A Mechanism of Resistance to 8-Azaguanine

I. Microbiological Studies on the Metabolism of Purines and 8-Azapurines*

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The problem of drug resistance is recognized by clinicians and experimentalists as one of the major difficulties in the advancement of the status of cancer chemotherapy (9, 43, 44, 51). The temporary control of certain human leukemias with metabolite antagonists such as the antifolics and antipurines is one of the more encouraging facets of cancer chemotherapy. The ultimate failure of these agents is associated with the emergence of populations of leukemic cells that no longer respond to drug treatment and are therefore generally termed drug-resistant. Law (27) has reviewed the problem of drug resistance in experimental neoplasms and has concluded that processes of selection of drug-resistant mutants are similar to those observed in bacterial chemotherapy.

Knowledge of the biochemical differences between the drug-susceptible population and the drug-resistant mutants that fail to respond to therapy, overgrow, and result in the refractory state might suggest means of circumventing this hurdle to more effective control of leukemia.

It has been observed by Elion et al. that mutations in Lactobacillus casei which lead to resistance to purine antagonists are accompanied by altered ability to metabolize one or more of the natural purines (16–20). Similar observations have been made on mutants of Streptococcus faecalis (1, 7, 24, 25). Since the discovery by Kidder and his associates (26, 39) of the anticancer activity of 8-azaguanine, azapurines have been the subject of intensive investigation. The experimental work of Smith and Matthews (46) on the metabolism of 8-azapurines and that of Mandel and his co-workers (29–34) on 8-azaguanine provide an excellent background for a detailed examination of resistance to this interesting class of purine analogs along the lines pointed out by Bennett et al. in an earlier study (4).

The present report, which elaborates a preliminary report (7), deals with studies designed to reveal the metabolic alterations associated with, and possibly responsible for, cellular resistance to 8-azaguanine. This study is divided into four major parts: (a) intermediary metabolism of natural purines and of 8-azapurines by wild S. faecalis and by 8-azaguanine-resistant mutants of this organism; (b) ability of these bacteria to incorporate 8-azaguanine into nucleic acids; (c) effects of 8-azapurines and derivatives on the growth of these bacteria; (d) intermediary metabolism of 8-azaxanthine and its incorporation into nucleic acid as 8-azaguanoylic acid by S. faecalis.

METHODS AND MATERIALS

The study of intermediary metabolism of radioactive purines and azapurines.—Streptococcus faecalis (ATCC 8043) was used as the parent strain from which the two 8-azaguanine mutants were selected in the manner previously described by Hutchison (25). The basal medium for growth and maintenance of all cultures was the F – PP + PGA modification (25) of the folic acid assay.

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medium of Flynn et al. (22). The 8-azaguanine-resistant mutants were maintained on the above medium which was supplemented with 100 μg of 8-azaguanine/ml. In certain growth experiments the F – PP + T medium (25) was used. The inoculum for tracer experiments consisted of twice-washed 34-hour broth cultures. In some experiments large flasks of medium were inoculated with S. faecalis and incubated at 37°C until half-maximum growth was approximated, as determined by optical density measurements; 12–14 hours’ incubation time was usually required. The flask cultures were then divided into 65-ml. portions so that several different tracer experiments could be run with the same bacterial culture.

In other experiments a 40-ml. culture was washed twice with physiological saline and was used to adjust the initial optical density of 500 ml. medium to 0.1–0.2. This inoculated medium was immediately subdivided into 65-ml. portions in 125-ml. Erlenmeyer flasks. For convenience in following the growth of the cultures, the flasks were fitted with 18 × 150 mm. culture tubes by means of a ground glass joint. This permitted inversion of the incubation flasks to determine the optical density at frequent intervals without necessitating removal of samples from the flasks. Logarithmic growth usually began about 1 hour after inoculation; radioactive substrates were added when half-maximum growth was approximated 2–3 hours after the massive inoculation. Under these cultural conditions the generation time of S. faecalis was calculated to be about 70 minutes.

Fifteen minutes after the addition of radioactive substrate (5 μc/65 ml medium), growth was stopped by pouring the 65-ml. cultures on 35 gm. of ice. Cells were then removed by centrifugation in a refrigerated centrifuge, and the packed cells were resuspended in 10 ml. of cold, distilled water. This suspension was immediately poured into 40 ml. of boiling absolute alcohol; the resulting 80 per cent alcohol was brought to boiling for 5 minutes. Centrifugation was repeated once and the supernatant was removed by centrifugation. The supernatant was dissolved in a small volume of 3-4 ml. in a Rinco rotating vacuum evaporator at a temperature of approximately 40°C., maintained by a water bath. This concentrate was lyophilized, and the residue was dissolved in 10 ml. of 0.5–0.6 ml. for chromatography. The volume of water used for redissolving the samples depended on the optical density of the culture at the time the radioactive substrate was added. In this manner comparable aliquots of extract could be used for chromatography even though some variation in optical density occurred in different experiments. Aliquots of 100 μl. and 80 μl. were placed near one corner of 18½ × 22½-inch sheets of unwashed Whatman No. 1 filter paper for chromatography in two dimensions (47). Each of the completed chromatograms was then placed in contact with two sheets of x-ray film. After 2 weeks the first film exposed to the 100-μl. chromatogram was developed; after 2 months’ exposure the second film was developed. Experience has shown that exposure for 2 months to the 100-μl. chromatogram was usually adequate to detect all but the very faintly radioactive areas on the chromatograms. The x-ray film exposed to 80-μl. chromatograms could be developed after intervals of 3 and 4 months as required by the radioautogram patterns.

The effects of purine antagonists on the intermediary metabolism of purines were studied by means of the technic described above. The non-radioactive purine antagonist was added to the exponentially growing bacterial culture 15 minutes prior to addition of the radioactive substrate. Fifteen minutes after addition of the labeled substrate, growth was stopped by pouring the culture on ice; subsequent treatment of the sample was then identical with that outlined above.

Confirmation of the identity of radioactive metabolites and quantitative comparisons of radioactivity.

