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# THE METABOLISM OF 6-MERCAPTOPURINE BY *BACILLUS CEREUS**

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**Abstract**—The growth-inhibitory purine analog, 6-mercaptopurine, was oxidized by resting cells of *Bacillus cereus* to the non-inhibitory catabolites, 6-thioxanthine and 6-thiouric acid. In growing cell cultures other pathways of drug metabolism appeared to be more important. Sulfur was rapidly cleaved from the drug, could be recovered as inorganic sulfite, and appeared finally in the proteins. The resulting purine skeleton was incorporated as adenine and guanine into both ribo- and deoxyribo-nucleic acids. No direct evidence could be found that 6-mercaptopurine was incorporated as such into nucleic acids. The upper limit calculated for such incorporation suggests that this pathway of metabolism was most probably insufficient to produce growth inhibition. A compound having the properties of 6-mercaptopurine ribonucleotide was present in the soluble fractions of cells. Metabolism to non-inhibitory compounds, principally the normal purines of the nucleic acids, appeared to be responsible for the cessation of growth inhibition characteristically observed with 6-mercaptopurine. When the rate of growth returned to normal, all the 6-mercaptopurine had been removed from the growth medium. The failure of increasing concentrations of 6-mercaptopurine to increase the magnitude of growth-inhibition, in contrast to the observed increase in the duration of inhibitory action, has been discussed.

## INTRODUCTION

THE purine analog 6-mercaptopurine has been of considerable interest since its introduction in the treatment of leukemias.[1] It has also been demonstrated to inhibit the growth of bacteria and of mammalian cells in culture.[2,3] The precise mechanism of action has not yet been elucidated, but the drug is known to interfere with the interconversion of purines in nucleic acid synthesis.[4,5] The possibility still exists that other biochemical pathways are affected also which may be even more closely related to the growth-inhibitory effect.

Studies on the metabolism of the drug in the mouse[6] and in *Streptococcus faecalis*[7,8] have revealed that some of the drug was converted to normal purines. Elion *et al.*[6] have suggested that the purine is incorporated into nucleic acids in mouse tissues, as is the case with certain other purine analogs.[9] In bacteria, however, such incorporation seemed to be slight.[10] The presence of 6-mercaptopurine ribonucleotide in microorganisms susceptible to the drug, in contrast to the absence of this ribonucleotide in resistant systems, has led Brockman *et al.*[11] to hypothesize that this anabolite is crucial in the drug's action. In the present study some of the routes by which the drug is metabolized have been investigated prior to studies on the mechanism of action of the

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drug. The micro-organism *Bacillus cereus* was selected because its growth is partially inhibited by the drug and because this micro-organism has been the object of quantitative studies in this laboratory.[12] Some of the present work has been reported previously.[13,14]

## MATERIALS AND METHODS

### *Compounds*

6-Mercaptopurine was obtained from Burroughs Wellcome and Co., Tuckahoe, N.Y. 6-Mercaptopurine-8-$^{14}$C (0·55 mc/m-mole) and hypoxanthine-8-$^{14}$C (0·81 mc/m-mole) were purchased from Isotopes Specialties, Inc., Burbank, Cal. 6-Mercaptopurine-$^{35}$S (7·1 mc/m-mole) and 6-mercaptopurine ribonucleoside were gifts of Dr. R. Wallace Brockman, Southern Research Institute, Birmingham, Ala. Both radioactive samples of 6-mercaptopurine were found to be pure by chromatography. 2-Hydroxy-6-mercaptopurine (thioxanthine) was purchased from Mann Research Laboratories, New York 6, N.Y. 2:8-Dihydroxy-6-mercaptopurine (thiouric acid), synthesized by Dr. Ti Li Loo, National Institutes of Health, Bethesda, Md.,[15] was kindly furnished to us. Purified milk xanthine oxidase and catalase were obtained from the Worthington Biochemical Co., Freehold, N.J. Trypsin came from the Difco Laboratories, Detroit, Mich. *Crotalus adamanteus* venom was obtained from the Ross Allen Reptile Institute, Silver Springs, Fla.

### *Enzyme methods*

To study the effect of xanthine oxidase on 6-mercaptopurine, 10 mg of the analog, 100 units of xanthine oxidase and about 0·2 mg of catalase were incubated in 1 ml of 0·025 M phosphate buffer of pH 7·5 at 37 °C for from 6 to 8 hr with occasional shaking.

To separate DNA from contaminating protein, 1—5 mg of the mixture was dissolved in 1·0 ml water, brought to pH 8·5, and 3 $\mu$g of trypsin was added; the mixture was incubated overnight at 37 °C, and the DNA reprecipitated as described below. Hydrolysis of nucleotides with *Crotalus* venom phosphatase was carried out by incubation at pH 9·0 for 6 hr, as described by Dunn and Smith.[16]

### *Micro-organism*

*Bacillus cereus*, strain 569H, was grown in polyethylene bottles at 37 °C and was shaken to produce optimal aeration. The growth medium consisted of salts and a casein hydrolyzate (vitamin-free casamino acids, Difco Laboratories, Detroit, Mich.) adjusted to pH 7·0 with potassium hydroxide.[17] Growth was measured as change in turbidity, determined as optical density at 540 m$\mu$ in the Beckman spectrophotometer model DU. Usually, 6-mercaptopurine was added to exponentially growing cultures at an optical density reading between 0·05 and 0·06 (i.e. about 40 $\mu$g dry weight of cells per ml).

For the resting cell incubations, cells which had been grown in the salts–amino acids medium were centrifuged, washed in the medium from which the amino acids had been omitted, and suspended in this deficient medium in Roux bottles at 37 °C.

