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THE ACTIONS OF THIOGUANINE IN *BACILLUS CEREUS**

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Abstract—The effects of the guanine analog, 6-thioguanine, have been evaluated in exponentially growing cultures of *Bacillus cereus*. The drug produced growth inhibition instantly, the duration but not the degree of which was related to the inhibitor concentration. Guanine prevented completely thioguanine-induced growth inhibition, in a competitive manner, whereas adenine and hypoxanthine were incapable of abolishing the inhibitory actions.

Although thioguanine was anabolized into the ribomononucleotide, the quantity of drug incorporated into nucleic acids was extremely small. It is unlikely that this incorporation was responsible for growth inhibition. The drug was readily desulfurated and the sulfur portion incorporated into proteins.

Thioguanine usually depressed the formation of protein and DNA in accordance with the decreased formation of cell mass, but RNA synthesis was decreased by a greater extent. Uracil incorporation into RNA pyrimidines was more specifically depressed than was RNA synthesis, whereas the conversion of orotic acid into RNA pyrimidines was enhanced during growth with thioguanine. The incorporation of amino acids into proteins was not specifically affected by drug treatment, but considerable fluctuations in replicate experiments were observed. The induction of penicillinase was unaltered by the analog. Bacterial flagella formation, on the other hand, was largely abolished, and the conversion of diaminopimelic acid into cell wall was decreased.

In comparison experiments with the structurally closely related drug 6-mercaptopurine, that analog produced very similar biochemical actions as did thioguanine on nucleic acid and protein biosynthesis. The two drugs differed markedly, however, in the ability of purines to antagonize their growth-inhibitory properties. Other dissimilarities between the drugs included a lack of inhibitory effect by mercaptopurine (but not thioguanine) on guanine incorporation and a lesser enhancement of orotic acid incorporation into RNA pyrimidines in the presence of mercaptopurine.

It is postulated that thioguanine and mercaptopurine produce a selectively damaging effect on the nucleic acids of *B. cereus*, perhaps on DNA, which leads to reduction of RNA synthesis and impairment of the formation of specific proteins.

6-THIOGUANINE, a structural analog of guanine, has been shown to be a useful agent in experimental leukemia therapy. Structurally, the compound is the 2-amino derivative of 6-mercaptopurine, an anticancer agent that has been found extremely valuable clinically. Although both drugs have been investigated extensively, the mechanisms by which the analogs act to inhibit growth have not been explained satisfactorily. It is most likely that the drugs interfere with nucleic acid synthesis, but the exact site or sites responsible for growth inhibition have been subject to controversy.[1–3] Recently it has been observed that both drugs prevent immune responses

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in mammals and inhibit antibody synthesis, indicating that some drug response on protein synthesis was also apparent.[4]

Certain actions of mercaptopurine have been investigated previously in bacteria.[5–8] It was thought desirable, therefore, to examine systematically the effects of thioguanine on growth and biosynthesis of a microbial system and to compare the two purine analogs.

Since LePage and colleagues[9–13] have been able to demonstrate a correlation between growth inhibition and incorporation of thioguanine into nucleic acids of tumor cells, this aspect was also examined in the present investigation. *Bacillus cereus* was chosen as the test system because this microorganism presents certain advantages in analysis.[14] Studies with *B. cereus* have also been extremely helpful in elucidating the inhibition of protein biosynthesis produced by another purine analog,[15–17] 8-azaguanine, which was incorporated extensively into nucleic acids.[18–20]

Several effects of thioguanine were uncovered during cell growth. The drug inhibited steps in cell wall formation and prevented normal flagellation of bacteria. Although the rates of formation of DNA and cellular protein were slowed, usually in relation to the slowing of turbidimetric increase of the cell cultures, RNA synthesis was retarded more specifically. A selective inhibition of the formation of specific proteins was postulated. Incorporation of thioguanine into nucleic acids was of such a low magnitude as to practically eliminate this potential explanation of the mechanism of growth inhibition.

Most of the actions of thioguanine were duplicated at least qualitatively by mercaptopurine, but some differences were noted. Whereas growth inhibition resulting from mercaptopurine could be completely overcome by hypoxanthine but not guanine, the growth-inhibitory effects of thioguanine were prevented by guanine and not hypoxanthine.

A recent report on some aspects of this work has been presented.[21]

MATERIALS AND METHODS

Bacterial growth and fractionation

Bacillus cereus 569 H cultures were grown as described previously.[19, 20] Growth was measured turbidimetrically in a Beckman spectrophotometer model DU at 540 mμ. Drugs and radioisotopes were added early during the logarithmic phase of growth, and measurements were made until turbidity had at least doubled. Cultures were sampled by removing 2-ml aliquots, measuring turbidity, and mixing with 2 ml saline, 10% trichloroacetic acid, or 1 N KOH prior to filtration on Schleicher and Schuell coarse membranes. For cells grown in the presence of radioisotopes, radioassay after filtration of saline-washed cells measured uptake into whole cells, whereas counting of cold-acid-washed cells measured radioactivity in all fractions of the cells except the acid-soluble pool. Correspondingly, filtration of cells washed in hot acid, a process that decomposes and solubilizes nucleic acids, led to recovery on the filter of protein and cell wall. The KOH treatment overnight at room temperature, which leads to loss of the RNA from cells, allowed for the measurement of DNA, protein, and cell wall on the filter.[14] By employing isotopic precursors specific for each cell fraction, utilization of compounds for biosynthesis was measured directly.

To isolate and identify radioactive compounds in cell fractions, 25-ml samples of cells were harvested and washed in 80% aqueous ethanol at 85° for 5 min to remove

the soluble pool and lipid fraction. This fraction was subjected to paper chromatography and paper electrophoresis for analysis of radioactive components, as described elsewhere.[20] The alcohol-insoluble residue was incubated with 1 N KOH overnight to hydrolyze RNA into mononucleotides. After perchloric acid neutralization at 0°, mononucleotides were separated by paper electrophoresis at pH 3·5 and molar activities calculated after counting radioactivity, elution in 0·1 N HCl, and measurement of u.v. absorption. The potassium perchlorate residue was treated to separate DNA bases as reported previously.[20]

Alternatively, in experiments measuring the metabolic fate of sulfur-labeled thioguanine, the alcohol-insoluble residue was extracted with 5% trichloroacetic acid at 100°. The residual protein fraction was hydrolyzed in 6 N HCl in a pressure cooker at 110° for 3 hr and the liberated amino acids separated by paper chromatography in isopropanol : HCl.[15, 22] A known amount of carrier thioguanine was added to the trichloroacetic acid extract containing nucleic acid degradation products and possibly drug anabolites. This mixture was then hydrolyzed in N HCl for 1 hr to free purine bases. After evaporation *in vacuo* the mixture was chromatographed first in isopropanol : water (7 : 3 v/v), followed by elution of the thioguanine area and rechromatography in isopropanol : 0·166 N HCl (2 : 3 v/v), and the molar activity of recovered thioguanine determined. The quantity of thioguanine present in the cell fraction was then calculated by isotope dilution of the labeled thioguanine used as the bacterial inhibitor. Spectrophotometric measurements with the characteristic absorption of thioguanine at 340 mμ were less sensitive than the experiments with thioguanine-^{35}S.

Bacterial content of nucleic acids was measured by extracting cold-acid-washed cells with hot perchloric acid or trichloroacetic acid, and analysis of the extract by colorimetry.[23–25] Incorporation of radiophosphorus served as a rough measure of total nucleic acids or DNA when cells were extracted with trichloroacetic acid or KOH respectively.[14]

Separation of RNA components

The procedure of Mandell and Hershey[26] was used to elute fractions from phenol-extracted cell-free preparations. Cells were disintegrated in a Mickle vibrator, extracted with phenol, and the aqueous phase containing the RNA extracted with ether to remove phenol. Nucleic acids were precipitated with 2·5 volumes of ethanol and the residue dialyzed before fractionation on a methylated albumin column.*

Estimation of penicillinase

For these experiments the inducible strain of *B. cereus* 569 was purchased from the American Type Culture Collection. Penicillinase was induced in these organisms during growth by the introduction of 3 μg penicillin-G/ml medium. The normal growth medium was supplemented with 5 mg citric acid and 5 mg gelatin/ml medium, at pH 7, to minimize destruction and adsorption of the enzyme.[27] The method of Citri[28] provided the most reproducible analysis for penicillinase present in the supernatant solution from cell suspensions.