—By means of the chromatographic-radioautographic technic of Tomisek (47), rather complete analysis of the cell components which are soluble in aqueous alcohol is possible. In the experiments reported here, the radioactive substrates used were the natural purines or 8-azapurines, and interest was focused on the free bases and their nucleosides and nucleotides. The two-dimensional Rf values permit tentative identification of the radioactive metabolites. Confirmation of the identity of purines and derivatives was obtained by eluting the radioactive areas of the 100-μl. chromatograms and rechromatographing the eluates in a single dimension; paper electrophoresis also proved useful in identification work. A 5 per cent sodium phosphate (pH 9)-isoamyl alcohol (3 vol.:1 vol.) solvent system was useful for purines; a 0.1 M sodium phosphate buffer (pH 6.8)-ammonium sulfate-n-propyl alcohol (100 vol.:60 vol.:2 vol.) solvent system proved useful for confirming

1 Maximum growth in experiments of this type corresponds to an optical density reading of about 1.0 measured in the Bausch and Lomb Spectronic 20 at 660 μm.

2 Aloe Scientific Division, A. S. Aloe Company, St. Louis, Mo.

3 In the experiments described, duPont 507, single-emulsion, blue-sensitive x-ray film was used throughout; the corresponding Eastman Kodak product has also proved satisfactory.
the identity of nucleosides and nucleotides. Acid hydrolysis of nucleosides and nucleotides to free purines and enzymatic hydrolysis of nucleotides to nucleosides was used. One-dimensional chromatography overnight on Whatman No. 3 MM paper with 70 per cent isopropanol-ammonia atmosphere followed by paper electrophoresis (33, 35) was used in conclusively identifying 8-azaguananyl acid. Direct chromatographic and electrophoretic comparison of 8-azaguananyl acid isolated from bacterial cells with enzymatically synthesized 8-azaguanosine-5'-phosphate (48) confirmed the identity of this compound.

The tentative identification of 8-azaguanosine di- and triphosphates (area A, Figures 2 and 3) in the soluble extracts is based on the chromatographic behavior in several solvent systems and the fact that partial acid hydrolysis yielded 8-azaguanic acid and complete acid hydrolysis yielded 8-azaguanine. Way et al. (49) prepared 8-azaguanosine di- and triphosphates chemically and enzymatically and found that these nucleotides could be separated in an isobutyric acid-ammonium isobutylate solvent system. Elution of the radioactive area A from two-dimensional chromatograms and rechromatography in Way's solvent system suggests the presence of 8-azaguanosine di- and triphosphates. It is possible that the area designated A on the radioautogram patterns (Figure 2) might contain some of the unidentified 8-azaguanine metabolite that Mandel and Markham (39) detected in medium in which Bacillus cereus was grown.

The total radioactivity of each metabolite was placed on a quantitative basis by eluting with water the radioactive areas from the 80-gl. chromatograms and evaporating the eluates on steel planchets. Radioactivity was then measured in counts/sec/planchet in internal gas-flow proportional counters to a probable error of 1 per cent. When the total radioactivity on planchets was quite low the probable error was greater, as indicated in the tabulated results.

Incorporation of 8-azaguanine-2-C\textsuperscript{14} into bacterial nucleic acids.—These studies were carried out with 1-liter cultures of bacteria which were incubated during logarithmic growth for at least 4 hours in the presence of 25 \(\mu\)c. of 8-azaguanine-2-C\textsuperscript{14}. The radioactive substrate was added in each case when the optical density of the culture reached approximately 0.8. After reaching maximum growth, the cells were removed by centrifugation, suspended in water, extracted in hot 80 per cent aqueous ethanol, and centrifuged. The debris was then suspended in 10 per cent sodium chloride and subjected to sonic vibration at 6°C. for 15 minutes in a Raytheon 50-watt, 9 Ke magnetostriiction oscillator.\textsuperscript{7} The sonicated suspension was heated at 95°–100°C. for 90 minutes, and the supernantant was filtered by gravity; extraction with hot 10 per cent sodium chloride was then twice repeated. The combined sodium chloride extracts were filtered by gravity and then poured into two volumes of alcohol and refrigerated overnight. The precipitate, consisting of sodium nucleate with some sodium chloride, was dissolved in warm water; two volumes of absolute alcohol and one volume of 0.8 N hydrochloric acid were added. The resulting precipitate of nucleic acid was separated by centrifugation and washed with ice water, alcohol, and ether. Ultraviolet absorption spectra and radioactivity were determined on samples of nucleic acids.

Proof of incorporation of 8-azaguanine-2-C\textsuperscript{14} into the nucleic acids of drug-susceptible .S. faecalis.—To prove that the radioactive activity present in the nucleic acids of bacteria was the result of incorporation of 8-azaguanine-2-C\textsuperscript{14}, the ribonucleic acid (RNA) portion of the combined nucleic acids was hydrolyzed enzymatically by incubation with yeast ribonuclease. Chemical hydrolysis was accomplished by treating the nucleic acids with 1 N KOH at 35°C. for 18 hours, followed by neutralization of KOH with HClO\textsubscript{4}. After refrigeration the potassium perchlorate was removed by centrifugation. The resulting mixture of 2',3'-mononucleotides obtained by enzymatic or by chemical hydrolysis was then analyzed chromatographically and electrophoretically (38). One-dimensional chromatography in 70 per cent isopropanol-ammonia atmosphere on Whatman No. 3 MM paper showed that a radioactive spot moved with guanylic acid; no radioactive 8-azaguanine could be detected. Electrophoresis\textsuperscript{8} in an ammonium formate buffer of pH 3.5 separated the four natural ribonucleotides; the radioactive compound again migrated with guanylic acid. When the RNA hydrolysate was subjected to electrophoresis in a 0.03 M horane buffer of pH 9.0, the radioactive compound was found to migrate ahead of guanylic acid and to exhibit fluorescence under ultraviolet light (Chart 2). The migration ratio of 8-azaguanine to this radioactive compound was 1:1.43. These data are in complete agreement with those of Matthews, who identified 8-azaguaninyl acid in nucleic acids from tobacco mosaic virus grown in the presence of 8-azaguanine (37).

