- Hokin, L. E. & Hokin, M. R. (1959b). *J. biol. Chem.* **234**, 1387.
- Ingle, D. J. (1954). *Acta endocr., Kbh.*, **17**, 172.
- Jones, M. E. (1962). In *Methods in Enzymology*, vol. 5, p. 912. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Knauff, H. G. & Windsheimer, F. (1960). *Arch. exp. Path. Pharmacol.* **239**, 442.
- Leevy, C. M., Hollister, R. M., Schmid, R., McDonald, R. A. & Davidson, C. S. (1959). *Proc. Soc. exp. Biol., N.Y.*, **102**, 672.
- Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
- McLean, P. (1961). *Nature, Lond.*, **191**, 1302.
- McLean, P. & Gurney, M. W. (1963). *Biochem. J.* **87**, 96.
- Neubert, D. & Maibauer, D. (1959). *Arch. exp. Path. Pharmacol.* **235**, 291.
- Oberling, Ch. & Rouiller, Ch. (1956). *Ann. Anat. path.* **1**, 401.
- Ratner, S. (1955). In *Methods in Enzymology*, vol. 2, p. 356. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Recknagel, R. O. & Litteria, M. (1960). *Amer. J. Physiol.* **36**, 521.
- Recknagel, R. O. & Lombardi, B. (1961). *J. biol. Chem.* **236**, 564.
- Recknagel, R. O., Stadler, J. & Litteria, M. (1958). *Fed. Proc.* **17**, 129.
- Rees, K. R. & Sinha, K. P. (1960). *J. Path. Bact.* **80**, 297.
- Rees, K. R., Sinha, K. P. & Spector, W. G. (1961). *J. Path. Bact.* **81**, 107.
- Reichard, H. (1959). *J. Lab. clin. Med.* **53**, 417.
- Richter, G., Frunder, H. & Börnig, H. (1958). *Hoppe-Seyl. Z.* **310**, 249.
- Rossi, F. & McLean, P. (1963). *Nature, Lond.*, **197**, 1207.
- Rossi, F. & Zatti, M. (1963a). *Experientia*, **19**, 197.
- Rossi, F. & Zatti, M. (1963b). *Brit. J. exp. Path.* **44**, 131.
- Schimke, R. T. (1961). *Cold Spr. Harb. Symp. quant. Biol.* **26**, 363.
- Schimke, R. T. (1962). *J. biol. Chem.* **237**, 459.
- Schimke, R. T. (1963). *J. biol. Chem.* **238**, 1012.
- Selye, H. (1950). *The Physiology and Pathology of the Exposure to Stress*, p. 161. Montreal: Acta Inc. Medical Publications.
- Smuckler, E. A., Iseri, O. A. & Benditt, E. P. (1961). *Biochem. biophys. Res. Commun.* **5**, 270.
- Smuckler, E. A., Iseri, O. A. & Benditt, E. P. (1962). *J. exp. Med.* **116**, 55.
- Thiers, R. E., Reynolds, E. S. & Vallee, B. L. (1960). *J. biol. Chem.* **235**, 2130.
- Tsuboi, K. K., Stowell, R. E. & Lee, C. S. (1951). *Cancer Res.* **11**, 87.
- Warren, R. L. (1959). *Colloq. spectrosc. int.* **8**, 213.

Biochem. J. (1964) **91**, 270

Action of 8-Azaguanine and 8-Azaxanthine on *Pseudomonas aeruginosa*

By F. BERGMANN, HANNA UNGAR-WARON AND HANNA KWIETNY-GOVRIN

Department of Pharmacology, The Hebrew University-Hadassah Medical School, Jerusalem, Israel

(Received 19 June 1963)

Bergmann, Ungar-Waron, Kwietny-Govrin & Leon (1962) found that 6-mercaptopurine does not inhibit the growth of *Pseudomonas aeruginosa*. These bacteria oxidize the antimetabolite very rapidly, at a rate exceeding that of hypoxanthine or xanthine, and thus prevent its incorporation into nucleic acids. This observation led to a study of other growth inhibitors of the purine series which because of structural peculiarities cannot serve as substrates of the bacterial purine-oxidizing system. The present paper deals with the action of 8-azaguanine and related compounds on the growth of *P. aeruginosa* and on the production of purine-catabolizing enzymes.