### *Isolation of nucleic acid components*

The cells were fractionated by the alkali digestion procedure of Studel and Paiser,[18] as described by Schmidt and Thannhauser,[19] and extracted with hot saline solutions as described by Jorpes.[20]

After washing, the cells were treated with 5% trichloroacetic acid (TCA), and the precipitate was extracted with 70% ethanol at 70 °C, 95% ethanol at 70 °C, and ethanol: ether 3:1 (v/v) at room temperature. The RNA was then hydrolyzed with 1 N KOH for 18 hr. Alternatively, the cells were treated with TCA, the precipitate was suspended in ethanol, disintegrated with glass beads, and the nucleic acids were extracted by treatment of the residue three times with 10% NaCl at 100 °C for 1 hr.[21] The sodium nucleates were treated with TCA and 2 vols. of ethanol in the cold, and then hydrolyzed with potassium hydroxide as above. The DNA, protein and potassium ions were removed from the ribonucleotides by precipitation with perchloric acid at 0 °C, and the ribonucleotides were separated by paper electrophoresis. The DNA was isolated from the alkali digest by precipitation with 0·06 ml of 6 N HCl to 1 ml of digest and sufficient TCA to give a final concentration of 5 per cent. Two volumes of ethanol were then added and the mixture was stored overnight at −10 °C to ensure complete precipitation. The precipitate was centrifuged, washed with ethanol and dried. It was then treated with trypsin to remove remaining protein, as described above, and reprecipitated. The purines were removed from this fraction by a 5 min hydrolysis in 0·1 N HCl at 100 °C.[22]

Compounds were chromatographed on Whatman No. 3 MM paper, with *iso*propanol-$H_2O$ (70:30 v/v) as solvent in an atmosphere of ammonia.[23] Material absorbing ultraviolet light was located on the chromatograms by ultraviolet light absorption prints according to the method of Markham and Smith,[24] using Remington Rand G-91 Portagraph paper. Radioactive spots were detected by placing the chromatogram against Kodak-Medical X-ray film (single coated, blue sensitive) for from 2 to 4 weeks. Compounds were separated by paper electrophoresis on strips of Whatman No. 3 MM paper, 2½ in. wide. A 1000 V power source (E.C. Apparatus Co., Swarthmore, Pa.) provided a potential gradient of approximately 20 V/cm. The strip was immersed in a bath of carbon tetrachloride placed between the electrode chambers, to prevent heating.[23] Solutions of 0·05 M formate, pH 3·5; 0·05 M acetate, pH 4·5 and 5·0; 0·05 M phosphate, pH 6·6 and 7·6; and 0·025 M borate, pH 8·5, 9·0 and 9·7 were used as buffers. The distribution of radioactivity and ultraviolet absorption along electrophoresis and chromatogram strips was measured by cutting the strips into 0·5 cm sections from which the compounds were eluted by immersion in 2 ml of 0·01 N HCl for 16 hr at 0 °C. After measurement of the ultraviolet absorption spectrum, 0·5 ml or 1 ml of each solution was transferred to an aluminum planchet, dried in a stream of cool air, and the radioactivity determined. Breakdown of some of the sulfhydryl compounds mentioned in this study has been observed to occur when the compounds were concentrated at one end of a chromatogram section by the capillary flow of an eluting fluid through the section, followed by evaporation from the end. This decomposition was avoided by immersing the spot in a large volume (10 ml or more) of water containing 1 drop of 1 N HCl in the cold, and lyophilizing the solution obtained.

*Conversion of cysteine to cysteic acid*

Cysteine in the bacterial protein fraction remaining after the sodium chloride extraction was oxidized to cysteic acid by a 5 min treatment with performic acid at room temperature.[25] The performic acid was removed by evaporation, and the protein hydrolyzed with 6 N HCl at 8 lb/in² (112 °C) for 3 hr. The cysteic acid was separated

readily from the amino acid mixture by paper electrophoresis (2 hr at 20 V/cm, pH 3·0 (0·2 M acetic acid)), since under these conditions it is the only compound migrating towards the anode.

*Membrane technique*

The uptake of radioactive materials by growing cells was measured by the membrane filter technique of Britten *et al.*,[26] a method which has been used regularly in this laboratory.[12] Samples of the culture were rapidly transferred into Beckman cells for turbidity measurements, and were then pipetted immediately for filtration through collodion membrane filters (Schleicher and Schuell Co., Keene, N.H.). These filters, which removed the cells quantitatively from the cultures, were attached with rubber cement to aluminum plates and allowed to dry. The radioactivity on the plates was determined in a proportional gas-flow counter usually to a standard error of 1 per cent.

The above filtration technique has been used by Roodyn and Mandel[27] as a rapid method of fractionation which provides the same results as those obtained by conventional techniques. Bacteria were treated with 5% TCA for 15 min at room temperature, or with 5% TCA for 30 min at 100°C. These suspensions were filtered onto the membranes and the residues washed with 1% TCA. The radioactivity in the "intact cells" was determined by filtering the untreated culture directly and washing with medium.

*Recovery of 6-mercaptopurine*

The percentage of 6-mercaptopurine remaining in the growth medium after various periods of incubation was estimated by the following procedure. A sample of the medium containing 6-mercaptopurine-8-$^{14}$C, after measurement of turbidity, was filtered through a collodion membrane and the filtrate collected. Carrier 6-mercaptopurine (at least 2000 times greater than the amount of the radioactive compound) was added to an aliquot of this filtrate. The mixture was then lyophilized, the residue taken up in a small volume of 0·01 N $NH_4OH$, and the 6-mercaptopurine was re-isolated by a two-dimensional combination of paper chromatography and paper electrophoresis at pH 9·0. 6-Mercaptopurine was the only detectable spot on the paper which absorbed ultraviolet light after this treatment. The specific activity of this material, calculated from the optical density at 325 mμ, was directly proportional to the concentration of 6-mercaptopurine remaining in the growth medium, since the ultraviolet absorption of the radioactive material was insignificant in comparison with that of the carrier.