* The author is indebted to Dr. F. E. Hahn, Dept. of Molecular Biology, Walter Reed Army Institute for Research, who supplied the procedure and facilities for carrying out this fractionation.

Measurements of bacterial dry weight

Five-ml cell samples from bacterial suspensions at selected turbidities were filtered through tared, washed membrane filters. The filters were then dried in a desiccator to constant weight and the dry weight of cells calculated. Reproducible values were easily obtained for control and drug-treated cells, and dry weight values were found to be identical for either culture at similar turbidimetric readings.

At OD_{540} of 0·4, 1 ml of cell suspension represented approximately 0·4 mg dry weight of cells. Microscopic examination revealed no major alteration in cell size caused by the drug treatment.

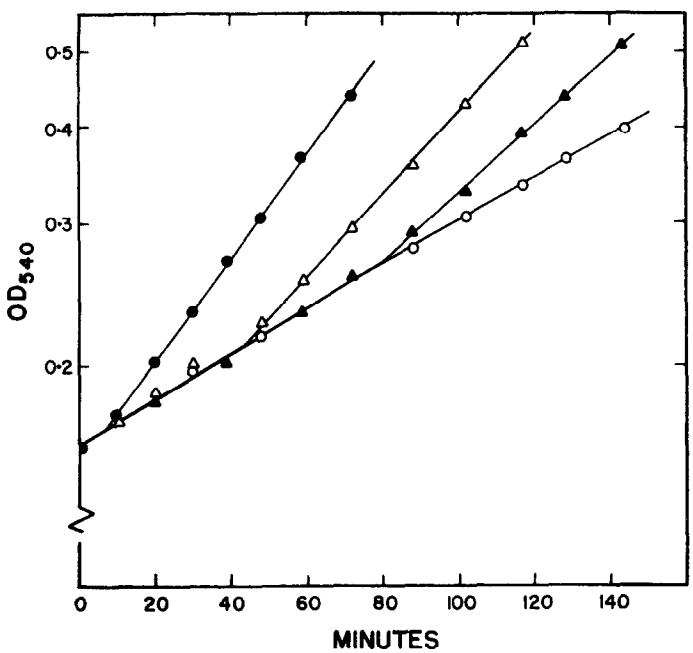

FIG. 1. Effect of 6-thioguanine on growth of *B. cereus*. Growth measured turbidimetrically at 540 mμ. Concentration of drug: ●, 0; △, 6 μM; ▲,12 μM; O, 30 μM.

Materials

Uracil-2-^{14}C, guanine-8-^{14}C, adenine-8-^{14}C, orotic acid-2-^{14}C, DL-valine-1-^{14}C, DL-leucine-1-^{14}C, acetate-1-^{14}C, and thioguanine-^{35}S from Isotopes Specialities Co., Burbank, Calif.; diaminopimelic acid-2,6-^{14}C from California Biochemicals, Inc., Los Angeles Calif.; DL-aspartic acid-4-^{14}C and DL-lysine-2-^{14}C from Tracerlab, Inc., Waltham, Mass.; acetate-2-^{14}C and glycine-2-^{14}C from Volk Laboratories, Skokie, Ill.; L-methionine-^{35}S and L-cystine-^{35}S from Schwartz Laboratories, Mount Vernon, N.Y.; and phosphate-^{32}P from Oak Ridge National Laboratories, Tenn. 6-Thioguanine, prepared by Francis Earle Laboratories, Peekskill, N.Y. (lot 516 M) was kindly furnished by the Cancer Chemotherapy National Service Center and was dissolved in 0·05 N HCl. Other chemicals were from commercial sources.

RESULTS

Effect on growth

The addition to exponentially growing cultures of *B. cereus* of concentrations of thioguanine greater than 6 μM resulted in an instantaneous retardation of growth. Increasing the concentration of the inhibitor had no further influence on the rate of growth but extended the period of growth inhibition before recovery took place. After the termination of inhibition, growth proceeded at almost the initial rate (Fig. 1). A concentration of 30 μM (5 μg/ml) provided a reproducible diminution in the growth rate for several hours and was used routinely in these experiments.

The diminution of the growth rate produced by thioguanine was considerably greater than that resulting from the introduction of mercaptopurine at equimolar concentration, as shown in Fig. 2. The combination of both drugs caused no additional

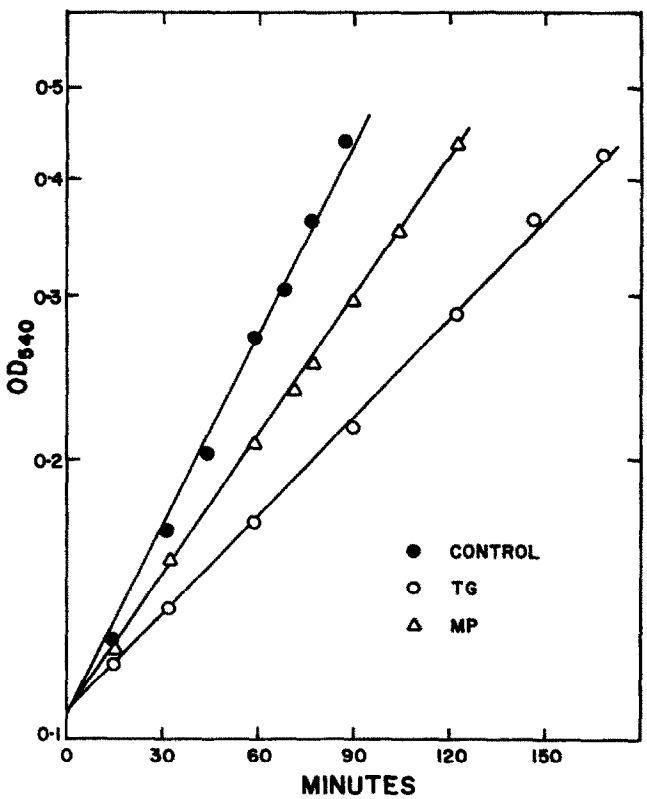

FIG. 2. Characteristic growth inhibition produced by 30 μM thioguanine or mercaptopurine. Higher concentrations did not alter the growth rate.

inhibition of growth over that shown by thioguanine alone. It has been reported previously[5, 7] that mercaptopurine also showed the characteristic lack of relationship between drug concentration and growth rate.

Effect of purines on preventing the drug-induced growth inhibition. The addition of guanine and thioguanine simultaneously altered the resulting rate of growth from

that of thioguanine alone and allowed intermediary growth rates, dependent on the relative concentrations of the two compounds. At molar concentrations ratios of guanine : thioguanine greater than 2, the growth-inhibitory action of the analog was completely prevented. The relationship was competitive, as demonstrated in Fig. 3 as