MATERIALS AND METHODS

Purines. The following derivatives were synthesized: 2- and 6-thioxanthine (Beaman, 1954); 6-thiouric acid (Levin, Kalmus & Bergmann, 1960); 2- and 8-thiouric acid (Loo, Michael, Garceau & Reid, 1959); 3-methyl-2-thioxanthine

and 3-methyl-2-thiouric acid (Bergmann, Levin, Kalmus & Kwietny-Govrin, 1961); 3-methyluric acid (Bergmann & Dikstein, 1955); 8-azaxanthine (Roblin, Lampen, English, Cole & Vaughan, 1945).

6-Mercaptopurine and uracil were obtained from California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A., and 8-azaguanine from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.

Other heterocyclic compounds. Benzimidazole and benzotriazole were prepared according to *Organic Syntheses* (1948, 1955).

Bacterial cultures. The strain of *P. aeruginosa* with high xanthine-oxidase activity was that described by Dikstein, Bergmann & Henis (1957). The organism was grown in a synthetic medium similar to that described by Davis & Mingioli (1950): KH_2PO_4 , 3 g.; K_2HPO_4 , 7 g.; sodium citrate dihydrate, 0.5 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g.; glucose, 5 g./l. The pH was adjusted to 7.0. The salt mixture was sterilized at 15 lb./in.² for 20 min. and the glucose passed separately through a Seitz filter.

The cells were grown by shaking at 30°. Samples were withdrawn at hourly intervals and the extinctions determined in a 1 cm. cell at 550 m μ in a Beckman spectrophotometer.

meter. When E_{550} exceeded 0.7, the bacterial suspension was diluted serially with 0.9% NaCl until the extinction was 0.4–0.7. By a colony-counting procedure a calibration curve was established relating the number of cells/ml. to E_{550} .

At the end of the growth period, the cells were collected and washed with 0.05M-phosphate buffer, pH 7.4, in a Lourdes refrigerated centrifuge at 5° until the supernatant exhibited an extinction less than 0.1 in the range of 260–300 m μ .

Enzyme experiments. Dikstein *et al.* (1957) found that the xanthine-oxidase activity of *P. aeruginosa* is not stable after lysis of the cells. The same has now been observed for the degradative action of the bacterium against uric acid. For this reason, oxidative activities were measured in washed resting cells, shaken with the substrate in 0.05M-phosphate buffer, pH 7.4, at 32°. Samples (4 ml.) were withdrawn at suitable intervals and were centrifuged at 10 000 rev./min. and 5° for 20 min. The supernatant was used for spectrophotometric readings. The oxidative activity against purines containing free CH groups in the ring ('xanthine-oxidase-like activity') was determined by following the initial linear increase in E_{305} and E_{315} of a solution of 3-methyl-2-thioxanthine (10 mg./l.) (Bergmann *et al.* 1962). This is a convenient one-step reaction, as the end product, namely 3-methyl-2-thiouric acid, is not further degraded by the bacterial cells. Similarly, the oxidation of uric acid (10 mg./l.) was measured by following the decline of E_{295} (Dikstein *et al.* 1957). Control vessels contained the bacterial suspension in the same buffer, but without substrate. These measurements, carried out with normal resting cells from an 8 hr. culture, served as a basis for the comparison of cultures that were of a different age or had undergone special treatments.

Rates are expressed as percentages of the rates of the standard substrates at the same cell count. A linear relationship exists between cell number and velocity of oxidation for the four compounds that were examined (xanthine, 6-thioxanthine, 3-methylthioxanthine and uric acid).

Thiouric acids were tested with cells collected after 8 hr. of growth and incubated as described above with 10 μ g. of the uric acid derivatives/ml. For 6-thiouric acid 8×10^9 cells/ml. were used and readings were taken at 330 and 347 m μ . For 2- and 8-thiouric acid, 1.2×10^{10} cells/ml. were employed; the oxidation of the former was measured at 308 m μ and of the latter at 307 and 315 m μ .

Paper chromatography. Mixtures of 8-azaguanine and 8-azaxanthine were analysed by descending paper chromatography on Whatman no. 1 paper, with 95% (v/v) ethanol-water-acetic acid (17:2:1, by vol.) as solvent. The R_F values were: azaguanine, 0.36; azaxanthine, 0.26. Spots were located by their fluorescence under a Mineralight ultraviolet lamp emitting light of about 255 m μ .