*Preparation of soluble fractions*

To prepare the soluble fraction of cells, 400 ml of culture was incubated with 1 μg of $^{35}$S- or $^{14}$C-6-mercaptopurine per ml of suspension. After 30–40 min, when the quantity of soluble metabolites was at a maximum, the culture was cooled in ice. The cells were centrifuged, suspended in water and then pipetted into 4 ml of ethanol at 85 °C. After 3 min, the precipitate was removed by centrifuging and the supernatant solution evaporated to dryness *in vacuo*.[28]

## RESULTS

*Metabolism of 6-mercaptopurine by resting cells*

Resting cells of *Bacillus cereus* (about 2 mg of dry weight per ml of culture) were incubated in the presence and absence of 20 μg of 6-mercaptopurine-8-$^{14}$C per ml of

suspension for 15—40 hr at 37 °C. An ultraviolet absorption print and radioautogram of a chromatogram of the medium containing 6-mercaptopurine, after removal of cells, is shown in Fig. 1. Two spots absorbing ultraviolet light were observed, A and B, which were not formed during incubation in the absence of 6-mercaptopurine. Of the five radioactive spots, two coincided with A and B; two others, C and D, although they contained as much radioactivity as A and B, did not absorb ultraviolet light. The fifth radioactive metabolite, E, could be distinguished from an ultraviolet absorbing metabolite which was present also in the control.

Metabolite A had the same ultraviolet absorption spectrum and chromatographic properties as authentic thiouric acid. This compound has been reported to be a metabolite of 6-mercaptopurine in man[15,29] and in the mouse.[6] The radioactive metabolite which had been purified by paper chromatography was mixed with carrier thiouric acid from which it was subsequently indistinguishable upon electrophoresis at pH 4·5, 5·0, 6·6 and 7·6. The metabolite is therefore presumed to be thiouric acid.

Metabolite B had the same ultraviolet absorption spectrum and chromatographic properties as thioxanthine, a potential intermediate in the conversion to thiouric acid. When carrier thioxanthine was added to the bacterial medium after incubation with 6-mercaptopurine-8-$^{14}$C, the re-isolated thioxanthine had a constant specific activity

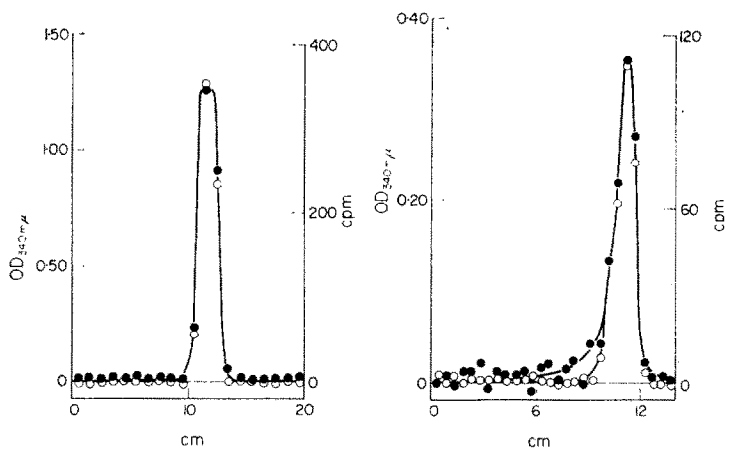


FIG. 2. Distribution of ultraviolet absorption (○——○) and radioactivity (●——●) from mixture of radioactive metabolite B and carrier thioxanthine. Left, paper chromatogram; right, paper electrophoresis strip, pH 7·6.

after the seventh and ninth recrystallizations from hot water. Since thioxanthine occasionally decomposed on recrystallization, reproducibility was improved by acidifying the incubation medium, lyophilizing and extracting the dry powder with hot pyridine. The pyridine was evaporated to dryness, and after carrier thioxanthine was added the mixture was recrystallized three times from hot water. Constant specific activity of the recrystallized thioxanthine was retained during chromatography followed by electrophoresis of the chromatographed compound at pH 6·6 and 7·6, as shown in Fig. 2, and therefore it was concluded that metabolite B is thioxanthine.

The high degree of sensitivity of the identification procedure shown in Fig. 2 was demonstrated by mixing radioactive thiouric acid, isolated from the medium, with carrier thioxanthine, and separating the mixture by electrophoresis at pH 6·6, at

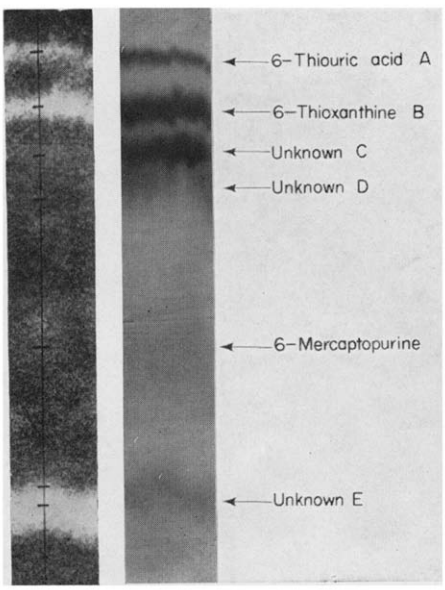


FIG. 1. Metabolites of 6-mercaptopurine-8-$^{14}$C in the medium of a resting *B. cereus* culture. Left ultraviolet absorption print and right, radioautogram of a chromatogram.

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which the compounds have similar mobilities. As shown in Fig. 3, the compounds could be differentiated from each other, since the specific activities of sections of the strip were not the same. Only a very slight difference in mobility is required, therefore, to distinguish a known compound from an unknown radioactive one.