Growth-inhibitory effects of purine antagonists on susceptible and drug-resistant strains of Streptococcus faecalis.—The effect of purine antagonists on the growth of S. faecalis was evaluated by aseptically adding a filter-sterilized solution of inhibitor to sterile F – PP + PGA medium. A twice-washed suspension of S. faecalis was adjusted to an optical density of 0.8 and diluted 1:100; 0.1 ml of this suspension served as a standard inoculum for 10 ml of medium. Growth was measured turbidimetrically after 18 hours' incubation at 37°C.

Radioactive substrates and nonradioactive compounds.—The following radioactive compounds were synthesized in the laboratories of Southern Research Institute: hypoxanthine-8-C\textsuperscript{14}, 5.1 mc/millimole;\textsuperscript{9} xanthine-8-C\textsuperscript{14}, 3.7 mc/millimole;\textsuperscript{9} 6-mercaptopurine-8-C\textsuperscript{14}, 2.5 mc/millimole;\textsuperscript{9} 2,6-diaminopurine-2-C\textsuperscript{14}, 2.3 mc/millimole;\textsuperscript{9} 8-azaguanine-2-C\textsuperscript{14}, 4 mc/millimole;\textsuperscript{9} 6-mercaptopurine-8-S\textsuperscript{14}, 0.3 mc/millimole. Radioactive substrates pur-

\textsuperscript{8} The electrophoresis apparatus used was similar to that described by Markham (35, 36). The authors are indebted to Dr. Roy Markham, Molteno Institute, University of Cambridge, and to Dr. George Mandel, George Washington University, Washington, D.C., for invaluable advice on the construction and application of this apparatus.

\textsuperscript{9} Synthesized by Dr. John A. Montgomery.

\textsuperscript{10} Synthesized by Dr. L. L. Bennett, Jr., and Mr. Harry T. Baker.

\textsuperscript{11} Synthesized by Mr. H. T. Baker using the procedure of English et al. (21). The 8-azaxanthine-2-C\textsuperscript{14} was freed of 8-azaguanine-2-C\textsuperscript{14} by passage through a Dowex 50 column at pH 5.
chased from Isotopes Specialties, Inc., were as follows: adenine-8-C\textsuperscript{14}, 1 mc/millimole; guanine-8-C\textsuperscript{14}, 1 mc/millimole; sodium formate-C\textsuperscript{14}, 1.4 mc/millimole.

Nonradioactive 6-mercaptopurine was obtained from the Wellcome Research Laboratories. Purine nucleosides and 5'-nucleotides were purchased from Pabst Laboratories. A sample of enzymatically synthesized 8-azaguanosine was a gift of Dr. Morris Friedkin, Washington University, St. Louis, and a chemically synthesized sample of this compound was obtained through the generosity of Dr. John Davoll, Parke, Davis and Company, Ltd., who also gave samples of 8-azaxanthosine, 8-azaalanosine, and 8-azaadenosine (14). Dr. James L. Way, University of Wisconsin, very kindly provided enough enzymatically synthesized 8-azaguanosine-5'-phosphate (48) to serve as a reference compound for chromatography and electrophoresis.

The 9-ethyl-8-azaguanine (3-ethyl-5-amino-7-hydroxy-3H-\textit{\textalpha}triazolo[4,3-d]pyrimidine) was synthesized in this laboratory by Dr. Fulmer Shealy; 4-amino-5-\textit{\textalpha}triazolecarboxamide was originally synthesized by Bennett and Baker (5), and an additional sample was synthesized by the organic preparations laboratory, Southern Research Institute, under the direction of Mr. W. E. Fitzgibbon.

The 8-azapurines used in the bacterial inhibition studies were purchased from the California Foundation for Biochemical Research or from Krishell Laboratories.

<table>
<thead>
<tr>
<th>TABLE 1</th>
</tr>
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<tr>
<td>COMPARISON OF THE GROWTH OF Streptococcus faecalis ON PURINES</td>
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<table>
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<th>PUPINE*</th>
<th>MOLECULAR CONCENTRATION</th>
<th>SF/O</th>
<th>SF/AZAG</th>
<th>SF/8-Aza</th>
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<tr>
<td>None</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Adenine</td>
<td>$1 \times 10^{-4}$</td>
<td>0.25</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Guanine</td>
<td>$1 \times 10^{-4}$</td>
<td>0.10</td>
<td>0</td>
<td>0</td>
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<td>Hypoxanthine</td>
<td>$1 \times 10^{-4}$</td>
<td>0.14</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Xanthine</td>
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<td>0</td>
</tr>
<tr>
<td></td>
<td>$3 \times 10^{-4}$</td>
<td>0.95</td>
<td>0</td>
<td>0</td>
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</tbody>
</table>

* The corresponding purine nucleosides are equivalent to the purine bases; purine nucleotides do not support the growth of SF/O.

† Incubation for 18 hours at 37°C on F-PP medium containing no folic acid but with 1 µg/ml of thymine added. Data for SF/MP have been reported (24, 25).

‡ Adenine inhibited growth.

RESULTS

Growth response of S. faecalis strains to natural purines and to 8-azapurines.—It has been observed that SF/O can utilize any one of the purines—adenine, guanine, hypoxanthine, and xanthine—or the corresponding nucleosides as its sole source of purines for growth on medium devoid of folic acid but with thymine added (24, 25). Mutants derived from SF/O that were resistant to 8-aza- guanine differed from the parent organism in their capacity to grow on natural purines. SF/AZAG, like SF/MP, could grow only on xanthine or xanthosine, and SF/8-Aza had lost the ability to utilize any of the purines or their ribosides for growth (Table 1). A mutant derived from SF/O that was resistant to 8-azaxanthine (SF/AZAX) was unable to grow on xanthine but could grow on adenine, guanine, and hypoxanthine.