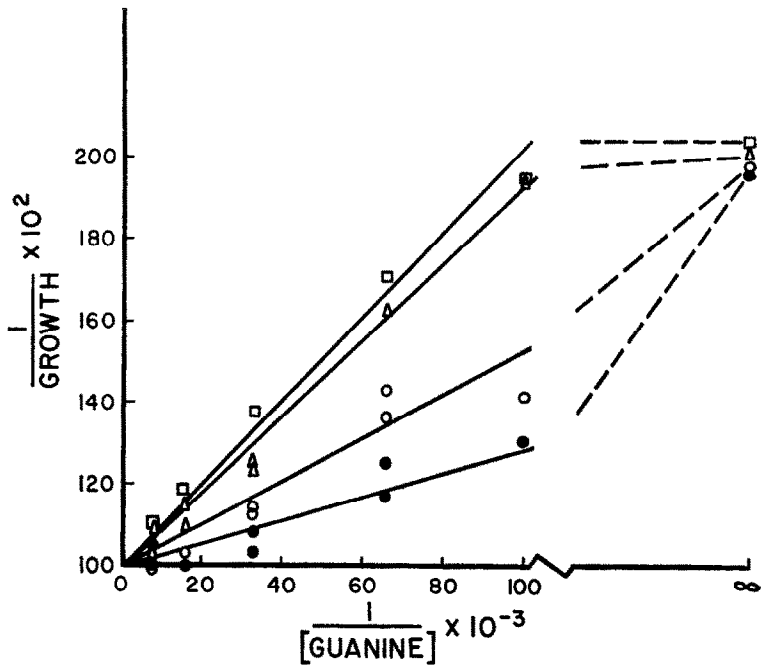

FIG. 3. Reciprocal plot indicating competitive reversal by guanine of growth inhibition produced by thioguanine. Concentrations of thioguanine: ●, 15 μM; O, 30 μM; △, 60μ M; □, 90 μM. Growth calculated from ratio of generation times in absence and presence of thioguanine. At these concentrations guanine had no effect on control growth rate.

a reciprocal plot[29] used previously for this purpose.[8] It is clear that thioguanine behaves as an analog of guanine in the *B. cereus* system.

Low concentrations of adenine or hypoxanthine partially prevented the inhibitory effect of thioguanine when added simultaneously, but higher purine concentrations were ineffective in completely preventing the growth inhibition produced by thioguanine (Fig. 4).

Effects on biosynthesis

In these experiments, comparisons have been made between drug-treated and control cells grown to similar turbidities in order to bring out more clearly drug-induced effects that differ from the overall effect of the drug on growth. Since growth was always inhibited by these drugs, a value of 100% would nevertheless represent a slower *rate* of biosynthesis compared to that of control cells, while values greater than 100% represent rates more rapid than that of turbidimetric increase and approaching those of untreated cells (approximately 200%). Values less than 100% therefore pinpoint specific inhibitory actions of the drug exceeding that on growth.

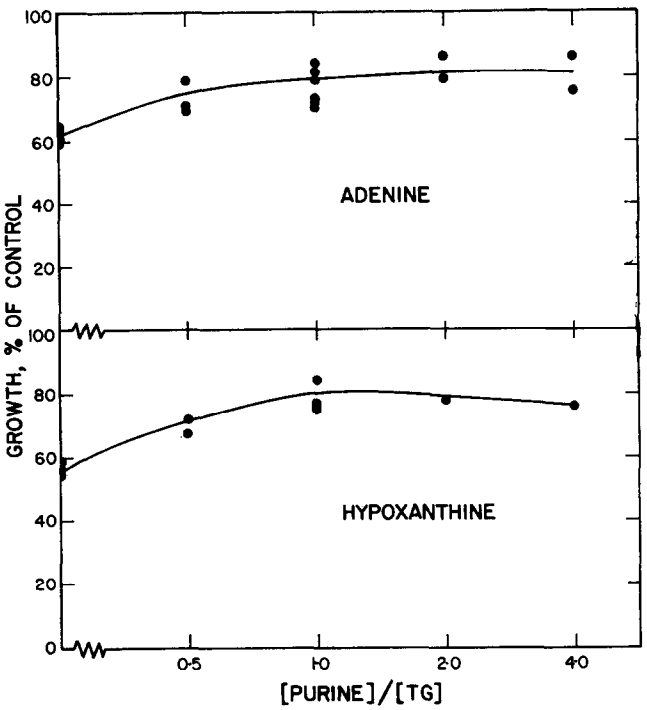

FIG. 4. Incomplete antagonism of thioguanine-induced growth inhibition by adenine (top) and hypoxanthine (bottom), at various ratios of concentration of purine to analog. In the absence of purines thioguanine allowed 65% of the control growth rate.

TABLE 1. EFFECT OF THIOGUANINE AND MERCAPTOPURINE ON NUCLEIC ACID CONTENT OF *Bacillus cereus* CULTURES

Cultures grown to similar turbidities in presence and absence of 30 μM drug, and contents of RNA and DNA measured as indicated. Approximately 15% and 1·5% of total drug weight of control cultures were RNA and DNA respectively. The estimated value for nucleic acid content at the beginning of the experiment (i.e. addition of drug) was subtracted from the total content at the time of harvest, so that the present values represent synthesis either in presence or absence of drug exclusively.

Experiment	Thioguanine		Mercaptopurine	
	RNA	DNA	RNA	DNA
	(% of control)			
1	52*	145†	48*	106†
2	49*	82†	66†	100†
3	65‡	76‡	70‡	77‡
4	71‡	98‡		
5	51‡	77§		
6	82‡			
7	95‡			
8	67†			
Mean (±S.E.)	67 ± 5·7	96 ± 13·0	61 ± 6·8	94 ± 8·8

* By orcinol method for RNA,[23] with hot trichloroacetic acid extract of cells.

† By indole method for DNA,[24] with hot trichloroacetic acid extract of cells.

‡ By ^{32}P incorporation measured by membrane filtration for RNA and DNA[14] periodically during incubation.

§ By diphenylamine method for DNA,[25] with hot perchloric acid extract of cells.

Nucleic acid content. Results in Table 1 indicate that, at similar increases in bacterial turbidity, the RNA content of thioguanine-treated cells usually was appreciably lower than that of control cells. Considerable variations in the extent of this diminution of content were observed in different experiments, however, and occasionally the effect was insignificant, even though growth inhibition was observed. Although there

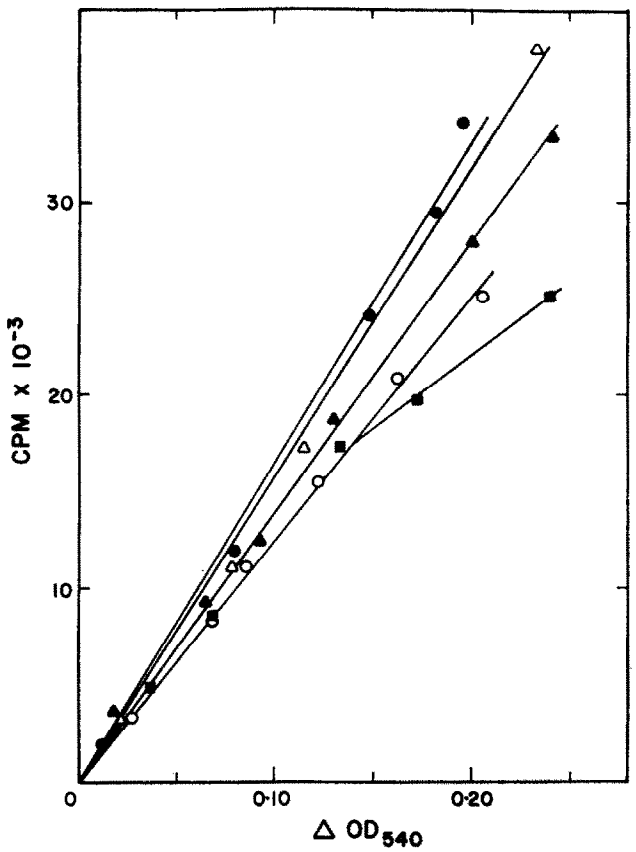

FIG. 5. Effect of thioguanine on incorporation of radiophosphate into bacterial cells. Almost all of radioactivity recovered in nucleic acid fraction. Cultures are compared at corresponding changes in turbidity. Incorporation is represented as total counts per minute in acid-washed cells present in 1 ml bacterial suspension. Concentration of thioguanine: ●, 0; △, 6 μ M; ▲, 12 μ M; O, 30 μM; ■ 120 μM.

also was considerable variation in the bacterial content of DNA resulting from thioguanine treatment, these effects were relatively minor compared to those of RNA. The effects of mercaptopurine resembled closely those of thioguanine. The variability in RNA content produced by mercaptopurine has been observed previously.[8]

Phosphate incorporation. Radiophosphate has served as a useful guide to nucleic acid synthesis, since almost all of it can be recovered from acid-washed cells in the form of ribonucleotides, after alkali hydrolysis. Figure 5 shows that with increasing concentrations of thioguanine the incorporation of ^{32}phosphate diminished, in accordance with the described decrease in nucleic acid content. The molar radioactivities

of the four major mononucleotides isolated from RNA were almost identical in any one culture, indicating that the drug had not produced a specific inhibitory effect on the biosynthesis of any particular nucleotide such as guanylic acid (Table 2). In experiment 1, all the molar activities were sharply depressed by thioguanine treatment, whereas in experiment 2 this effect was almost negligible. The variability in drug response on nucleic acid content accounts for this difference between the two experiments.