Incubation of resting cells of *B. cereus* (2 mg of dry weight per ml) for 16 hr, in the presence of 20 $\mu$g/ml of thioxanthine led to the formation of a metabolite with the chromatographic and ultraviolet-absorbing properties of thiouric acid. Incubation

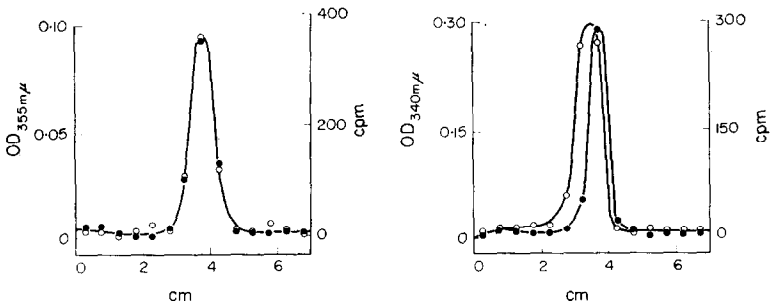


FIG. 3. Sensitivity of analysis method. Distribution of ultraviolet absorption (○——○) and radioactivity (●——●) along paper electrophoresis strips (pH 6·6). Left, radioactive metabolite A plus carrier thiouric acid; right, radioactive metabolite A plus carrier thioxanthine.

of 6-mercaptopurine with commercial xanthine oxidase, in the presence of catalase, gave rise to two products with the chromatographic and spectrophotometric properties of thioxanthine and thiouric acid. Table 1 gives an indication of the amount of the metabolites produced after two periods of incubation using the separation procedure shown in Fig. 1. Metabolite C was not distinguished clearly from thioxanthine in this experiment. It appears that thiouric acid was produced at a slower rate than

TABLE 1. RADIOACTIVE METABOLITES OF 6-MERCAPTOPURINE-8-$^{14}$C IN THE MEDIUM OF RESTING *B. cereus* CULTURES

| Compound | Code | Per cent of total radioactivity | |
|---|---|---|---|
| | | 16-hr incubation | 40-hr incubation |
| 6-Thiouric acid | A | 5 | 33 |
| 6-Thioxanthine + unknown | B + C | 50 | 42 |
| Unknown | D | — | 4 |
| 6-Mercaptopurine | — | 42 | 12 |
| Unknown | E | 3 | 9 |

thioxanthine, consistent with the hypothesis that 6-mercaptopurine was converted first to thioxanthine, which was subsequently oxidized to thiouric acid, Neither thioxanthine nor thiouric acid was found to inhibit the growth of *B. cereus* at concentrations up to 20 $\mu$g/ml. Since 6-mercaptopurine is inhibitory to growth at less than one-twentieth of this concentration, this metabolic route apparently involved detoxication. The three other metabolites produced by resting cells remain unidentified.

*Metabolism of 6-mercaptopurine by growing cells*

A dose-response curve with 6-mercaptopurine (Fig. 4) demonstrated that the generation time of *B. cereus* was essentially constant during the inhibition of growth at all the levels tested, whereas the duration of inhibition was related to the drug concentration. Similarly, Bolton and Mandel[10] have reported that the inhibition of the growth of *E. coli* by 6-mercaptopurine terminated spontaneously after some time, and

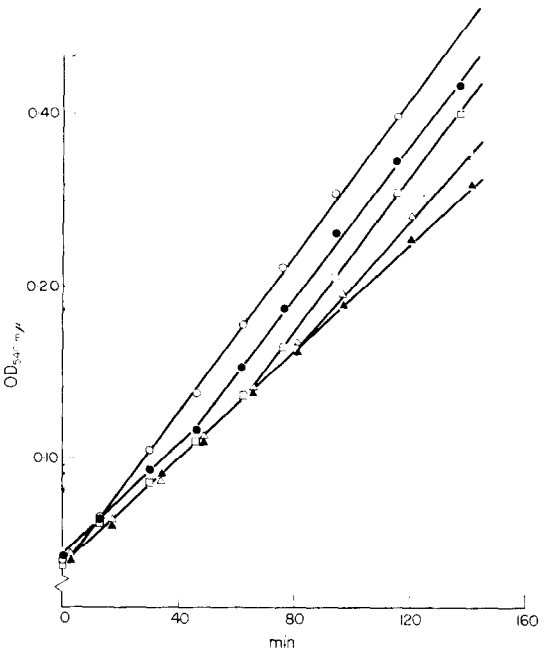


FIG. 4. Effect of various concentrations of 6-mercaptopurine on the growth rate of *B. cereus*. ○——○, 0 μg/ml; ●——●, 0·5 μg/ml; □——□, 1·0 μg/ml; △——△, 2·0 μg/ml; ▲——▲, 5·0 μg/ml.

normal growth resumed. Since metabolism of the drug could have been responsible for this cessation of inhibition, the metabolic pathways of 6-mercaptopurine were studied with growing bacteria. The conversion to thioxanthine and thiouric acid appeared to be of little importance in growing cells, since after 10 hr of growth in the presence of an excessive dose of 6-mercaptopurine (50 μg/ml) less than 10 per cent of the radioactivity remaining in the bacterial medium was recovered in the form of these metabolites.

Cells growing in the presence of 1 μg of either 6-mercaptopurine-8-$^{14}$C or 6-mercaptopurine-$^{35}$S per ml of medium were fractionated by the membrane technique. Fig. 5 shows the incorporation of the radioisotopes into the various fractions of the growing cells. It is clear that the fate of the two radioactive isotopes was quite different, implying that the sulfur was rapidly cleaved from the purine ring. Radioactive carbon was largely incorporated into the fraction soluble in hot TCA, which contained all of the nucleic acids. $^{35}$S from 6-mercaptopurine-$^{35}$S, on the other hand, was incorporated in the residue insoluble in hot TCA, which contained essentially all the protein of the cell,[27] and only a small amount of radioactivity could be extracted with hot TCA. The relatively high incorporation of radioactive carbon into the hot TCA-insoluble

residue soon after the addition of the drug was observed repeatedly. Although the nature of this substance is unknown, it is tempting to speculate that it may represent a purine which is closely associated with protein. The proportion of radioactivity from the carbon-labeled drug in the acid-soluble fraction was much smaller than that of the $^{35}S$ from the sulfur-labeled drug, a finding which implies that the removal of the sulfur was rapid and extensive. The decrease in the total radioactivity inside the cells, particularly with the $^{35}S$-labeled drug, was apparently due to the elimination of metabolites from the cell into the medium after the splitting of the sulfur-purine bond.