GROWTH EXPRESSED AS PERCENT OF CONTROL

It was observed that any one of the azapurines inhibited growth of SF/O when grown on F-PP + PGA medium (Chart 1). At the inhibitor level used, 10 µg/ml, SF/AZAG was inhibited only by 8-azaxanthine; SF/8-Aza, on the other hand, was not inhibited by any of the azapurines (Chart 1). Growth of SF/MP, like that of SF/AZAG, was inhibited by 8-azaxanthine (24). SF/AZAX was found to be susceptible to inhibition by all the azapurines except azaxanthine.

It is of interest that SF/AZAG and SF/8-Aza were also resistant to 8-azaguanosine (Table 2). This result paralleled the observation that SF/MP is also resistant to the nucleoside of 6-mercaptopurine (45).

Alkylation of 8-azaguanine in the triazole ring,
as in the case of 9-ethyl-8-azaguanine, resulted in complete loss of activity as an inhibitor of growth of SF/O. As would be expected, the compound had no effect on SF/AZAG. It is known that the triazole analog of 4-amino-5-imidazolecarboxamide can be converted to 8-azaguanine in some biological systems (46). This compound inhibited growth of SF/O at a concentration 1000 times the inhibitory concentration of 8-azaguanine but was without effect on SF/AZAG and SF/8-Aza at a concentration 5000 times that at which 8-azaguanine inhibited SF/O.

Comparison of the conversion of purines to nucleotides by susceptible and drug-resistant bacteria.—By means of the chromatographic-radioautographic technic, a rather complete qualitative analysis of the intermediary metabolism of \textit{S. faecalis} strains was possible. SF/O, SF/MP, SF/AZAG, and SF/8-Aza readily accomplished the synthesis of purines \textit{de novo}, as evidenced by their capacity to grow in a folic acid medium that was devoid of purines and to metabolize formate-C\textsubscript{14} to radioactive purine nucleotides. SF/O was observed to metabolize each of the radioactive purines to the corresponding nucleotides and to interconvert these derivatives. The corresponding purine nucleosides were also frequently found in the soluble extracts. Thus, when any one of the radioactive purines was used as a substrate by SF/O, a rather complete radioautographic pattern of purines, nucleosides, and nucleotides was obtained (Table 3). In contrast to this ready capacity of SF/O to metabolize purines, 8-azaguanine-resistant \textit{S. faecalis} was unable to metabolize guanine-8-C\textsubscript{14} or

### TABLE 2

**Effects of 8-Azaguanine and 8-Azaguanosine on the Growth of SF/O, SF/AZAG, and SF/8-Aza**

<table>
<thead>
<tr>
<th>Molar Concentration</th>
<th>SF/O</th>
<th>SF/AZAG (8\text{-Aza} )</th>
<th>SF/8-Aza</th>
<th>SF/O</th>
<th>SF/AZAG</th>
<th>SF/8-Aza</th>
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<tr>
<td>Control</td>
<td>0.50</td>
<td>0.92</td>
<td>0.76</td>
<td>0.51</td>
<td>0.98</td>
<td>0.74</td>
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<td>(1 \times 10^{-8})</td>
<td>0.46</td>
<td>0.95</td>
<td>0.74</td>
<td>0.48</td>
<td>0.94</td>
<td>0.74</td>
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<tr>
<td>(5 \times 10^{-8})</td>
<td>0.36</td>
<td>0.95</td>
<td>0.74</td>
<td>0.40</td>
<td>0.92</td>
<td>0.75</td>
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<tr>
<td>(1 \times 10^{-7})</td>
<td>0.02</td>
<td>0.95</td>
<td>0.72</td>
<td>0.04</td>
<td>0.95</td>
<td>0.72</td>
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<td>(5 \times 10^{-7})</td>
<td>0</td>
<td>0.94</td>
<td>0.76</td>
<td>0</td>
<td>0.95</td>
<td>0.72</td>
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<td>(1 \times 10^{-6})</td>
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<td>0.75</td>
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<td>0.75</td>
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<td>0.76</td>
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<td>0.78</td>
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* Incubation for 20 hours at 37°C on Flynn's medium containing no purines or pyrimidines but with 1 mg. of folic acid/ml.

† 9-Ethyl-8-azaguanine was without effect on SF/O or SF/AZAG at a concentration of \(3 \times 10^{-8}\) M.

‡ 4-Amino-5-carboxamido-\(r\)-triazole was without effect on SF/O and SF/AZAG at a concentration of \(3 \times 10^{-8}\) M.

### TABLE 3

**Comparison of the Intermediary Metabolism of Purines by SF/O and SF/AZAG**

<table>
<thead>
<tr>
<th>Radioautogram</th>
<th>Adenine-8-C\textsubscript{14}</th>
<th>Guanine-8-C\textsubscript{14}</th>
<th>Hypoxanthine-8-C\textsubscript{14}</th>
<th>Xanthine-8-C\textsubscript{14}</th>
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</table>

* A zero indicates no activity detected by radioautography; a bracket indicates that the compounds were not separated by chromatography.

† Predominantly ATP; other di- and triphosphates may be present in small amounts.
hypoxanthine-8-C_14 (Figure 1; Table 3). On the other hand, adenine-8-C_14 and xanthine-8-C_14 were converted to purine nucleotides by SF/AZAG (Table 3). SF/MP was observed to metabolize purines in the same manner as SF/AZAG (5). Therefore, it was concluded that the drug-resistant organisms were unable to grow on hypoxanthine and guanine as a result of their inability to form nucleotides of these purines.