RESULTS

Effect of 8-azaguanine on the growth rate of *Pseudomonas aeruginosa*. Azaguanine, in concentrations up to 100 μ g./ml., did not retard growth (Fig. 1 and Expt. 2 in Table 1). The negative result may be due to inability of the cells to absorb the antimetabolite or to convert it into 'active' derivatives, such as the corresponding nucleoside or nucleotide, that can be incorporated into the nucleic acids (Smith & Matthews, 1957). However, in view of previous experience with 6-mercapto-

Fig. 1. Influence of various inhibitors on the growth of *Pseudomonas aeruginosa*. The cells were grown in the synthetic medium described in the Materials and Methods section. Samples were withdrawn from the cultures at suitable intervals, and their extinctions were measured in a Beckman spectrophotometer at 550 m μ . The following antimetabolites were added: O, none; +, 8-azaguanine (100 μ g./ml.) (this curve coincides with the control); ▲, benzimidazole (250 μ g./ml.); △, benzotriazole (250 μ g./ml.); ●, 8-azaxanthine (100 μ g./ml.).

purine (Bergmann *et al.* 1962), the possibility of metabolic degradation of 8-azaguanine was investigated. Indeed, when the culture medium was examined after incubation for 24 hr., the antimetabolite had been converted quantitatively into 8-azaxanthine. On the other hand, after incubation for 9 hr., i.e. at the end of the logarithmic phase, the antimetabolite was essentially unchanged. After 11 hr., some azaxanthine could already be detected, and after 17 hr. most of the 8-azaguanine had undergone deamination. 8-Azaxanthine was identified both spectrophotometrically and by paper chromatography.

Influence of 8-azaxanthine on the growth of Pseudomonas aeruginosa. When 8-azaxanthine was added to the culture medium at 10–100 mg./l., it produced marked effects. After a short initial growth period of about 3 hr., complete standstill ensued for about 10 hr. (Fig. 1). Growth was then resumed at such speed that the slope of the new logarithmic phase exceeded that of the control. After about 26 hr. the cell count reached the same level that had been attained by the control within 8 hr.

The strain emerging from such a culture medium had a normal growth rate, whether subcultured in the presence or absence of the antimetabolite (Expts. 5 and 6 in Table 1). Likewise, growth remained unaffected if after several passages through pure culture medium the cells were exposed a second time to azaxanthine (Expt. 7 in Table 1). Clearly the resistance to this antimetabolite has become hereditary after a single exposure, suggesting selection of refractory cells that had already been present in the original strain.

Deamination of 8-azaguanine proceeds so slowly that during the logarithmic phase only very low concentrations of 8-azaxanthine can accumulate. Although these minute amounts of inhibitor have no measurable effect on the growth rate, they may cause a gradual selection of resistant cells, i.e. a

strain exposed to 8-azaguanine may be resistant to 8-azaxanthine. However, as shown in Table 1 (Expt. 3), exposure of *P. aeruginosa* to azaguanine followed by passage through a medium containing azaxanthine results in full inhibition by the latter.

Resting cells of *P. aeruginosa* are not affected by azaxanthine. Only during active cell division does a resistant strain emerge.

Possible mechanisms of growth inhibition by azaxanthine. The growth-retarding effect of azaxanthine may be due to an action on some stage of purine metabolism. Although this compound is a strong inhibitor of mammalian urate oxidase (Norris & Roush, 1950), it did not affect the activity of xanthine or urate oxidase of normal resting cells at concentrations up to 100 mg./l., which affect growth very strongly.

Since the antimetabolite may interfere in the biosynthesis of enzymes concerned with purine metabolism, the oxidation rates of 3-methyl-2-thioxanthine and of uric acid were measured at various stages of growth in normal cells and in cells exposed to azaxanthine for the first time (Table 2). In the controls, xanthine-oxidase activity is highest at the onset of the logarithmic phase, declines gradually during growth and further during the subsequent stationary phase, whereas urate-oxidase activity is maintained throughout at an approximately constant level. In cells exposed for the first time to azaxanthine, xanthine-oxidase activity is greatest during the initial inhibitory phase and decreases steadily when growth is resumed and likewise during the stationary phase, but remains always at a level about twice as high as that in the original strain. On the other hand, the urate-oxidase activity of cells growing in the presence of azaxanthine reaches its lowest level near the end of the inhibitory phase and recovers progressively during the following logarithmic phase, without, however, attaining the control level.