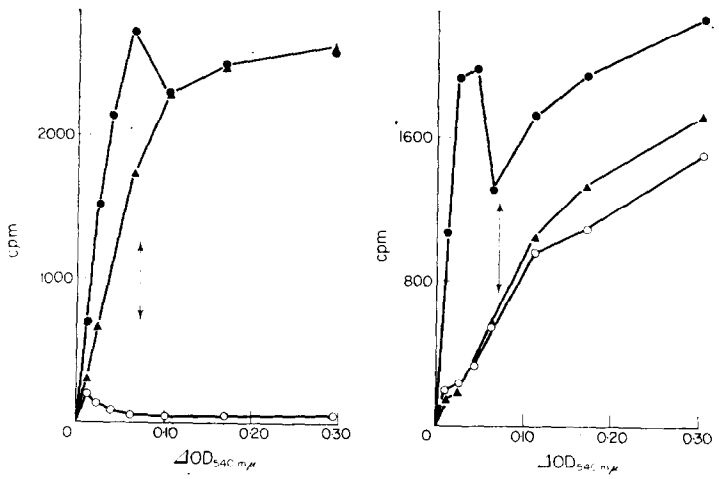


FIG. 5. Uptake of radioactivity from labeled 6-mercaptopurine by growing cells of *B. cereus*. Left, 8-$^{14}C$; right, $^{35}S$. ●——● intact cells; ▲——▲ residue after treatment with cold TCA; ○——○ residue after treatment with hot TCA. Inhibition stopped at arrow.

Fig. 5 demonstrates that when the growth inhibition ceased, radio-carbon was no longer present in the acid-soluble fraction, while the radioactivity in the residue insoluble in cold TCA remained constant. However, radioactive sulfur continued to be incorporated into all fractions at approximately the same rate, even after the inhibition had ceased. It is interesting that the initial phase of uptake of radiosulfur into the acid-soluble fraction had been completed prior to the cessation of inhibition.

The decrease in the concentration of 6-mercaptopurine in the growth medium during incubation was estimated after various periods of incubation, using the procedure described under Methods. The cells were grown with 6-mercaptopurine-8-$^{14}C$ at an initial concentration of 0·5 $\mu$g/ml. As is shown in Fig. 6, 6-mercaptopurine could no longer be detected in the medium at a time which coincided with the cessation of inhibition of cell growth.

*Metabolites of 6-mercaptopurine-8-$^{14}C$ in the nucleic acid fraction.* The mononucleotides derived from the RNA of cells which had been incubated for 2 hr in the presence of 2$\mu$g of 6-mercaptopurine-8-$^{14}C$ per ml were separated by paper electrophoresis at pH 3·5. Fig. 7 shows the distribution of ultraviolet absorption and radioactivity along the electrophoresis strip. Two major radioactive peaks were observed which coincided exactly in position with adenylic and guanylic acids and had the same ultraviolet absorption spectra. It was calculated that approximately 55 per cent of the

radioactivity from 6-mercaptopurine-8-$^{14}$C incorporated into the nucleic acids was present as adenine and 45 per cent as guanine. A small additional peak corresponding to another compound was also observed at about 8·5 cm from the origin. This product lacked the characteristic absorption at 320 m$\mu$ of a 6-mercaptopurine derivative, and its mobility was less than that expected for 6-mercaptopurine ribonucleotide. Since such a

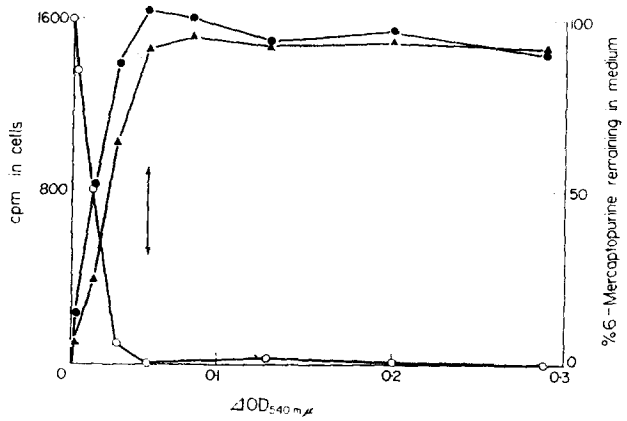


FIG. 6. Uptake of radioactivity from 6-mercaptopurine-8-$^{14}$C into growing cells of *B. cereus*; intact cells ●——●; residue after treatment with 5% TCA ▲——▲. Percentage of original 6-mercaptopurine remaining in the growth medium ○——○. Growth inhibition stopped at arrow.