TABLE 2. RELATIVE MOLAR ACTIVITIES OF RNA MONONUCLEOTIDES FROM *BACILLUS CEREUS**

Precursor	Expt.	Thio-guanine	Mercapto-purine	Relative molar activities of 2′- and 3′-nucleotides				Molar activities (cpm/mμmole)
				AMP	GMP	UMP	CMP	
^{32}Phosphate	1	−	−	99	102	99	101	34·2
		+	−	69	68	68	72	
	2	−	−	100	94	97	107	3·7
		+	−	93	86	90	87	
Glycine-^{14}C		−	−	100	(100)			13·6
		+	−	56·3	5·5			
		−	+	61·9	52·4			
Guanine-^{14}C	1	−	−	45·6	(100)			144
		+	−	42·0	68·4			
		−	+	19·2	91·0			
	2	−	−	23·5	(100)			152
		+	−	23·5	62·2			
		−	+	11·1	103·5			
Uracil-^{14}C		−	−			(100)	90	11·2
		+	−			46	45	
Orotate-^{14}C	1	−	−			(100)	88	3·4
		+	−			141	138	
	2	−	−			(100)	116	1·9
		+	−			195	190	

* Grown with labeled precursors in presence and absence of drugs for similar turbidimetric increases. Values in parentheses arbitrarily set at 100, for which molar activities are indicated.

Glycine, adenine, and guanine incorporation. The molar activities of the RNA purine mononucleotides formed during growth in the presence of glycine-^{14}C and the drugs are shown in Table 2. The radioactivity of RNA adenine was depressed by thioguanine treatment compared to control values, undoubtedly because of the customary decrease in RNA content; the value for the corresponding guanine fraction, however, was diminished to a far greater extent. While the utilization of adenine-^{14}C by bacteria was not materially affected by thioguanine, the drug was capable of impairing severely the incorporation of guanine-^{14}C into nucleic acids (Fig. 6). The molar activities of the isolated purine mononucleotides show that the conversion of added guanine to RNA guanine was decreased specifically (Table 2). At a constant concentration of thioguanine, lowering the concentration of exogenous guanine-^{14}C enhanced the drug effect (Fig. 6).

The decreased formation of RNA guanine from both the endogenous precursor glycine-^{14}C and the exogenous labeled guanine, as well as the direct desulfuration of thioguanine to guanine or a guanine-like product as described below, suggests that thioguanine diluted out the various pathways of RNA guanine formation by supplying a catabolite that was more readily incorporated into this product. It is most likely that the desulfuration of thioguanine released a purine intracellularly which then competed favorably with extracellular guanine for biosynthesis.

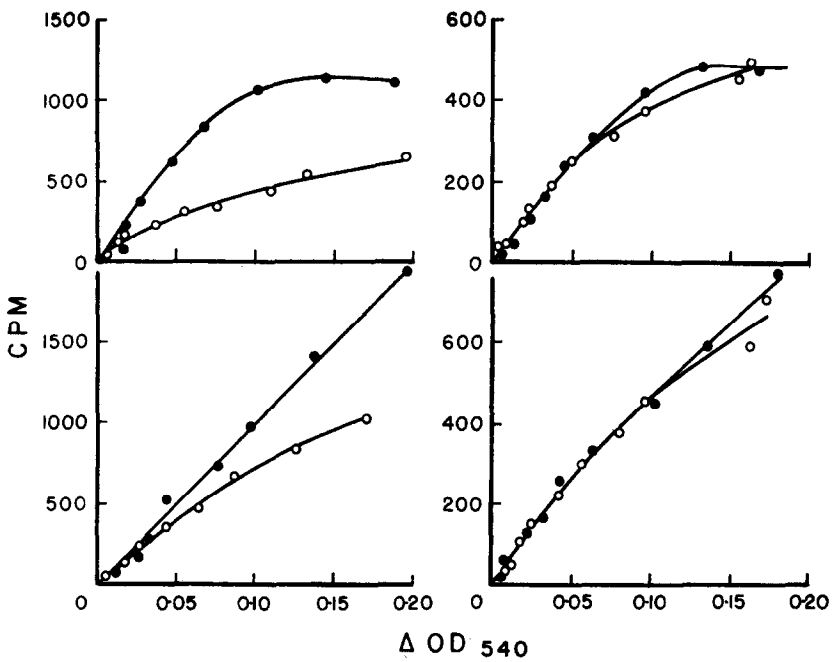

FIG. 6. Effect of thioguanine on incorporation of ^{14}C-guanine (left) and ^{14}C-adenine (right) into acid-washed cells. All of radioactivity recovered in nucleic acid purines. Incorporation represented as in Fig. 5. Concentration of thioguanine: ●, 0; O, 30 μM. Concentration of purines: top left, 6 μM guanine; bottom left, 30 μM guanine; top right, 15 μM adenine, bottom right, 35 μM adenine.

Results with mercaptopurine differed appreciably. Although adenine-^{14}C incorporation was not affected significantly, the molar activities of RNA adenine and guanine derived from labeled glycine were decreased by similar extents, probably indicating that catabolism of that drug supplied both purines. This result is in agreement with data on the desulfuration of mercaptopurine and incorporation of the resulting purine skeleton similar to the incorporation of hypoxanthine.[7] A significant difference between thioguanine and mercaptopurine can be demonstrated in the effect of the drugs on incorporation of guanine-^{14}C (Fig. 7). Although mercaptopurine decreased the conversion of guanine-^{14}C to RNA adenine, this pathway accounts for only a small share of the total incorporated radioactivity (Table 2).

Uracil incorporation. The depressant effect of thioguanine on nucleic acid synthesis was expected to be reflected in a decreased conversion of uracil-^{14}C into nucleic acid pyrimidines. Actually, the inhibitory effect of thioguanine on uracil incorporation,

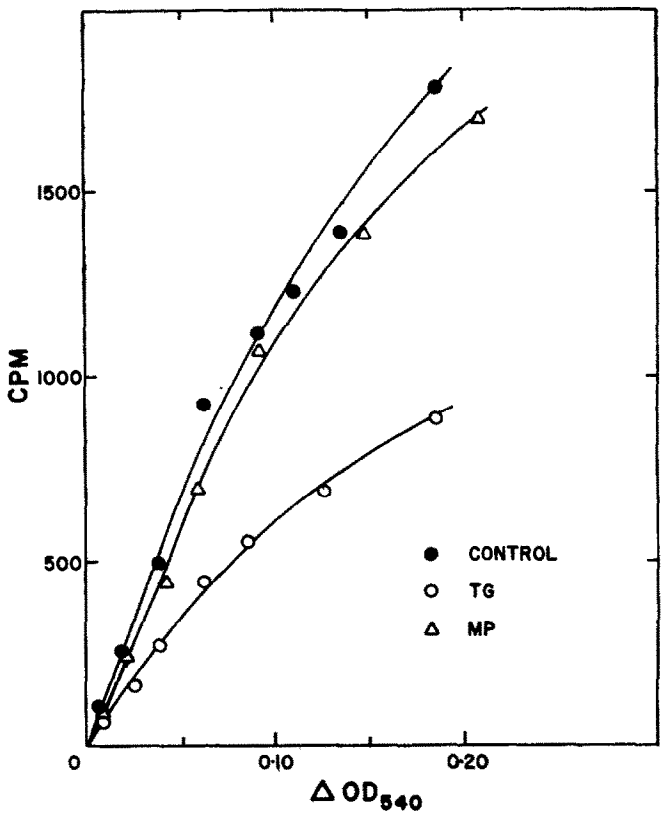

FIG. 7. Differential effects of thioguanine and mercaptopurine on incorporation of guanine-^{14}C into nucleic acids of *B. cereus*. Concentration of mercaptopurine and thioguanine, 30 μM; concentration of guanine, 18 μM. Experimental details as in Fig. 6.

compared to parallel runs where the label was ^{32}P, usually exceeded that on nucleic acid synthesis, roughly by a factor of 2. Comparison of the characteristic effect shown in Fig. 8 with Fig 5 and the molar activity values after labeling with phosphate or uracil (Table 2), confirms that thioguanine had produced a specific inhibitory effect on the incorporation of uracil into nucleic acids. Complete recovery of isotope uptake was observed upon termination of growth inhibition (Fig. 8).