Table 1. *Influence of 8-azapurines on the growth of Pseudomonas aeruginosa*

Cells were grown in the synthetic medium described in the Materials and Methods section and supplemented with purines as shown below. The cell density was determined by measuring E_{550} after 8 hr., i.e. at the end of the logarithmic phase of the control culture. 8-Azaxanthine exhibits its strongest inhibitory action after incubation for 8 hr. (see Fig. 1).

Expt. no.	No. of passages through			Growth of final culture
	8-Azapurines (100 mg./l.)	Synthetic medium	8-Azaxanthine (100 mg./l.)	
1	—	1	—	Normal
2	1 (8-Azaguanine)	—	—	Normal
3	1 (8-Azaguanine)	—	1	Full inhibition
4	1 (8-Azaxanthine)	—	—	Full inhibition
5	1 (8-Azaxanthine)	1–6	—	Normal
6	3 (8-Azaxanthine)	—	—	Normal
7	1 (8-Azaxanthine)	6	1	Normal
8	1 (Xanthine* and 8-azaxanthine)	1	1	Full inhibition

* Xanthine concn. 100 mg./l.

Table 2. *Oxidative activities of bacterial cells grown under various conditions*

Cells were grown in the synthetic medium described in the Materials and Methods section. They were collected after the growth periods specified, washed repeatedly and incubated with substrate at 32° in 0.05M-phosphate buffer, pH 7.4. All values in the Table represent rates relative to those (designated as 100) of an equal number of cells of the normal strain after 8 hr. of growth. Each value was obtained as the average of three experiments.

Growth period (hr.)	Control	After first exposure to 8-azaxanthine (100 mg./l.)	After three consecutive passages through 8-azaxanthine (100 mg./l.)
Oxidation of 3-methyl-2-thioxanthine			
4	280	470	200
8	100	350	100
24	80	200	65
48	40	90	70
Oxidation of uric acid			
4	170	80	170
8	100	10	100
24	80	40	75
48	80	45	85

Table 3. *Antagonism between 8-azaxanthine and various purines*

Cells were grown in the synthetic medium described in the Materials and Methods section, in the presence of 8-azaxanthine (100 mg./l.) and of the purine indicated in column 1. Growth was measured after incubation for 4 hr., i.e. during the logarithmic phase of the control culture, by measuring E_{550} . In this Table the extinction of the control culture after 4 hr. of growth is designated as 100.

Purine added to synthetic medium	Relative growth (%)
None	19
Hypoxanthine (100 mg./l.)	135
6-Mercaptopurine (100 mg./l.)	43
Xanthine (100 mg./l.)	160
2-Thioxanthine (100 mg./l.)	75
3-Methyl-2-thioxanthine (100 mg./l.)	80
6-Thioxanthine (100 mg./l.)	85
Uric acid (50 mg./l.)	30
3-Methyluric acid (100 mg./l.)	35
6-Thiouric acid (250 mg./l.)	30
3-Methyl-2-thiouric acid (100 mg./l.)	30
Uracil (100 mg./l.)	20

The marked changes in enzymic activities noted in Table 2 are observed only during the first exposure to azaxanthine. In an established resistant strain the potency of either enzyme is identical with that of the control within the limits of accuracy of the analytical method (see column 4 of Table 2).

The above results may suggest an imbalance in the formation and removal of uric acid as the source of growth inhibition by azaxanthine. It was found that uric acid in the highest concentration

attainable (50 mg./l.) has no effect whatsoever on growth. However, it is not known whether uric acid can penetrate through the membrane or undergoes degradation at the cell surface. Therefore the lack of effect of added uric acid does not exclude the possibility that metabolic imbalance is a factor in inhibition by azaxanthine.

Antagonism between various purines and 8-azaxanthine. Although rapidly oxidized, hypoxanthine or xanthine reverses the growth inhibition by 8-azaxanthine, when supplied to the culture medium in large concentrations, and significantly accelerates cell multiplication (Table 3). Uric acid, at 50 mg./l., counteracts the growth inhibition only slightly.

Simultaneous incubation of the *Pseudomonas* with xanthine and azaxanthine prevents the selection of resistant cells. When the strain was subsequently exposed to azaxanthine alone, the full inhibitory effect of the latter was obtained (Expt. 8 in Table 1).