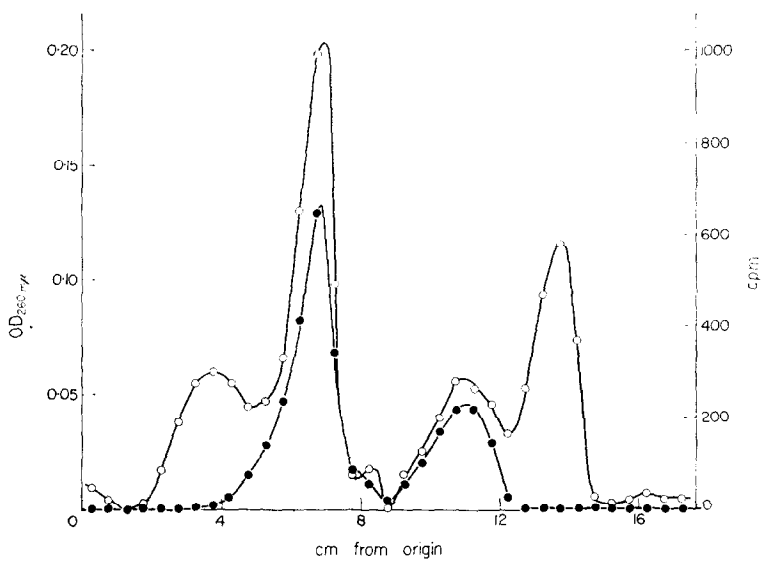


FIG. 7. Distribution of ultraviolet absorption (○——○) and radioactivity (●——●) along paper electrophoresis strip. Ribonucleotides from RNA of cells incubated with 6-mercaptopurine-8-$^{14}$C separated at pH 3·5.

compound has also been observed in experiments with labeled hypoxanthine, it was concluded that the product was derived from 6-mercaptopurine only after the cleavage of the mercapto group.

To determine whether or not 6-mercaptopurine was converted into the purines of DNA, bacteria were grown in the presence of 6-mercaptopurine-8-$^{14}$C, as is described

above, and DNA was isolated. After hydrolysis to obtain the purine bases, carrier 6-mercaptopurine was added and the mixture was subjected to electrophoresis at pH 9·7. Adenine and guanine were found to be radioactive, whereas no radioactivity could be detected in the 6-mercaptopurine area of the strip. Therefore, the presence of radioactivity from 6-mercaptopurine-8-$^{14}$C in the fraction soluble in hot TCA may be attributed to the conversion of the drug to adenine and guanine derivatives.

For purposes of comparison, bacteria were incubated for 1 hr with hypoxanthine-8-$^{14}$C at a concentration of 5 $\mu$g/ml and the RNA fraction was hydrolyzed and subjected to electrophoresis as before. The distribution of radioactivity along the paper strip was very similar to that shown in Fig. 7. Of the radioactive material in the nucleic acids, 60 per cent was recovered in the adenine and 40 per cent in the guanine components.

*The incorporation of* $^{35}$S *from 6-mercaptopurine-*$^{35}$S *into the protein fraction.* The distribution of $^{35}$S between the various cell fractions indicated that most of the radioactivity was incorporated into the proteins (Fig. 5). Cells which had been grown in the presence of 3 $\mu$g of 6-mercaptopurine-$^{35}$S per ml of culture for 2 hr were harvested and extracted to prepare the protein residue. The protein was treated with performic acid to oxidize the cysteine to cysteic acid and was then hydrolyzed. Carrier cysteic acid was added and the amino acid mixture was separated by paper electrophoresis. The isolated cysteic acid was radioactive, implying that the sulfur from the drug had been used by the cells in the synthesis of proteins. It seems reasonable to suppose that all the radio-sulfur in the residue insoluble in hot TCA was present as the sulfur amino acids formed from the sulfur split from 6-mercaptopurine.

*Metabolites of 6-mercaptopurine-*$^{35}$S *in the nucleic acid fraction.* Since a trace of radioactive sulfur was solubilized during the extraction of the nucleic acid fractions, attempts were made to establish an upper limit of the possible incorporation of 6-mercaptopurine-$^{35}$S as such into the nucleic acids. The extraction with hot TCA is known to remove more than nucleic acids alone, which do not contain sulfur. For instance, from *B. cereus* grown in the presence of cystine-$^{35}$S, about 10 per cent of the cellular radioactivity is extracted by this reagent.[30]

The mononucleotides derived from the RNA of a culture grown for 2 hr in the presence of 6-mercaptopurine-$^{35}$S at a concentration of 3 $\mu$g/ml, were chromatographed and the radioactivity along the paper was measured. In the areas to which the nucleotides migrated, two small unidentified peaks of radioactivity were observed. By assuming that both of these compounds contained 6-mercaptopurine itself, even though they were probably derived from contaminating non-nucleic acid material, and from a knowledge of the specific activity of the 6-mercaptopurine-$^{35}$S, a maximum of $2 \times 10^{-4}$ $\mu$mole of the drug could have been associated with 0·5 $\mu$mole of RNA guanine, or one residue of 6-mercaptopurine for 2500 of guanine. In all likelihood, therefore, there would be less than 1 mole of drug present in 10,000 normal ribonucleotide units, or in RNA having a molecular weight of $3 \times 10^6$.

The incorporation of 6-mercaptopurine-$^{35}$S into DNA also was examined, si ance trace of radioactivity was associated with this fraction. Treatment with trypsin of DNA prepared from cells incubated as above with 6-mercaptopurine-$^{35}$S reduced the radioactivity in the precipitable material by about 60 per cent, presumably due to the release of soluble peptides. After hydrolysis to liberate the purines, carrier 6-mercaptopurine was added and the mixture was subjected to paper electrophoresis

at pH 9·0. Since the 6-mercaptopurine area of the strip differed in radioactivity from that of the background by less than 3 counts/min, it was calculated that no more than $1{\cdot}6 \times 10^{-5}$ μmole of 6-mercaptopurine could have been present. Since the strip contained 0·157 μmole of guanine, the incorporation of 6-mercaptopurine did not exceed 1 mole for each 10,000 moles of guanine, or one 6-mercaptopurine residue for every 40,000 normal base residues.

