties of TG but sensitive to 2′-deoxythioguanosine, contained relatively large amuonts of 6-thioGMP after exposure to TG; little TG, however, was incorporated into the DNA, while 2′-deoxythioguanosine was well incorporated into these macromolecules. These studies have resulted in the alternative suggestion that the growth-inhibitory effects of TG are the result of such incorporation. A possible decrease in the sensitivity of one or more of the various enzymes depicted in Fig. 1 to 6-thioGMP, however, could also account for the resistance to TG of neoplastic cell lines that accumulate 6-thioGMP.

The related compound, 6-mercaptopurine (6-MP), is converted *in vivo* to 6-thioIMP; this analog nucleotide attains intracellular concentrations that are inhibitory to both PRPP amidotransferase and IMP dehydrogenase.[16] As indicated in Table 1, 6-thioIMP did not inhibit the activity of hog brain ATP:GMP phosphotransferase; the sensitivity of the tumor enzyme to this antimetabolite remains to be tested. That 6-MP is converted to 6-thioGMP is shown by the finding of 6-thioGMP in the DNA of 6-MP-treated neoplastic cells.[27] It is doubtful, however, that concentrations of 6-thioGMP are achieved in cells exposed to 6-MP that are inhibitory to ATP:GMP phosphotransferase.

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Division of Biological and Medical Sciences, Brown University, Providence, R.I., U.S.A.
R. P MIECH
R. E. PARKS, JR.

Department of Pharmacology, Yale University School of Medicine, New Haven, Conn., U.S.A.
J. H. ANDERSON, JR.
A. C. SARTORELLI

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The central pharmacological effect of chlorpromazine in rats with alloxan-diabetes

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IN PREVIOUS investigations the authors found that administration of insulin increased the velocity of penetration across cell membranes of many drugs, as well as their tissue level and potency.[1–7] The