6-Mercaptopurine and 6-thioxanthine also counteract the growth inhibition by azaxanthine, thioxanthine being the more powerful. 6-Thiouric acid, like uric acid itself, has only a feeble action. Similarly, 3-methyl-2-thioxanthine antagonizes azaxanthine effectively, whereas its oxidation product 3-methyl-2-thiouric acid has only small potency (Table 3). These results clearly indicate that only purines with an imidazole ring, but not those with an imidazolone moiety, can avert growth inhibition by azaxanthine effectively. Uracil and other pyrimidines did not check the action of 8-azaxanthine.

Influence of various purines on growth and enzyme production. None of the derivatives tested in Table 3 affects the growth rate of the native strain, with the exception of 3-methyl-2-thioxanthine which curbs it slightly, but they do enhance the production of purine-oxidizing enzymes. Thus either hypoxanthine or xanthine, when present in the culture medium, causes a large increase in the xanthine-oxidase-like activity of normal cells (Table 4). Uric acid, 3-methyluric acid, or 6- or 8-thiouric acid have no effect. 6-Mercaptopurine and 6-thioxanthine augment the xanthine-oxidase activity to a comparable degree. 2-Thioxanthine has no measurable influence on enzyme formation, whereas its 3-methyl derivative is effective. However, the oxidation product of the latter, namely 3-methyl-2-thiouric acid, is again devoid of any influence.

Comparison of Tables 3 and 4 indicates that a certain parallelism exists between stimulation of xanthine-oxidase production and the antagonism of purines to azaxanthine-induced growth retardation; however, quantitative differences are apparent. On the other hand, column 3 in Table 4

Table 4. *Rate of oxidation of 3-methyl-2-thioxanthine by Pseudomonas aeruginosa after growth in the presence of various purines*

Cells were grown for 8 hr. in the synthetic medium described in the Materials and Methods section, in the presence of the purine indicated in column 1. The xanthine-oxidase-like activity of washed cell suspensions was measured with 3-methyl-2-thioxanthine (10 mg./l.) as substrate. This compound also served as standard for determination of the relative rate of oxidation of various purines by washed bacterial cells. The values in column 3 are taken from the work of Bergmann *et al.* (1962).

Purine added to synthetic medium	Relative xanthine-oxidase-like activity (%)	Relative rate of oxidation of purine by resting cells (%)
None	100	—
Hypoxanthine (100 mg./l.)	1300	22
Xanthine (100 mg./l.)	1700	33
Uric acid (50 mg./l.)	100	—
3-Methyluric acid (100 mg./l.)	130	—
2-Thioxanthine (100 mg./l.)	120	25
3-Methyl-2-thioxanthine (100 mg./l.)	630	100
3-Methyl-2-thiouric acid (100 mg./l.)	130	—
6-Mercaptopurine (100 mg./l.)	400	47
6-Thioxanthine (100 mg./l.)	280	15
6-Thiouric acid (100 mg./l.)	100	—
8-Thiouric acid (100 mg./l.)	130	—
8-Azaxanthine (100 mg./l.)	350	—
8-Azaxanthine (100 mg./l.) + hypoxanthine (100 mg./l.)	1300	—
Uracil (100 mg./l.)	170	—
Benzimidazole (250 mg./l.)	40	—
Benzotriazole (250 mg./l.)	50	—

reveals that enhancement of enzyme production by a given purine does not parallel its rate of oxidation by the same enzyme.

The influence of various purines on urate-oxidase-like activity of *P. aeruginosa* is demonstrated in Table 5. Again hypoxanthine or xanthine, when added to the active medium, enhances this activity immensely. 6-Thioxanthine is less powerful and uracil has only a small effect. Even uric acid stimulates the urate-oxidase activity only moderately. The results in column 3 of Table 5 express again the lack of any definite relationship between the affinity of a substrate to urate oxidase and its effect on the formation of this enzyme.

Effect of benzimidazole and benzotriazole on Pseudomonas aeruginosa. The specificity observed in Table 3 suggests that the imidazole ring of purines may be important for their competition with azaxanthine. If so, benzimidazole and benzotriazole might also be expected to affect cell growth.

Both substances proved to be only weak growth inhibitors (Fig. 1), benzotriazole being somewhat

superior. However, the mechanism of their action is fundamentally different from that of azaxanthine, as shown by the experiments in Table 6.