*Stability of 6-mercaptopurine in alkali.* It was observed that 6-mercaptopurine is considerably less stable in alkaline than in acid solutions. The possibility existed that the digestion with potassium hydroxide used in the above experiment destroyed 6-mercaptopurine, thus precluding the possibility of detecting the original drug or its anabolites. After incubation of 6-mercaptopurine or its ribonucleoside in 1 N potassium hydroxide for 16 hr at room temperature, chromatography revealed a single ultraviolet-absorbing component, the spectrum of which was identical to that of the untreated material. Thus, it seems reasonable to assume that any 6-mercaptopurine ribonucleotide present in the nucleic acids would have been similarly unaffected by the digestion.

*Metabolites in the soluble fraction of the cells.* Curve 1 of Fig. 8 shows the distribution of radioactivity along a chromatogram of the metabolities in the soluble fraction from cells incubated with 6-mercaptopurine-$^{35}$S, as described in Methods. Since, by analogy with the metabolism of cysteine,[31] sulfite may be expected to be a metabolite of 6-mercaptopurine, the soluble fraction was dissolved in water, and 10 mg of sodium sulfite and excess hydrochloric acid were added. The mixture was then allowed to stand in a closed container near an open dish of barium acetate solution to trap volatile sulfur dioxide. The resulting barium sulfite precipitate was isolated and was found to be radioactive. Chromatography of the soluble fraction which had been subjected to the above acid-treatment indicated that the large slow-moving radioactive peak was reduced in size (Fig. 8 curve 2,).

To exclude the possibility that the extract contained radioactive metabolites other than sulfite which would be rendered volatile by acid, the ethanol extract derived from cells incubated with 6-mercaptopurine-$^{35}$S was divided into two equal portions. One portion was evaporated to dryness and 0·05 ml of saturated sodium bisulfite solution was added, followed by an equimolar amount of benzaldehyde. The benzaldehyde–bisulfite complex which formed was recrystallized from ethanol or *iso*propanol, and was found to be radioactive. To the second portion of the ethanol extract, 10 mg of sodium bisulfite and excess hydrochloric acid were added and sulfur dioxide was allowed to distill into barium acetate, as is described above. The solution was then evaporated to dryness and the benzaldehyde–bisulfite complex formed. The total radioactivity of this complex was considerably lower than in the first case. The amount of radioactivity lost from the extract by treatment with acid was essentially constant when determined in three ways (Table 2): (1) by direct counting of the radioactivity in the extract before and after the acid treatment; (2) by determining the amount of activity in the barium sulfite precipitate; and (3) by calculation from the difference in specific activities of the benzaldehyde bisulfite complexes formed with and without acid treatment. These results imply that inorganic sulfite was one of the metabolites of 6-mercaptopurine-$^{35}$S. In view of the probable reutilization of some of the sulfite for protein synthesis, an exact estimate of the extent of this desulfuration at any one time, as in the sampling procedure described here, would have little significance.

The presence of 6-mercaptopurine ribonucleotide was suggested by the work of Brockman *et al.*[11] and of Paterson.[32] In the present experiments, the chromatographic distribution of radioactive metabolites in the soluble fraction was compared using cells incubated with 6-mercaptopurine-$^{35}$S (curve 1, Fig. 8) and 6-mercaptopurine-8-$^{14}$C

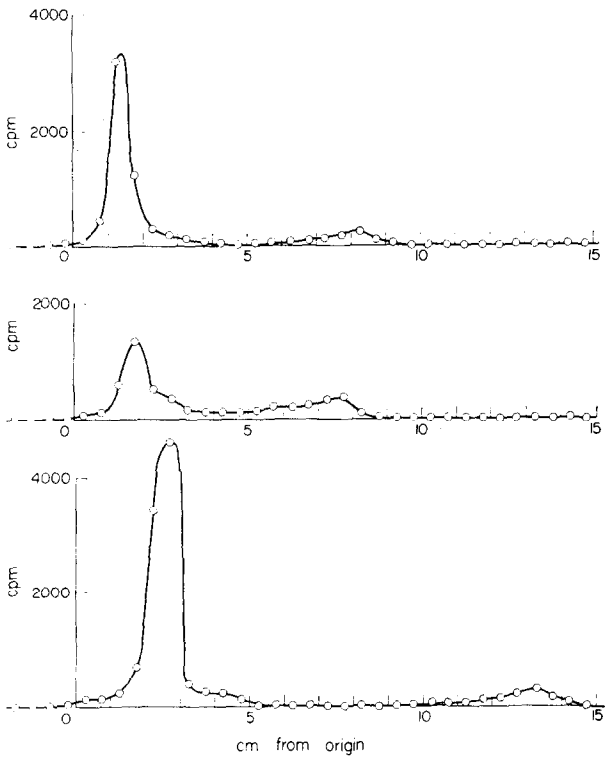


FIG. 8. Distribution of radioactivity along chromatograms of the soluble fraction of cells. Curve 1 (top), cells incubated with 6-mercaptopurine-$^{35}$S; curve 2 (center), cells incubated with 6-mercaptopurine-$^{35}$S after removal of sulfite; and curve 3 (bottom), cells incubated with 6-mercaptopurine-8-$^{14}$C.

TABLE 2. RADIOACTIVITY DERIVED FROM THE SOLUBLE FRACTION OF *B. cereus* AFTER INCUBATION WITH 6-MERCAPTOPURINE-$^{35}$S

(Acid-treatment volatilized sulfite as sulfur dioxide which was trapped as barium sulfite.)

| | Radioactivity (counts/min) | | |
|---|---|---|---|
| | Before acid-treatment | After acid-treatment | Volatilized by acid-treatment |
| Total activity of extract | 58,150 | 49,830 | 8320 |
| Total activity of barium sulfite precipitate | — | 8060 | 8060 |
| Total activity of benzaldehyde-bisulfite complex | 14,640 | 4440 | 10,200 |

(curve 3, Fig. 8). The major peaks in both fractions had a low $R_f$ (1·5–3·5 cm from origin), which is characteristic of nucleotides. These peaks were eluted and resolved by paper electrophoresis at pH 3·5. Apart from material remaining at the origin, there was only one other peak which coincided exactly in both paper strips. The properties of this material (low $R_f$, high electrophoretic mobility at pH 3·5, labeled with both $^{35}S$ and $^{14}C$) were similar to those expected of 6-mercaptopurine ribonucleotide.