A discrepancy exists between the data on the growth response to purines and the metabolic studies with C_14-labeled purines. That discrepancy is the inability of SF/MP and SF/AZAG to grow on adenine as contrasted to their capacity to metabolize adenine-8-C_14 to AMP, ADP, and ATP. An explanation for this difference was had little, if any, effect on the conversion of adenine-8-C_14 or xanthine-8-C_14 to nucleotides (5). When 8-azaguanine was studied in a similar manner the result was unexpected. Not only did high levels of 8-azaguanine fail to inhibit metabolism of exogenous purines in exponentially growing cultures, but there were significant increases in the amounts of radioactive nucleotides formed in the 8-azaguanine-treated cultures as compared with untreated controls. One possible explanation for this result is that the formation of polynucleotides is altered by 8-azaguanylic acid or by the incorporation of 8-azaguanylic acid into the nucleic acid in such a way that mononucleotides accumulate. In considering these experimental results it is important to note that cultures in the exponential growth phase were exposed to 8-azaguanine for only 15 minutes prior to the addition of the radioactive substrate. Under such conditions it might be possible for nucleotide synthesis to continue for a short time in the inhibited cells, even though cell division was slowed or stopped. It will be necessary to couple studies of the effects of 8-azaguanine on nucleotide synthesis with the effects of 8-aza-guanine on the synthesis of nucleic acids from pre-formed purines and by biosynthesis de novo.

It has been observed in Bacillus cereus that 8-azaguanine can replace guanine in RNA (30, 46) and that the cells grown in the presence of 8-azaguanine contain more RNA than do untreated cells (33). Attention has recently been focused on the effects of 8-azaguanine on protein synthesis in this organism by Mandel (31) and by Chantrenne (10). It is not clear whether the observed effect of

<table>
<thead>
<tr>
<th>TABLE 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>COMPARISON OF THE INTERMEDIARY METABOLISM OF 8- AZAGUANINE-2-C_14 AND 8- AZAXANTHINE-2-C_14 BY Streptococcus faecalis</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Radioactivity (counts/sec) of compounds eluted from two-dimensional chromatograms†</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Radioautogram designation</strong></td>
</tr>
<tr>
<td><strong>F</strong></td>
</tr>
<tr>
<td><strong>C</strong></td>
</tr>
<tr>
<td><strong>D</strong></td>
</tr>
<tr>
<td><strong>E</strong></td>
</tr>
<tr>
<td><strong>G</strong></td>
</tr>
<tr>
<td><strong>B</strong></td>
</tr>
<tr>
<td><strong>AB</strong></td>
</tr>
<tr>
<td><strong>A</strong></td>
</tr>
</tbody>
</table>

* Small amounts of a compound tentatively identified as 8-azaosine have been detected accompanying 8-azaguanosine.
† Tentative identification.
‡ Some hydrolysis of azaguanylic acid di- and triphosphates sometimes takes place upon chromatography in the second dimension (n-butanol-propionic acid-water) yielding 8-azaguanosine di- and triphosphates.
§ Tentatively identified as 8-azaguanylic acid di- and triphosphates.

brought out in the study by Balis et al. (1) on the incorporation of exogenous purines into the nucleic acid of susceptible and resistant S. fae G. These authors observed that SF/MP was the only one of eight strains of S. fae G that was extremely inefficient in converting exogenous adenine to nucleic acid guanine. Consistent with this result is the observation that SF/MP was the only mutant which could not grow on adenine in the absence of folic acid (1). It appears that SF/AZAG is like SF/MP in that it can convert adenine to adenyl acid but presumably cannot efficiently convert adenyl acid to guanylic acid.

The effect of purine antagonists on the intermediary metabolism of purines.—It was previously observed that 6-mercaptopurine blocked the metabolism of hypoxanthine-8-C_14 and guanine-8-C_14 to the corresponding nucleotides in SF/O, whereas it had little, if any, effect on the conversion of adenine-8-C_14 or xanthine-8-C_14 to nucleotides (5). When 8-azaguanine was studied in a similar manner the result was unexpected. Not only did high levels of 8-azaguanine fail to inhibit metabolism of exogenous purines in exponentially growing cultures, but there were significant increases in the amounts of radioactive nucleotides formed in the 8-azaguanine-treated cultures as compared with untreated controls. One possible explanation for this result is that the formation of polynucleotides is altered by 8-azaguanylic acid or by the incorporation of 8-azaguanylic acid into the nucleic acid in such a way that mononucleotides accumulate. In considering these experimental results it is important to note that cultures in the exponential growth phase were exposed to 8-azaguanine for only 15 minutes prior to the addition of the radioactive substrate. Under such conditions it might be possible for nucleotide synthesis to continue for a short time in the inhibited cells, even though cell division was slowed or stopped. It will be necessary to couple studies of the effects of 8-azaguanine on nucleotide synthesis with the effects of 8-aza-guanine on the synthesis of nucleic acids from pre-formed purines and by biosynthesis de novo.

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8-azaguanine on protein synthesis is a result of the formation of fraudulent nucleotides or of the formation of an altered RNA.

Comparison of the metabolism of C\textsuperscript{4}-labeled 8-azapurines by susceptible and resistant bacteria.—Early in this study it was observed that SF/O metabolized 8-azaguanine-\textsuperscript{2}C\textsuperscript{14} to several derivatives, identified as 8-azaguanosine and 8-azaguanine nucleotides, whereas SF/AZAG, SF/8-Aza, and SF/MP failed to do so (7). Figure 2 illustrates this striking metabolic difference disclosed by means of the chromatographic-radioautographic technic; this result is expressed quantitatively in Table 4. There was no significant degradation of 8-azaguanine to 8-azaxanthine in either the sensitive or resistant strains of \textit{S. faecalis}. It is of interest that Remy has recently observed that a mutant of \textit{Escherichia coli} which was resistant to \textit{x},\textit{d}-diaminopurine failed to metabolize this compound to the nucleotide, whereas the parent strain of \textit{E. coli} did so (41).

### Table 5

<table>
<thead>
<tr>
<th>Combined nucleic acid†</th>
<th>Specific activity of nucleic acid (counts/sec/500 (\mu)g)</th>
<th>8-Azaguanine concentration resulting in 50 per cent growth inhibition ((\mu)g/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SF/O</td>
<td>1235</td>
<td>0.01</td>
</tr>
<tr>
<td>SF/AZAG</td>
<td>7.08</td>
<td>&gt;300</td>
</tr>
<tr>
<td>SF/8-Aza</td>
<td>1.09 ± 7%</td>
<td>&gt;300</td>
</tr>
<tr>
<td>SF/MP</td>
<td>26.2</td>
<td>20</td>
</tr>
</tbody>
</table>

*See Chart 2.
†RNA+DNA; \(\lambda_{max} = 259 \text{ m}\mu\).