No change was produced in the relative labeling of RNA uracil and cytosine (Table 2). Since guanosine triphosphate is a cofactor in this pyrimidine interconversion,[30] apparently thioguanine or its nucleotides do not antagonize the normal function of the guanine derivative. Measurement of radioactivity in cytosine and thymine of bacterial DNA, or of the various uridine nucleotides of the alcohol-soluble fraction,[20] revealed no specific biochemical step selectively sensitive to the analog which might account for the overall decrease in incorporation of uracil. Instead, all of these fractions and their labeled components showed a similar decrease in radioactivity. Experiments with mercaptopurine provided results essentially similar to those with thioguanine. It appears that less uracil entered the cells in the presence of the drugs, hence reducing the size of the labeled pool and the utilization of radiocarbon for nucleic acid pyrimidine synthesis.

Aspartic acid and orotic acid conversion to pyrimidine nucleotides. The conversion of aspartic acid into isolated pyrimidine nucleotides was reduced in the presence of thioguanine in relation to the lowered RNA content under these conditions. On the other hand, incorporation of orotic acid-^{14}C into nucleic acid pyrimidines was considerably enhanced in the presence of thioguanine. The increased incorporation of this pyrimidine precursor associated with a concomitant reduction of RNA synthesis resulted in a considerable increase in molar activities of the isolated RNA cytidylic and uridylic acids (Table 2). The total incorporation of orotic acid into DNA was essentially doubled when cells were grown to the same final turbidity with thioguanine as control cells.

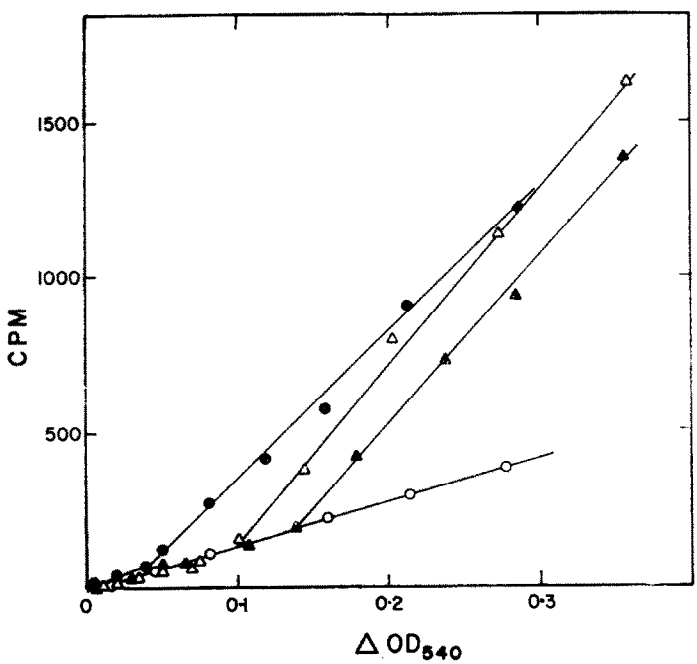

FIG. 8. Effects of thioguanine on incorporation of uracil-^{14}C into acid-washed cells. All of radioactivity recovered in nucleic acid pyrimidine fraction. Incorporation represented as in Fig. 5. Concentration of thioguanine: ●, 0; △, 6 μM; ▲, 12 μM; O, 30 μM.

By contrast, mercaptopurine did not produce such a marked effect. As shown in Fig. 9, a noticeable discrepancy existed between the action of the two drugs on the incorporation of orotic acid. Since mercaptopurine reduces nucleic acid synthesis, however, the eventual increase in incorporation of orotic acid into nucleic acids, compared to control cells, although slight, suggests that a quantitative rather than qualitative difference exists between the actions of mercaptopurine and thioguanine with respect to the extent of increased utilization of this precursor.

Effect on RNA fractions. Since thioguanine exerted unexpected effects on the formation of RNA pyrimidines from uracil and orotic acid, it was desirable to isolate different RNA fractions to determine if the incorporation effects were associated with particular subcellular components. Cells grown in the presence and absence of

thioguanine and labeled simultaneously with either uracil-^{14}C or orotic acid-^{14}C for 2 hr were harvested and cell-free preparations fractionated on methylated albumin columns as described. In preliminary experiments the characteristic patterns of elution of transfer and ribosomal RNA were not altered to any noticeable extent by exposure to thioguanine. After uracil or orotic acid labeling of cells, the ratio of radioactivity

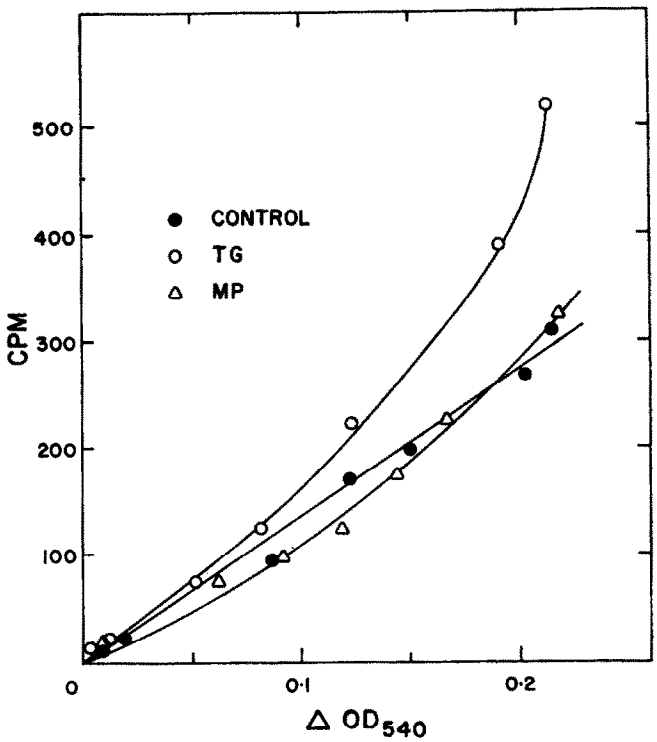

FIG. 9. Differential effects of thioguanine and mercaptopurine on incorporation of orotic acid-2-^{14}C into acid-washed cells. Almost all radioactivity recovered in nucleic acid pyrimidines. Incorporation represented as in Fig. 5. Thioguanine and mercaptopurine at 30 μM.

to optical density did not deviate markedly for any of the component peaks in each individual preparation, but thioguanine decreased all specific activities after uracil labeling and increased them after orotic acid-^{14}C. Thus, thioguanine curtailed the incorporation of uracil for the major RNA components to similar extents and did not cause orotic acid to be used for preferential labeling of any particular RNA fraction.