For the identification of the ribonucleotide of 6-mercaptopurine, the major peak from curve 2 of Fig. 8 (cm 1·5–3·5) was eluted and treated with *Crotalus* venom to hydrolyze any ribonucleotides present to the corresponding ribonucleoside.[16] A known amount of authentic 6-mercaptopurine ribonucleoside was added as carrier and the mixture was chromatographed in the *iso*propanol–ammonia solvent, in which ribonucleosides have a higher $R_f$ than ribonucleotides. The ribonucleoside was eluted and subjected to paper electrophoresis using borate buffers at pH 8·3 and 9·0. The specific activities of sections of the ribonucleoside spot were constant along the length of the strip. It was concluded that the material which had a low $R_f$ in curve 2 of Fig. 8 contained 6-mercaptopurine ribonucleotide. Assuming 100 per cent hydrolysis by the phosphatase, about one-fifth of the total radioactivity of the soluble fraction was associated with the ribonucleotide, or about 1 $\mu$mole of the metabolite per g of dry weight of cells.

## DISCUSSION

The conversion of 6-mercaptopurine to thiouric acid in resting cells suggested that the enzyme xanthine oxidase was partly responsible for the metabolism of the drug, as had been demonstrated earlier for mammalian cells.[6,15,29] The similarity to the oxidation of hypoxanthine by this enzyme has been further demonstrated here with the observation that 6-mercaptopurine is converted by resting cells first to thioxanthine, which is then oxidized to thiouric acid. Thioxanthine has recently been shown to be a metabolite of thioguanine in mammalian tumor cells.[33]

This katabolic pathway is characteristic only of resting cells. Growing cells rapidly converted most of the drug into normal purines which were incorporated subsequently into nucleic acids. Thus, after a brief period of incubation, very little of the original compound remained to be degraded by the route characteristic of resting cells.

This conversion to normal purines is of importance, since a number of studies have suggested[34] that 6-mercaptopurine inhibits purine synthesis and purine interconversions because of an observed decrease in incorporation of a radioactive precursor into the nucleic acids or a lowering of the specific activity of the purines in the nucleic acids. It is clear that at least some of these decreases could be due to a dilution of the radioactivity from the precursor with non-radioactive purines derived from the drug. In those studies in which the total amount of nucleic acids was measured, however,[10,35] an actual decrease in nucleic acid content was shown to have been caused by the drug. Furthermore, since normal purines will reverse the growth inhibition, the metabolism of the drug itself produces compounds which will tend to overcome the inhibition.

Radioactive sulfur which had been cleaved metabolically from 6-mercaptopurine was lost rapidly into the growth medium, but later it re-entered the cell and was incorporated into the protein from which some of it was recovered as cysteic acid. Sulfite, which was concluded to be a product of metabolism of the drug, was present in the soluble fraction of the cells. It is possible that in the cell 6-mercaptopurine was

converted to purine-6-sulfinic acid which was then split to give sulfite and hypoxanthine. Since some of the radioactivity from cells grown in the presence of $^{35}$-S-amino acids which are incorporated mainly into proteins can be extracted by procedures which extract principally nucleic acids,[30] caution must be exercised in deciding whether or not radioactive sulfur from 6-mercaptopurine-$^{35}$S which is liberated during such extraction is, in fact, due to incorporation of the unchanged drug.

The results presented indicate that little if any 6-mercaptopurine was incorporated into the nucleic acids of *B. cereus*, in agreement with earlier investigations with *E. coli*,[10] but apparently at variance with those on animal tissues.[6] The upper limit of incorporation was calculated to be less than one molecule of 6-mercaptopurine in a molecule of RNA having a molecular weight of $3 \times 10^6$. It seems unlikely that such a low level of fraudulent nucleic acid, containing the unnatural base, could account for the biological activity of the drug, at least in micro-organisms, especially since inhibition of growth by 6-mercaptopurine begins almost immediately (which, therefore, would not allow for any such incorporation into nucleic acids) and ceases abruptly when all the drug has been metabolized to other compounds.

An alternative suggestion, namely that 6-mercaptopurine inhibits growth by interfering with cell metabolism at the nucleotide level, is supported here by the identification of 6-mercaptopurine ribonucleotide in the soluble fraction, as in the work of Brockman *et al.*[36] with *Streptococcus faecalis* and by Paterson[32] with a tumor. Since inability to synthesize this ribonucleotide has resulted in resistance to 6-mercaptopurine,[36] it has been suggested that the ribonucleotides of the unnatural purines may be, or are closely related to, the true inhibitors of metabolism and therefore of growth.

It is clear that the relatively short duration of inhibition of *B. cereus* (also observed in *E. coli* by Bolton and Mandel[10]) is due to the metabolism of the drug to inactive compounds. In the initial stages of inhibition sufficient 6-mercaptopurine is present to allow production of the inhibitory metabolite. Thereafter, the competition between these anabolic processes and the catabolism which results in the formation of normal purines may antagonize the inhibitory action of the drug, and reduce the concentration of 6-mercaptopurine below that required for inhibition of growth. Thus, the duration of inhibition produced by decreasing concentrations of 6-mercaptopurine will be shorter as bacterial enzymes can catabolize the smaller quantities of the drug more readily.

Since increasing concentrations of the drug in the bacterial medium did not produce greater inhibition, it is possible that the enzyme systems which produced the inhibitory metabolite became saturated. Concentrations as low as 0·1 $\mu$g of 6-mercaptopurine per ml were definitely growth inhibitory for short periods of time and thus must have been adequate to saturate this enzyme system.

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