If SF/O, the growth of which in culture tubes was markedly inhibited by as little as 0.01 \(\mu\)g/ml of 8-azaguanine, metabolizes 8-azaguanine to 8-azaguanylic acid, then SF/O might also be expected to incorporate 8-azaguaname into its nucleic acid. Conversely, SF/AZAG and SF/8-Aza, organisms which were not inhibited by 300 \(\mu\)g/ml of 8-azaguanine, and SF/MP, which was inhibited by 20 \(\mu\)g/ml of 8-azaguanine, did not form significant amounts of 8-azaguanylic acid and therefore would not be expected to incorporate 8-azaguanine into nucleic acid. Experiments designed to compare the incorporation of 8-azaguanine into bacterial nucleic acid provided experimental evidence supporting this postulate. As the data of Table 5 show, the nucleic acid from SF/O was much more radioactive than was that from SF/AZAG, SF/8-Aza, or SF/MP.

Proof that 8-azaguanine-\textsuperscript{2}C\textsuperscript{14} was actually incorporated into the nucleic acid of SF/O was obtained by enzymatic and chemical degradation of RNA to a mixture of \(\text{2}',\text{3}'\)-mononucleotides. 8-Azaguanylc acid-\textsuperscript{2}C\textsuperscript{14} was conclusively identified by the chromatographic and electrophoretic methods described by Markham (33, 35) and Matthews (37). No free 8-azaguanine-\textsuperscript{2}C\textsuperscript{14} was associated with the nucleic acids (Chart 2).

A comparison of the metabolism of 8-azaxanthine-\textsuperscript{2}C\textsuperscript{14} was made after it was demonstrated that 8-azaxanthine inhibited the organisms that are capable of metabolizing xanthine (SF/O, SF/AZAG, and SF/MP), but failed to inhibit SF/8-Aza, the mutant that is unable to grow on xanthine. The 8-azaxanthine-\textsuperscript{2}C\textsuperscript{14} was metabolized to 8-azaxanthosine, 8-azaguanosine, 8-azaguanylic acid, and to 8-azaguanosine di- and triphosphates by SF/O, SF/AZAG, and SF/MP (Figure 3, Table 4); no 8-azaxanthilic acid was found in extracts from \textit{S. faecalis}. The 8-azaxanthine-\textsuperscript{2}C\textsuperscript{14} was not significantly metabolized by SF/8-Aza and was recovered unchanged in the soluble extracts from the cells (Table 4).

A comparison of the ability of these organisms to incorporate 8-azaxanthine-\textsuperscript{2}C\textsuperscript{14} into their
nucleic acids was made. The results, paralleling those obtained with 8-azaguanine-2-C\textsuperscript{14}, showed that 8-azaxanthine-2-C\textsuperscript{14} was incorporated as 8-azaguanic acid into the nucleic acids of SF/O, SF/AZAG, and SF/MP, the organisms which metabolize 8-azaxanthine to 8-azaguanic acid, whereas the nucleic acids of SF/8-Aza were devoid of radioactivity (Table 6). Proof that 8-azaxanthine-2-C\textsuperscript{14} was incorporated into the nucleic acids of SF/O and SF/AZAG as 8-azaguanic acid was obtained, as before, by hydrolysis of RNA and identification of 8-azaguanic acid-2-C\textsuperscript{14} as the sole radioactive compound in the resulting mixture of 2',3'-mononucleotides.

**Experimental evidence that 8-azaguanine nucleotides are probably the active inhibitors.**—Indirect evidence that 8-azaguanic acid is the inhibitory intermediate rather than 8-azaguanine itself was obtained by studies with 9-ethyl-8-azaguanine. Since the ethyl group blocks the 9-position, one would predict that this compound could not inhibit growth if a "lethal synthesis" to a nucleotide is a necessary step. Experimental results demonstrated that 9-ethyl-8-azaguanine was without effect on the growth of \textit{S. faecalis} at levels more than 1000 times the inhibitory level for 8-azaguanine. The results obtained from the study of metabolism of 8-azaxanthine-2-C\textsuperscript{14} also point to 8-azaguanic acid as the active inhibitor.

If it could be demonstrated that 8-azaguanic acid inhibited the 8-azaguanine-resistant organisms or interfered with nucleotide metabolism in cell-free enzyme preparations, then direct evidence that the nucleotide is the active form of the inhibitor would be provided. Chemical and biosynthetic preparations of 8-azaguanic acid are being made for assay against 8-azaguanine-resistant biological systems.

**Resistance to 6-mercaptopurine.**—Preliminary studies comparing the metabolism of 6-mercaptopurine by susceptible and resistant bacteria suggest that a picture similar to that drawn for 8-azaguanine may emerge (7). When the metabolism of 6-mercaptopurine-8-C\textsuperscript{14} was compared in SF/O, SF/MP, and SF/AZAG, it was observed that SF/O extensively metabolized 6-mercaptopurine; SF/MP and SF/AZAG failed to do so. Hypoxanthine, inosine, inosinic acid, and other natural purine derivatives were identified as metabolites of 6-MP-8-C\textsuperscript{14} in extracts from SF/O; therefore, it was evident that some 6-mercaptopurine-8-C\textsuperscript{14} was being converted to hypoxanthine-8-C\textsuperscript{14}. Balis \textit{et al.} (2) also have observed that SF/O metabolizes 6-MP-8-C\textsuperscript{14} to hypoxanthine-8-C\textsuperscript{14}, which was then used as an exogenous purine source for synthesis of nucleic acid purines. To eliminate this difficulty, a study was made using 6-mercaptopurine-S\textsuperscript{35} as a substrate; SF/O metabolized 6-MP-S\textsuperscript{35}, but SF/MP and SF/AZAG did not. One of the derivatives of 6-MP-S\textsuperscript{35} formed by SF/O behaved chromatographically like a ribotide. Balis \textit{et al.} (1, 2) have speculated that one mechanism of resistance to 6-mercaptopurine might be the result of failure to convert 6-MP to the nucleotide.