Protein precursor incorporation. Experiments in which the growth medium was supplemented with radioactive amino acids demonstrated that incorporation of radioactivity from these compounds usually was not specifically affected by thioguanine or mercaptopurine. Figure 10 represents the results of an experiment on the incorporation of lysine-^{14}C, a precursor specific for protoplasmic proteins of *B. cereus*[16], in the presence and absence of the drugs. In this group of experiments with labeled amino acids, however, small drug-induced deviations from control values were observed frequently. With lysine-^{14}C, subtle variabilities from one experiment to the next and ranging up to 20% in either direction of control values were noted, especially when

growth inhibition was allowed to proceed for more than one doubling of bacterial turbidity. With other labeled amino acids that are also condensed exclusively into protein of *B. cereus*,[16] such as methionine-^{35}S, cystine-^{35}S, valine-^{14}C, or leucine-^{14}C, at turbidities corresponding to those of control cells, incorporation was either unchanged or slightly depressed by the drugs.

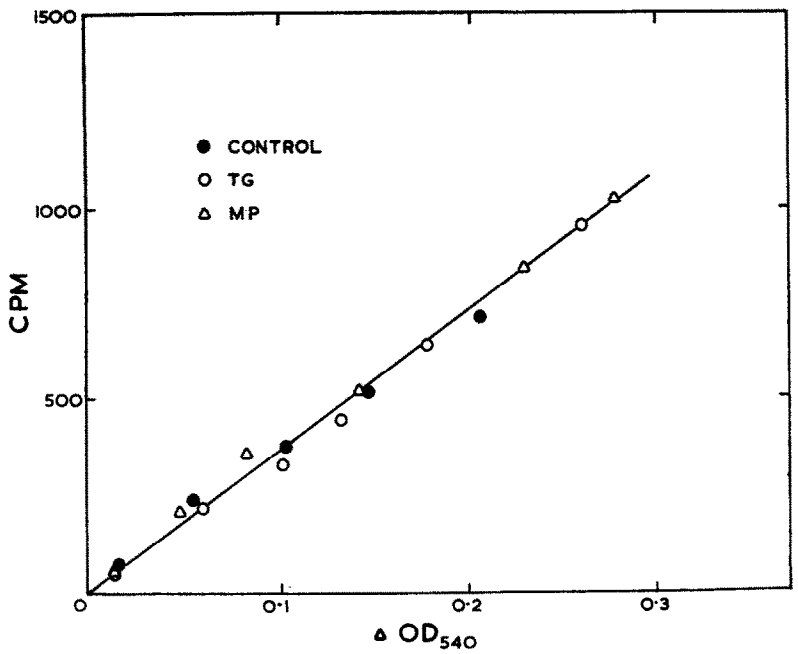

FIG. 10. Lack of effect of either thioguanine (30 μM) or mercaptopurine (30 μM) on incorporation of lysine-^{14}C into hot-acid-washed cells. All of radioactivity in bacterial proteins. Incorporation represented as in Fig. 5.

Results with radioactive acetate showed even greater fluctuations.* In most of the experiments with acetate-1-or -2-^{14}C, about 50% more radiocarbon was incorporated into the cells in the presence of thioguanine than in its absence, for the same increase in bacterial turbidity. Most of the increase was in the cell fraction insoluble in hot trichloroacetic acid, i.e. cell wall and protein. After hydrolysis with 6 N HCl the label was recovered mainly in glutamic acid. During inhibition by mercaptopurine, incorporation of acetate was enhanced also, but not quite to the same extent as observed with thioguanine. On the other hand, in several other experiments, in the presence of the drugs, 20% less radioactivity was incorporated from acetate-^{14}C than was the case in control cells. Since labeled acetate supplies radiocarbon for numerous bacterial cell components including cell wall, it is difficult to estimate how much of the variability in incorporation was directly associated with protein synthesis.

* It should be mentioned that this variation in effect was not observed in experiments with *Escherichia coli* grown in the casein hydrolysate medium as described for *B. cereus*. Both drugs regularly diminished the utilization of acetate-^{14}C for biosynthesis of *E. coli* (see also Ref. 5), and mercaptopurine usually produced a more profound depression of acetate incorporation than did thioguanine.

Induction of penicillinase. Since the above precursor incorporation experiments involved only total bacterial protein, an attempt was made to investigate the drugs' effects on the production of a single protein, penicillinase. This enzyme was induced in control cultures of *B. cereus* 569 and in cultures which had received thioguanine or mercaptopurine. Ten min after the introduction of the analogs, penicillin was added to the cultures during continued incubation, and growth medium was removed at 10-min intervals to assay for the presence of penicillinase. Penicillinase was induced by all cultures at essentially the same rate, and growth resumed rapidly after a brief penicillin-induced slowing. The thermal stability, during heating to 55°, of the penicillinase produced during growth in the presence or absence of the analogs was unchanged.

Formation of flagella. The effect of thioguanine and mercaptopurine on flagellation of *B. cereus* was examined, since flagella have been demonstrated to consist almost entirely of protein.[31] For these experiments test tubes containing a small amount of growth medium and, as required, thioguanine or mercaptopurine (30–60 μM) were inoculated with a dilute suspension of bacteria and the cells allowed to grow overnight at 25° without agitation. A noticeable difference in turbidity of the drug-treated tubes compared to the control was evident. The cells were then stained with fuchsin–tannic acid for flagella.* Whereas almost all control cells were flagellated, most of the cells formed in the presence of thioguanine or mercaptopurine lacked flagella. Characteristic stains of bacteria are shown in Fig. 11.

Cell wall formation, Diaminopimelic acid has been shown to be incorporated exclusively into cell walls in *B. cereus* from which it is recoverable in unchanged form.[16, 32] The incorporation of this labeled precursor, therefore, has served to indicate drug-induced effects on specific components of cell wall. In the presence of thioguanine or mercaptopurine, a diminished quantity of the labeled precursor was incorporated compared to control cells (Fig. 12), implying that the drugs had inhibited the formation of bacterial cell wall.

Metabolic fate of thioguanine-^{35}S

Radiosulfur distribution in cell fractions. Bacillus cereus cultures were grown in the presence of thioguanine-^{35}S (6·7 mc/mmole) for 2 hr and radioactivity in various cell fractions measured by the membrane filtration technique.[14] Approximately 50% of the total radiosulfur in cells washed with fresh growth medium was extractable with cold trichloroacetic acid, signifying that the pool of acid-soluble derivatives of the drug was relatively large. Extraction with hot trichloroacetic acid to degrade and solubilize nucleic acids removed only another 5% of the radioisotope, and the residual radioactivity was associated with the protein and cell wall fraction.

Ethanol-soluble fraction. Radioautography of paper electrophoresis strips used for the resolution of this fraction revealed at least twelve compounds. One of these components corresponding to less than 5% of the total radioactivity in the fraction had an electrophoretic mobility at pH 9·2 of 1·15 times that of guanylic acid. Upon hydrolysis of this compound with carrier thioguanine in N HCl and chromatography, radioactivity fingerprinted with the carrier. Since this metabolite had the correct electrophoretic mobility[33] and was identical by electrophoresis and chromatography

* Courtesy of Dr. Rudolph Hugh, Dept. of Microbiology, The George Washington University School of Medicine.

with an authentic sample of thioguanylic acid isolated from mouse tissue,* it was concluded to be thioguanosine 5′-monophosphate. Isotope dilution measurements indicated that cells contained approximately 0·02 μg thioguanosine monophosphate/mg dry weight of cells. (By contrast, cells grown with guanine-^{14}C contain about 1 μg guanine nucleotides in this quantity of cells.[14])

A major component of the sulfur-labeled ethanol-soluble fraction was indistinguishable during electrophoresis from glutathione.