During successive passages through a culture medium containing benzotriazole, growth retardation becomes more and more pronounced (Expts. 1-3 in Table 6). After exposure to the inhibitor, the strain returns only gradually to its normal growth rate when transferred to pure synthetic medium (Expt. 4 in Table 6). Cells that have been grown in the presence of benzotriazole retain their full sensitivity to azaxanthine (Expt. 7 in Table 6). Accordingly, simultaneous exposure to benzotriazole and azaxanthine produces a very strong inhibition that is still marked after incubation for 24 hr. (Expt. 8 in Table 6). The combination of these two inhibitors also prevents the selection of an azaxanthine-resistant mutant (Expt. 10 in Table 6). In addition, the growth impairment due to benzo-

Table 5. *Rate of oxidation of uric acid by Pseudomonas aeruginosa after growth in the presence of various purines*

The conditions were the same as in Table 4. The urate-oxidase-like activity of washed-cell suspensions was determined with uric acid (10 mg./l.) as substrate. The relative rates of oxidation of thiouric acids refer to the rate of uric acid degradation with the same number of cells/ml. The substrate concentrations were 10 mg./l. throughout. The oxidation of 2-thiouric acid was measured with 1.2×10^{10} cells/ml., by the decline of E_{308} . Degradation of 8-thiouric acid was also assessed with 1.2×10^{10} cells/ml., by following the decrease of E_{307} and E_{315} . The oxidation of 6-thiouric acid was measured with 8×10^9 cells/ml., by following changes in E_{330} and E_{347} .

Purine added	Relative urate-oxidase-like activity (%)	Relative rate of oxidation of purine by urate oxidase (%)
None	100	—
Hypoxanthine (100 mg./l.)	1900	—
Xanthine (100 mg./l.)	1900	—
Uric acid (50 mg./l.)	300	100
3-Methyluric acid (100 mg./l.)	100	0
2-Thioxanthine (100 mg./l.)	100	—
3-Methyl-2-thioxanthine (100 mg./l.)	135	—
3-Methyl-2-thiouric acid (100 mg./l.)	100	0
2-Thiouric acid (100 mg./l.)	80	0.05
6-Mercaptopurine (100 mg./l.)	100	—
6-Thioxanthine (100 mg./l.)	430	—
6-Thiouric acid (100 mg./l.)	80	1
8-Thiouric acid (100 mg./l.)	80	0.25
8-Azaxanthine (100 mg./l.)	10	—
8-Azaxanthine (100 mg./l.) + hypoxanthine (100 mg./l.)	1800	—
Uracil (100 mg./l.)	180	—
Benzimidazole (250 mg./l.)	80	—
Benzotriazole (250 mg./l.)	85	—

Table 6. *Effect of benzotriazole on the growth of Pseudomonas aeruginosa*

The cell density was determined as in Table 1. At any given moment the cell counts are expressed as percentages of the number of cells/ml. in a control culture of the same age. The concentration of benzotriazole was 250 $\mu\text{g./ml.}$, and that of 8-azaxanthine was 100 $\mu\text{g./ml.}$ All experiments, with the exception of Expt. 5, were carried out with the azaxanthine-sensitive strain. In Expts. 4, 7, 9, 10 and 11, the values in the Table represent the growth obtained in the last subculture indicated.

Expt. no.	Growth inhibitor added	Relative growth (%) after incubation for					
		2 hr.	3 hr.	4 hr.	5 hr.	6 hr.	12 hr.
1	Benzotriazole	73	62	45	39	45	68
2	Benzotriazole, second passage	43	28	17	12	12	72
3	Benzotriazole, third passage	35	20	13	8	8	53
4	Benzotriazole $\rightarrow$ pure medium*	58	57	50	55	73	100
5	Benzotriazole on azaxanthine-resistant strain	64	40	28	25	27	63
6	Azaxanthine, first exposure	57	37	19	10	7	75
7	Benzotriazole $\rightarrow$ azaxanthine	41	26	13	8	7	85
8	Benzotriazole and azaxanthine combined†	67	32	17	8	5	44
9	Benzotriazole and azaxanthine $\rightarrow$ pure medium	90	82	79	70	69	65
10	Benzotriazole and azaxanthine $\rightarrow$ pure medium $\rightarrow$ azaxanthine	58	37	21	14	8	83
11	Benzotriazole and azaxanthine $\rightarrow$ pure medium $\rightarrow$ benzotriazole	58	49	39	38	42	62

* Horizontal arrow indicates that the bacteria were first exposed to benzotriazole and subsequently transferred to the pure synthetic medium described in the Materials and Methods section. The values represent the growth rate under the latter conditions.