**DISCUSSION**

The results of this study, when coupled with knowledge of purine biosynthesis (8, 23), permit construction of a rather complete diagram depicting the metabolism of natural purines, of 8-azaguanine, and of 8-azaxanthine by \textit{S. faecalis} (Chart 3). Using this diagram as a basis for discussion, the mechanism postulated for resistance to 8-azaguanine—failure to metabolize 8-azagu
nine to 8-azaguanylic acid—becomes apparent. The capacity of SF/AZAG to convert 8-azaxanthine to 8-azaguanylic acid and thus circumvent the 8-azaguanine resistance in SF/AZAG points to 8-azaguanylic acid as the inhibitory derivative of 8-azaguanine. The lack of inhibition of SF/O by 9-ethyl-8-azaguanine supports the view that 8-azaguanine must be metabolized to 8-azaguanylic acid in order to become inhibitory. By the terminology of Peters (40), the conversion of 8-azaguanine to 8-azaguanylic acid might then be looked on as a "lethal synthesis." The selection, from a population of susceptible bacteria, of mutants that are unable to convert 8-azaguanine to 8-azaguanylic acid is considered to be the manner by which the cultures designated SF/AZAG and SF/8-Aza arose.

Welch and Handschumacher (50) first observed that resistance of S. faecalis to a pyrimidine antagonist, 6-azauracil, was associated with a diminished ability to convert uracil to uridine and suggested that perhaps such resistant cells could not convert 6-azauracil to 6-azauridine. Subsequent studies by Welch and his associates have shown that 6-azauracil-resistant S. faecalis were indeed inhibited by 6-azauridine.

Davis and Maas have carefully considered mechanisms by which resistance to a drug might arise (12, 13). Of the seven theoretical mechanisms listed by these authors, two appear to be possible for 8-azaguanine: (a) decreased conversion of the administered compound into a more active inhibitor; and (b) decreased penetration of the cell by the inhibitor.

The experimental evidence gathered in the course of this work has led us to accept the first of these mechanisms. Initial results from work in progress in this laboratory on the conversion of purines to nucleotides by cell-free enzyme preparations from SF/O, SF/AZAG, and SF/MP have shown that the pyrophosphorylases normally present in SF/O which catalyze the conversion of guanine, hypoxanthine, 6-mercaptopurine, and 8-azaguanine to the corresponding nucleotides are missing, or are inactive, in SF/AZAG and SF/MP. Davidson (11) has recently presented the results of experiments in which he observed no differences in permeability to 8-azaguanine of 8-azaguanine-susceptible or -resistant L1210 ascites cells in vitro. Altered cell permeability does not appear to be a factor in resistance to 8-azaguanine.

Since resistance to 8-azaguanine is accompanied by inability to convert guanine to guanylic acid it appears possible that guanine pyrophosphorylase is the enzyme that converts 8-azaguanine to 8-azaguanic acid (Chart 4). Results showing that 8-azaguanine-resistant S. faecalis were also resistant to 8-azaguanosine (Table 2) suggest that conversion of 8-azaguanosine to 8-azaguanylic acid by the action of ATP and a kinase may not be significant.

Results of a study of the in vivo metabolism of 8-azaguanine-2-C\(^{14}\) by mouse neoplasms that are susceptible or resistant to growth inhibition by 8-azaguanine have been reported by Bennett et al. (4). This study has recently been extended (6), and the results show that the growth of those neoplasms which convert significant amounts of 8-azaguanine to 8-azaguanylic acid is inhibited by 8-azaguanine. Conversely, those neoplasms which fail to accomplish the formation of 8-azaguanylic acid are resistant to growth inhibition by 8-azaguanine. The consistent results obtained in biological systems as diverse as bacterial and mammalian cells provide the basis for the hypothesis that the formation of 8-azaguanic acid, or derivatives, is a necessary prerequisite for growth inhibition and for the corollary hypothesis that resistance to 8-azaguanine stems from failure to form nucleotides of 8-azaguanine.

Smith and Matthews, as a result of correlation of growth inhibition by 8-azaguanine with incor-
poration of the drug into the nucleic acids of several biological systems, have concluded that 8-azaguanine is inhibitory as a result of its incorporation into nucleic acids (46). As the present study shows, one can equally well correlate growth inhibition with 8-azaguanic acid formation which, in turn, is directly related to ability to incorporate 8-azaguanine into nucleic acids. The present study has shown that 8-azaguanine is probably metabolized as depicted in Chart 5. The resistance mechanism proposed in the case of 8-azaguanine is that the first step in this sequence (Chart 5) does not occur to any significant extent in the drug-resistant organisms examined in this study. If resistance arises as a result of gene mutation, independent of treatment, and subsequent selection of the mutants by the drug, it is possible that other mechanisms of resistance to 8-azaguanine will be found. For example, other resistant organisms might convert 8-azaguanine to 8-azaguanic acid but then be unable to carry out further steps on the route to fraudulent co-factors or fraudulent nucleic acids.12

Considerable evidence has been accumulated justifying the conclusion that the genetic mechanism in bacteria is similar to that in higher organisms and that a genetic mechanism is responsible for the origin and transmission of resistance (15, 28). From the studies made on the metabolism of 8-azaguanine in bacteria and experimental neoplasms that are resistant to the drug it appears that the same genetic mechanism of resistance to 8-azaguanine exists in these biological systems.

SUMMARY

1. The parent, 8-azaguanine-susceptible strain of Streptococcus faecalis (SF/O), which is able to utilize any one of the purines—adenine, guanine, hypoxanthine, or xanthine—for growth, was found to metabolize 8-azaguanine-2-C\(^{14}\) to 8-azaguanosine-3'-phosphate. The nucleic acids isolated from SF/O grown in the presence of 8-azaguanine-2-C\(^{14}\) yielded 8-azaguanonic acid upon enzymatic and chemical hydrolysis.