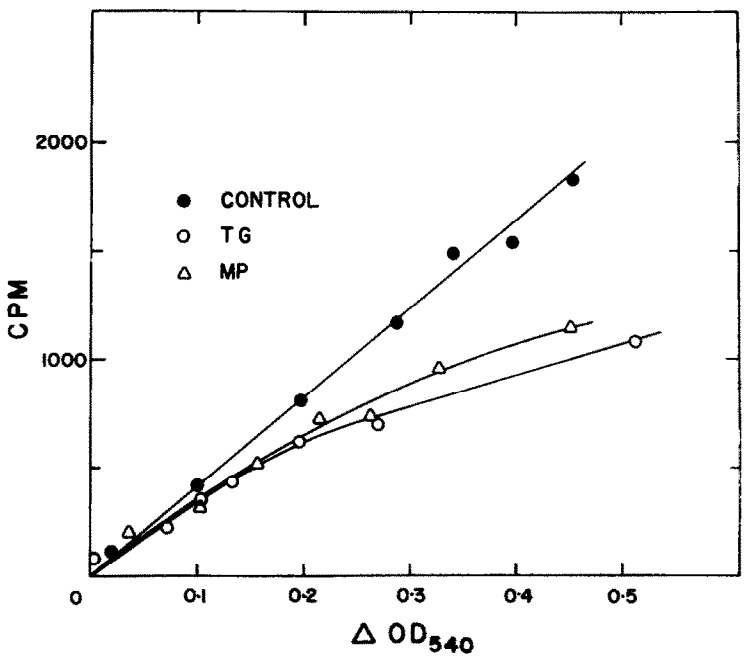

FIG. 12. Effect of thioguanine (30 μM) and mercaptopurine (30 μM) on incorporation of diaminopimelic acid-^{14}C into acid-washed cells. All of radioactivity in cell wall. Incorporation represented as in Fig. 5.

Nucleic acid fraction. Digestion of cell residues after the hot ethanol extraction in 0·5 N KOH at 37° for 3 hr produced essentially complete hydrolysis of RNA to mononucleotides,[14] and did not lead to decomposition of thioguanine, as examined chromatographically. Radioautography following the paper electrophoretic resolution at pH 9·2 of the neutralized alkali digest revealed seven major components. The trace of thioguanosine-2′,3′-monophosphate was characterized by electrophoretic mobility and, after hydrolysis, fingerprinting with carrier thioguanine.

For quantitation of the incorporation of the analog into nucleic acids, drug-labeled cells were extracted three times with cold trichloroacetic acid to remove the acid-soluble pool and adsorbed free drug. The cells were then mixed with carrier thioguanine and extracted with hot trichloroacetic acid. This extract was mixed with an equal volume of 2 N HCl and heated at 100° to ascertain complete hydrolysis to

* Courtesy of Dr. G. A. LePage, Stanford Research Institute, Menlo Park, Calif.

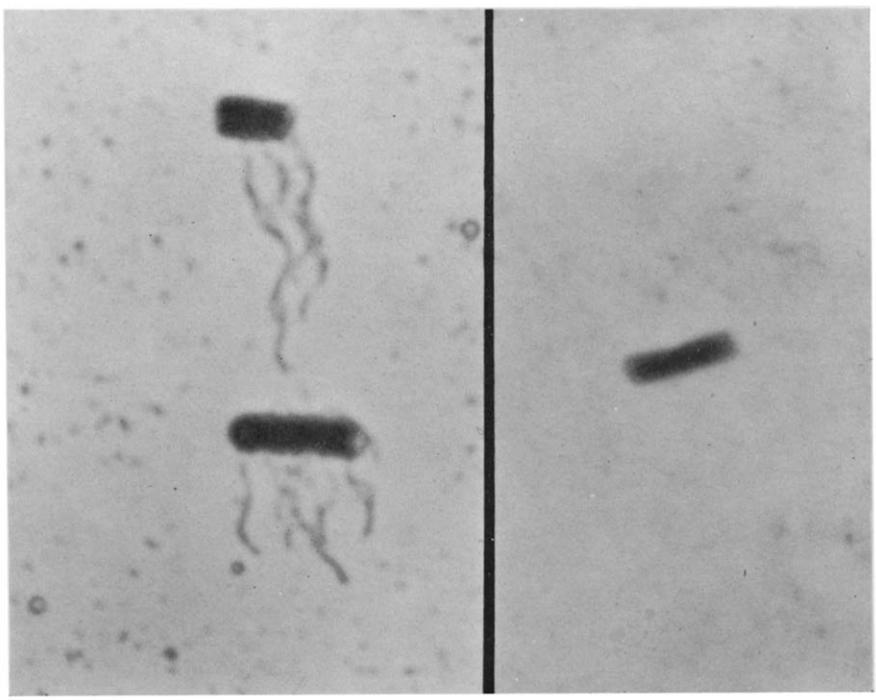

FIG. 11. *B. cereus* stained to show flagella. Left, control cells; right, cells treated with thioguanine. Mercaptopurine-treated cells looked like those after thioguanine. Magnification about $\times 2000$.

the purine bases. The hydrolysate was subjected to chromatography and the thioguanine component rechromatographed before measurement of molar activity. It was estimated that 0·04 μg thioguanine was incorporated per mg total nucleic acids.

Protein and cell wall fraction. The residue after extraction of thioguanine-^{35}S-labeled cells with hot trichloroacetic acid was hydrolyzed as described. Radioactivity was recovered mainly in cystine, cysteic acid, and an area corresponding to sulfate. Thus, the distribution pattern of radioactivity closely resembled that of labeling cells with sulfate-^{35}S or cystine-^{35}S,[15] indicating that the radiosulfur had been cleaved from thioguanine and had been utilized for protein synthesis.

DISCUSSION

Effect on growth

Examination of the effect of thioguanine on the change in turbidity of exponentially growing cultures of *B. cereus* revealed an immediate decrease in the growth rate. The extent of this effect was not related to the concentration of the drug added. Growth resumed after a period of inhibition, but it is possible that some irreversible damage may have taken place, since the rate of growth after recovery differed very slightly from that of control cells. The duration of the inhibitory effect was related to the concentration of drug added. The closely related drug, mercaptopurine, showed a similar pattern of growth inhibition,[5, 7] and both drugs apparently saturated their receptor sites or the sites of interaction with anabolic enzymes so readily that increased concentration of drug produced no additional responses. Catabolism by desulfuration of mercaptopurine and undoubtedly thioguanine[7] then depleted the receptor of active drug and led to restoration of growth.

Reversal of the effects of thioguanine in different biological systems is related to the ease of interconversion of purines or their utilization. For example, adenine was superior to guanine in reversing the action of thioguanine in *Streptococus faecalis*, whereas both purines effectively antagonized the drug in *Lactobacillus casei*.[34] In *B. cereus* the complete and competitive blockage by exogenous guanine (but not adenine or hypoxanthine) of the inhibitory effect of thioguanine implies that this analog acts by replacing guanine in some important biochemical reaction. Since normal growth occurs in the absence of guanine, the usual calculations resulting from the reciprocal plot (Fig. 3) cannot be made. It is likely that guanine antagonizes the formation of thioguanosine monophosphate, which was identified in *B. cereus* and which may be the active inhibitor, by competing with the base analog for nucleotide pyrophosphorylase. Such an effect has been suggested for hypoxanthine in overcoming the action of mercaptopurine.[35]

Effect on biosynthesis

It must be emphasized that in the present experiments comparisons between treated and control cells have been made at similar turbidities. Thus, any drug effect which proceeds at a *rate* identical with that of growth is not apparent. On the other hand, it can readily be calculated that thioguanine inhibited the rate of RNA synthesis by 67%, whereas that of DNA, protein, and growth were slowed by only 50%.

Nucleic acid biosynthesis. The reason for the increased utilization of orotic acid-^{14}C for pyrimidine nucleotide formation during growth inhibition by thioguanine, involving a decreased nucleic acid content, is not clear. It indicates however, that at least in the bacterial system, exogenous orotic acid incorporation does not serve as an accurate index of *de-novo* nucleic acid pyrimidine formation. In addition, the stimulatory effect of thioguanine on orotic acid incorporation, relative to RNA formation, contrasted sharply with the depression of uracil utilization. Divergent effects on the two nucleic acid pyridimine precursors have also been established for other drugs in the *B. cereus* system, such as 8-azaguanine,[20] chloramphenicol,[36] and amobarbital.[37]

Beckwith *et al.*[38] have reported the reduction in dihydroorotic acid dehydrogenase activity of *E. coli* by thioguanine, related to either curtailed synthesis or activity of this enzyme. If this effect is characteristic of *B. cereus* also, the resulting decrease of endogenous orotic acid synthesis may allow cells to incorporate more exogenous orotic acid.