† In Expt. 8, *P. aeruginosa* was exposed to a mixture of benzotriazole and azaxanthine and then subcultured in pure medium (Expt. 9) and subsequently in medium containing azaxanthine alone (Expt. 10) or benzotriazole alone (Expt. 11).

triazole is also decreased by the simultaneous exposure to azaxanthine. Expt. 9 in Table 6 shows that a strain that had been grown first in the presence of benzotriazole and azaxanthine, and was subsequently passed through pure medium, recovered much faster than cells exposed to benzotriazole alone (compare Expts. 1 and 4 in Table 6). Benzotriazole appears to be more effective against the azaxanthine-resistant than against the native strain (Expts. 1 and 5 in Table 6).

Both benzimidazole and benzotriazole decrease the xanthine-oxidase-like activity of *P. aeruginosa* to about one-half, whereas they have relatively little effect on the urate-oxidase-like activity (see Tables 4 and 5).

DISCUSSION

In sensitive cells, 8-azaguanine is converted into the corresponding nucleosides and nucleotides and is then incorporated into the nucleic acids, predominantly into RNA (Smith & Matthews, 1957; Mandel, 1957, 1961). The resulting impairment of protein synthesis is the most common mechanism by which azaguanine interferes with growth (Chantrenne & Devreux, 1958, 1960). Azaguanine-resistant strains, as a rule, are unable to accomplish the chain of reactions leading to incorporation of the antimetabolite (Brockman, Sparks, Hutchison & Skipper, 1959). Azaxanthine has no effect on azaguanine-sensitive strains, unless it can be converted by the cell into azaguanine or its derivatives,

notably 8-azaguanic acid (Smith & Matthews, 1957).

In view of these observations, the behaviour of *P. aeruginosa* towards the azapurines is unusual. The strain employed in the present experiments is completely refractory towards azaguanine, but is strongly inhibited by azaxanthine. The lack of any growth-retarding effect of azaguanine cannot be explained by deamination of the antimetabolite because this reaction, as shown in the present studies, lags considerably behind growth. Presumably the cells of the *Pseudomonas* are unable to absorb azaguanine.

Under the influence of azaxanthine a resistant mutant of *P. aeruginosa* emerges rapidly. The resistant cells resemble the native strain in all respects so closely that their refractoriness may be ascribed provisionally to lack of penetration of azaxanthine. Thus, in contrast with its effect on the native strain, azaxanthine does not induce increased xanthine-oxidase activity in the resistant cells.

Although benzotriazole and benzimidazole potentiate the growth-inhibitory effect of azaxanthine, they prevent selection of an azaxanthine-resistant mutant. This may be explained by the assumption that the benzo derivatives penetrate both into azaxanthine-sensitive and azaxanthine-resistant cells and suppress the multiplication of either cell type alike. Indeed, Expt. 5 in Table 6 demonstrates the great susceptibility of the

azaxanthine-resistant mutant to the inhibitory effect of benzotriazole.

Hypoxanthine or xanthine, when added to the standard medium, does not influence the growth of *P. aeruginosa*, but in the presence of azaxanthine each stimulates growth above that of the control level. It is suggested that azaxanthine improves the direct utilization of these purines, perhaps by opening up metabolic pathways that normally are not available or are only of minor importance. This unique corollary of azaxanthine inhibition needs further investigation.

The antagonism of various purines to growth inhibition by azaxanthine (Table 3) does not correspond in detail to their action on xanthine-oxidase formation (Table 4). A similar discrepancy is evident for their effect on urate-oxidase formation (Table 5). Thus the activity of the cells towards uric acid is strongly depressed when azaxanthine is added to the culture medium, and is powerfully stimulated by hypoxanthine or xanthine, whereas uric acid produces only a slight enhancement. Similarly, 6-thioxanthine stimulates urate-oxidase formation markedly, whereas 6-thiouric acid is ineffective. 2-Thioxanthine is inert and its 3-methyl derivative enhances enzyme production only very slightly, although both are converted into 2-thiouric acids with considerable speed (Bergmann *et al.* 1962). Apparently, sulphur in position 2 greatly decreases the affinity of purines to the receptor.