2. An 8-azaguanine-resistant mutant of S. faecalis (SF/AZAG) was observed to grow on xanthine as a purine source but was unable to grow on other purines. SF/AZAG failed to convert 8-azaguanine to 8-azaguanic acid and failed to incorporate significant amounts of the fraudulent purine into nucleic acids.

3. A different 8-azaguanine-resistant mutant (SF/8-Aza) was unable to grow even on xanthine and, like SF/AZAG, failed to form 8-azaguanic acid or to incorporate 8-azaguanine into nucleic acids.

4. 8-Azaxanthine was inhibitory to SF/O and SF/AZAG but not to SF/8-Aza; SF/O and SF/AZAG metabolized 8-azaxanthine-2-C\(^{14}\) to 8-azaguanic acid-2-C\(^{14}\), whereas SF/8-Aza failed to do so. After incubation with 8-azaxanthine-2-C\(^{14}\), the nucleic acids of SF/O and SF/AZAG contained 8-azaguanic acid-2-C\(^{14}\), whereas those of SF/8-Aza did not. SF/MP metabolized 8-azaxanthine-2-C\(^{14}\) in the same manner as did SF/AZAG.

5. It was observed that high concentrations of 9-ethyl-8-azaguanine were without effect in inhibiting growth of S. faecalis, an organism the growth of which is inhibited by very low concentrations of 8-azaguanine. Growth of 8-azaguanine-resistant organisms was not inhibited by 8-azaguanosine.

6. From these results it is concluded that, in order to become inhibitory, 8-azaguanine must first be metabolized to 8-azaguanic acid, which may then exert its growth-inhibitory effect by interfering with nucleotide metabolism, by being

12 It has recently been observed that 6-thioguanine-sensitive and -resistant Ehrlich ascites carcinoma cells can metabolize 6-thioguanine to 6-thioguanic acid (38, 42). At higher doses of thioguanine the resistant cells were not so efficient in converting thioguanine to thioguanic acid. This difference was attributed to increased degradation of thioguanine by the resistant cell line.
incorporated into nucleic acids, or by forming a fraudulent co-factor.

Resistance to inhibition by 8-azaguanine in the microbiological systems studied can be interpreted as resulting from inability of the resistant mutants to metabolize 8-azaguanine to 8-aza- guanylic acid. Those organisms that were unable to metabolize 8-azaguanine were also incapable of metabolizing guanine or hypoxanthine to nucleo- tidies. The hypothesis is set forth that the enzymes that convert these bases to nucleotides are missing, or are inactive, in the drug-resistant mutants studied.

ACKNOWLEDGMENTS

The authors wish to acknowledge the able technical assistance of Mrs. Margarette Simpson and Mrs. Ava Wilson in the chromatographic and electrophoretic analyses. We wish to thank Dr. Glynn P. Wheeler, Dr. L. L. Bennett, Jr., and Dr. George G. Kelley for valuable suggestions and discussions during the course of this work.

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11. DAVIDSON, J. D. Permeability of Resistant L1210 Leuke-


Fig. 1.—Radioautograms comparing the intermediary metabolism of hypoxanthine-8-C14 and guanine-8-C14 by SF/O and SF/8-Aza.

<table>
<thead>
<tr>
<th>Acid</th>
<th>Identification</th>
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<tbody>
<tr>
<td>51, 53</td>
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</tr>
<tr>
<td>59</td>
<td>Adenine</td>
</tr>
<tr>
<td>25</td>
<td>Adenosine</td>
</tr>
<tr>
<td>3</td>
<td>Hypoxanthine</td>
</tr>
<tr>
<td>5</td>
<td>Inosine</td>
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</table>

<table>
<thead>
<tr>
<th>GMP</th>
<th>Guanine</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>Guanosine</td>
</tr>
<tr>
<td>17</td>
<td>Xanthine</td>
</tr>
<tr>
<td>11</td>
<td>IMP</td>
</tr>
<tr>
<td>34</td>
<td>ATP</td>
</tr>
<tr>
<td>12</td>
<td>GTP</td>
</tr>
</tbody>
</table>


Fig. 2.—Radioautograms comparing the metabolism of 8-aza-8-C$^{14}$ by SF/0, SF/AZAG, SF/8-Aza, and SF/MP.

A 8-Azaguanosine di- and triphosphates
B 8-Azaguanic acid
C 8-Azaguanosine
D 8-Azaguanosine (tentative)

E 8-Azaxanthosine
F 8-Azaguanine
G 8-Azadenosine

Legend:
1. Unidentified
2. Guanine
3. GMP
4. Adenine
5. Guanosine
6. AMP
7. Adenosine
8. Xanthine
9. ADP
10. Hypoxanthine
11. IMP
12. GTP

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Fig. 3.—Radioautograms comparing the intermediary metabolism of 8-azaxanthine-2-C¹⁴ by SF/O, SF/AZAG, and SF/8-Aza.

| A 8-Azaguanosine di- and triphosphates | E 8-Azaxanthosine |
| B 8-Azaguanic acid | F 8-Azaguanine |
| C 8-Azaxanthine | G 8-Azaadenosine (tentative) |
| D 8-Azaguanosine | H Unidentified |


**Figure 1.** Radioautograms comparing the intermediary metabolism of hypoxanthine-8-C14 and guanine-8-C14 by SF/O and SF/8-Aza. 51, 53 Unidentified 4 Guanine 16 GMP 52 Adenine 45 Guanosine 29 AMP 25 Adenosine 44 Xanthine 17 ADP 3 Hypoxanthine 11 IMP 34 ATP 5 Inosine 12 GTP.
A Mechanism of Resistance to 8-Azaguanine I. Microbiological Studies on the Metabolism of Purines and 8-Azapurines

R. W. Brockman, Carolyn Sparks, Dorris J. Hutchison, et al.


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