The rapid cleavage of thioguanine in *B. cereus* to a guanine-like product which then antagonized the incorporation of exogenous guanine probably accounted for the inhibitory effect of thioguanine on the incorporation of exogenous guanine. In tumor cells, however, desulfuration of the drug to normal purines was not extensive,[39] and the observed reduction of guanine incorporation is believed to be due to competition between drug and guanine for ribomononucleotide formation.[40]

Protein biosynthesis. Although there is little information on the effects of thioguanine on protein synthesis, it is likely that they resemble those of mercaptopurine. In *E. coli* no effect on total protein synthesis was produced by mercaptopurine,[5] and the drug affected leucine incorporation into leukemic leukocytes inconsistently *in vitro*.[41] A specific mercaptopurine-induced depression in the incorporation of amino acids into leukocyte protein, independent from the drug-related fall in leukocyte count, was claimed for the cells from two leukemic patients.[41] Mercaptopurine has been reported to inhibit the induction of tryptophan pyrrolase in rat liver,[42] but produced no effect on the induction of certain drug-metabolizing enzymes[43] or of β-galactosidase in *E. coli*.[5] The suppression of antibody synthesis by both mercaptopurine and thioguanine apparently is not a direct effect on protein biosynthesis, since administration of the drugs during the height of the immune response had no effect on the titer of antibody in serum.[44] Furthermore, drugs like 8-azaguanine, which inhibit protein formation quite extensively, are not particularly effective in suppressing immune responses (reviewed in Ref. 45). The specificity with respect to time of administration of thioguanine in the formation of various hemagglutinins has been described.[46] The selective alteration by mercaptopurine and thioguanine of the protein composition of rabbit serum[47] or of the enzyme composition of tumor homogenates[48] suggests that the analogs can exert preferentially inhibitory effects on the formation of certain proteins but not on others.

Results with *B. cereus* are in general agreement with the reported observations. The normal induction of penicillinase and the unaltered incorporation of amino acids in the presence of thioguanine contrast sharply with the small but frequently observed depressions of amino acid incorporation and inhibition of flagella formation, probably involving the decreased formation of the protein, flagellin. These results suggest that alterations in the pattern of protein biosynthesis are being produced by thioguanine, which can only be demonstrated by studying the synthesis of particular

proteins. It is known from studies with 8-azaguanine that the synthesis of specific proteins can be depressed selectively.[49]

Anabolism of thioguanine

After 2-hr growth inhibition by thioguanine, bacterial nucleic acids were found to contain 1 molecule of thioguanine for approximately 2,500 of guanine. If only the nucleic acids actually synthesized during growth inhibition are considered, only 1 molecule of thioguanine was calculated for each 1,200 of newly formed guanine. This minute extent of incorporation and the instantaneous onset of growth inhibition by thioguanine in *B. cereus* practically exclude the possibility of accumulation of the analog in nucleic acids from the most likely mechanisms of growth inhibition.

These results differ sharply from those carried out in tumor cells. LePage[10] has reported that a total of 3–6 μg analog could be incorporated into DNA and RNA per ml tumor cells in 1 hr. This quantity of analog incorporation is so great as to approximate that of exogenous guanine or adenine incorporation into nucleic acids under normal conditions.[50, 51] LePage and Jones[11] also showed evidence that thioguanine inhibited tumor growth by incorporation into DNA and that this process prevented replication of normal DNA by the cells.

It is important to stress, in addition to the species variation observed, that potent inhibition of growth has been demonstrated in the bacterial cell systems where incorporation of thioguanine was almost nonexistent.

Relationship between thioguanine and mercaptopurine

The general similarity in the actions of these structurally closely related purine analogs has long been apparent, and the question has been raised whether the two drugs act by the same mechanism and through a common intermediate. For instance, there is usually cross-sensitivity and cross-resistance to the two drugs, and similar catabolic products have been recovered. A number of differences between the analogs has been reported, however.[13] In mammals, the drugs produced different toxicities.[52] A tumor subline resistant to thioguanine was only partially cross-resistant to mercaptopurine, and combinations of the two drugs produced potentiation of their carcinostatic actions.[53] The two drugs differed in their effect on glycine incorporation into tumor cells,[40, 54] and the ribomononucleotides of the two drugs differed quantitatively in their ability to inhibit the conversion of inosinic acid to adenylosuccinic acid.[55] The greatest discrepancy is the incorporation of thioguanine into tumor DNA, associated with growth inhibition,[13] and either the complete lack of such incorporation of mercaptopurine[56] or else possible incorporation in the absence of growth inhibition.[57]

The present experiments on *B. cereus* again point out the many biochemical similarities between the two drugs but also indicate marked differences. There was a strong similarity in the pattern of growth inhibition produced by the analogs, and drug combinations caused no enhancement of growth inhibition. Both drugs curtailed RNA synthesis more than that of DNA, and both inhibited cellular flagellation, diaminopimelic acid incorporation into cell wall, and uracil incorporation. Effects on protein formation were identical, and incorporation of either analog into polynucleotides was either extremely minute or too small to be measurable.[7]

A major contrast in behavior was the ability of guanine to prevent completely and competitively growth inhibition by thioguanine but not mercaptopurine. Conversely, hypoxanthine overcame completely and competitively growth inhibition by mercaptopurine[8] but not thioguanine. Other differences included the decreased conversion of exogenous guanine into RNA-guanine by thioguanine but not mercaptopurine (Fig. 7) and the appreciably greater effect of thioguanine than mercaptopurine on the incorporation of orotic acid into RNA pyrimidines (Fig. 9).

It appears that the mechanisms of growth inhibition of the two analogs on bacterial cells are almost identical but do involve a number of divergent intermediary steps.

Mechanism of growth inhibition of the two drugs in B. cereus

Bennett *et al.*[3] have reported that, of the several possible means by which mercaptopurine has been recognized to inhibit purine synthesis, the most sensitive drug-susceptible step occurs prior to the formation of 4-aminoimidazole-5-carboxamide ribotide. These authors concluded that the interconversion of purine ribonucleotides was not a primary site of action. Thioguanine has been reported to block the *de-novo* purine pathway prior to the formation of α-N-formylglycinamide ribotide,[40] but this drug response, related to feedback inhibition, was not considered responsible for growth inhibition.[58] In tumor systems the closest relationship of a response produced by thioguanine has been the drug's incorporation into DNA and the formation of nucleotide polyphosphates,[13] but this has not been found with mercaptopurine.[57] Although thioguanine incorporation into the nucleic acids of *B. cereus* is minute, a major alteration in biological function cannot be excluded absolutely. A drug-induced effect on DNA synthesis or function without changing drastically DNA content might be expected to reduce RNA synthesis and to result in alterations in the synthesis of specific proteins.

Other mechanisms of growth inhibition may also be considered, particularly because of the almost instantaneous inhibition of growth after thioguanine treatment. Since *B. cereus* has no requirement for exogenous purines, competition between the analog and normal purines for entry into the cell is as unlikely a mechanism of action of thioguanine as it was for mercaptopurine.[8] An effect of drugs on the synthesis or function of some purine-containing cofactor essential for some vital step in growth might rapidly produce growth inhibition and might at the same time give rise to a series of other biochemical derangements. Although in mammalian cells mercaptopurine ribonucleoside triphosphate has been reported to inhibit the formation of NAD,[59,60] in *B. cereus* mercaptopurine had no effect on the synthesis or activity of NAD or CoA.[8] The report that the sulfhydryl group of mercaptopurine binds through metal ions to RNA[61] suggests another possible action of the drug that could exert rapid effects. It is not known whether these interactions demonstrate any selectivity to particular nucleotide sequences of nucleic acids. Alterations in the effective concentration of metals through this complexing may produce alterations in macromolecular association and are known to affect bacterial flagella formation.[62]

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