It cannot be decided at present whether the higher efficiency of hypoxanthine or of xanthine derivatives in the above examples is related to a structure of the receptor site that fits an imidazole ring better than an imidazolone moiety, or whether the xanthine derivatives serve as precursors that can penetrate more easily into the cells and supply the corresponding uric acids at an intracellular site.

The present experiments show that 8-azaxanthine affects a number of different sites. According to Smith & Matthews (1957), azapurines are incorporated by *Bacillus cereus* mainly into RNA. From the present experiments with *P. aeruginosa* it cannot be decided whether azaxanthine acts as such or is converted inside the cell into one or more active forms, the latter competing with normal metabolites during RNA synthesis. It is noteworthy that certain unnatural purines, which presumably are not capable of incorporation, antagonize various actions of azaxanthine.

SUMMARY

1. 8-Azaguanine does not inhibit the growth of *Pseudomonas aeruginosa*, but undergoes slow deamination.

2. 8-Azaxanthine arrests the growth of this species temporarily. This growth retardation is

abolished by hypoxanthine, xanthine and a number of unnatural purines.

3. During growth inhibition by azaxanthine, the xanthine-oxidase-like activity of the bacterial cells is enhanced. Much larger increments of enzymic activity are obtained by the addition of hypoxanthine, xanthine or certain unnatural purines, which all contain an unsubstituted imidazole ring.

4. During growth inhibition by 8-azaxanthine, the urate-oxidase-like activity of the bacteria is strongly depressed. On the other hand, the addition of hypoxanthine or xanthine to the culture medium produces a huge increase in the enzymic activity of the normal strain.

5. After the first exposure to 8-azaxanthine a resistant strain emerges. This strain shows normal xanthine-oxidase and urate-oxidase activity, even when growing in the presence of the antimetabolite.

6. Benzimidazole and benzotriazole are weak growth inhibitors. They depress xanthine-oxidase activity of the bacterial cells, but leave their urate-oxidase activity unaffected.

This work was supported by Grant no. GM 06631-04 from the National Institutes of Health of the United States of America. Some of this work was included in a Ph.D. Thesis submitted by H.U.-W. to the Faculty of Science, The Hebrew University, Jerusalem, Israel.

REFERENCES

- Beaman, A. G. (1954). *J. Amer. chem. Soc.* **76**, 5633.
- Bergmann, F. & Dikstein, S. (1955). *J. Amer. chem. Soc.* **77**, 691.
- Bergmann, F., Levin, G., Kalmus, A. & Kwietny-Govrin, H. (1961). *J. org. Chem.* **26**, 1504.
- Bergmann, F., Ungar-Waron, H., Kwietny-Govrin, H. & Leon, S. (1962). *Biochim. biophys. Acta*, **55**, 512.
- Brockman, R. W., Sparks, C., Hutchison, D. J. & Skipper, H. E. (1959). *Cancer Res.* **19**, 177.
- Chantrenne, H. & Devreux, S. (1958). *C.R. Soc. Biol., Paris*, **152**, 1202.
- Chantrenne, H. & Devreux, S. (1960). *Biochim. biophys. Acta*, **39**, 486.
- Davis, B. D. & Mingioli, E. S. (1950). *J. Bact.* **60**, 12.
- Dikstein, S., Bergmann, F. & Henis, Y. (1957). *J. biol. Chem.* **224**, 67.
- Levin, G., Kalmus, A. & Bergmann, F. (1960). *J. org. Chem.* **25**, 1752.
- Loo, T. L., Michael, M. E., Garceau, A. G. & Reid, J. C. (1959). *J. Amer. chem. Soc.* **81**, 3039.
- Mandel, G. (1957). *J. biol. Chem.* **225**, 137.
- Mandel, G. (1961). *J. Pharmacol.* **133**, 141.
- Norris, E. R. & Roush, A. (1950). *Arch. Biochem.* **29**, 124.
- Organic Syntheses (Coll. Vol.)* (1948). **2**, 65.
- Organic Syntheses (Coll. Vol.)* (1955). **3**, 106.
- Roblin, R. O., jun., Lampen, J. O., English, J. P., Cole, Q. P. & Vaughan, J. R., jun. (1945). *J. Amer. chem. Soc.* **67**, 290.
- Smith, J. D. & Matthews, R. E. F. (1957). *Biochem. J.* **66**, 323.