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Mechanism of Uptake of Purines by *Pseudomonas aeruginosa**

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ABSTRACT: During growth inhibition, 8-azaxanthine is incorporated into the ribonucleic acid of *Pseudomonas aeruginosa*. The azaxanthine-resistant strain, which emerges after about 10-hr exposure, does not incorporate the antimetabolite any more. Although xanthine or hypoxanthine does not stimulate growth, they undergo incorporation into the ribonucleic acid. The experiments with labeled purines indicate that a common mechanism serves for the uptake of xanthine, 6-thioxanthine, and 8-azaxanthine from the external medium into the protoplasm, and that this mechanism has changed in the azaxanthine-resistant strain. Incorporation of hypoxanthine and 6-mercaptopurine into the ribonucleic acid proceeds along a different path, which is not altered by 8-azaxanthine. In each of the two different mechanisms, only purines of related structure compete with each other.

3-Methylxanthine is not absorbed by the *Pseudomonas* but suppresses the uptake of xanthine or 6-thioxanthine; suppression is more effective in the azaxanthine-resistant strain than in the wild type. Therefore, competition most likely takes place on the cell surface.

The growth of *Pseudomonas aeruginosa* is insensitive to a concentration of 100 μg/ml of 8-azaguanine, but is strongly inhibited by 8-azaxanthine (Bergmann *et al.*, 1964; Clarke and Meadow, 1966). When the latter antimetabolite is added to a synthetic culture medium from the start, growth retardation becomes manifest after about 3 hr. However, the inhibitory effect persists only for a period of 10–12 hr. Subsequently, growth is resumed at a rate similar to the log phase of the control (Figure 1), and after 24 hr the cell density reaches the value of control cultures, multiplying in the absence of 8-azaxanthine. It is thus indicated that a new strain has emerged. Indeed the cells, collected after a single exposure to the antimetabolite, are resistant to 8-azaxanthine and this property has become hereditary as shown by transfers over 2 years. The azaxanthine-resistant strain resembles in its general properties, including growth rate, the original wild type.

Resistance to an antimetabolite can result from a variety of biochemical changes. In the present case, it was assumed that the azaxanthine-resistant strain is unable to absorb or to incorporate 8-azaxanthine. This hypothesis has been substantiated in the present study by the use of labeled antimetabolite. The results obtained raised the question whether the change, represented by transformation of the wild type to an azaxanthine-resistant strain, concerns only the mechanism regulating the uptake of 8-azaxanthine or affects also other purines.

Materials and Methods

Purines. The following purines were synthesized according to known methods: 6-thioxanthine (Beaman, 1954), 3-methylxanthine (Bredereck *et al.*, 1950), 8-methylxanthine (Bergmann *et al.*, 1961), 3-methyluric acid (Bergmann and Dikstein, 1955), and 8-azahypoxanthine (Roblin *et al.*, 1945).

[^{35}S]-6-Mercaptopurine and [8-^{14}C]hypoxanthine were purchased from the Radiochemical Centre, Amersham, England; [8-^{14}C]xanthine was supplied by International Chemical and Nuclear Corp., Calif.; [8-^{14}C]-6-mercaptopurine by California Foundation for Biochemical Research; and [2-^{14}C]-8-azaguanine by Nichem Inc., Bethesda, Md. The chemical purity of the labeled purines was checked by paper chromatography and ultraviolet spectrophotometry.

Preparation of Labeled Purines. SYNTHESIS OF [8-^{14}C]-3-METHYLXANTHINE. An intimate mixture of 4,5-diamino-

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3-methyluracil sulfate (Traube, 1900) (70 mg, 0.33 mmole), sodium [^{14}C]formate (17 mg, 0.25 mmole, 1 mCi), and potassium formate (63 mg, 0.75 mmole) was heated to 220° for 15 min, with occasional stirring with a glass rod. After cooling, the whole mass was dissolved in 2 ml of ammonia under slight warming. Acidification of the solution with glacial acetic acid precipitated the 3-methylxanthine, which was filtered and washed with water. The material was again dissolved in warm ammonia and streaked on Whatman No. 3 MM. The chromatogram was developed with a mixture of isopropyl alcohol–dimethylformamide–25% ammonia (65:25:10, v/v). The material was extracted from the paper with dilute ammonia; this solution contained 51 mg of 3-methylxanthine, λ_{max} (pH 8) 272 mμ (log ε 4.04). The yield was 90% on the basis of the uracil used. The overall activity of the preparation was 5.1×10^8 dpm, corresponding to 23% of the radioactivity used (1 mCi, 2.2×10^9 dpm). Since labeled formate represented only 25% of the total formate added, the radioactive yield was 92%.

SYNTHESIS OF [8-^{14}C]-6-THIOXANTHINE. A mixture of potassium formate (38 mg, 0.45 mmole), sodium formate (13 mg), and sodium [^{14}C]formate (17 mg, 1 mCi; total sodium formate used 0.45 mmole) was heated to 200° and again cooled to 180°. Then 4,5-diamino-6-thiouracil (Levin *et al.*, 1960) (53 mg, 0.3 mmole) was added under stirring with a fine glass rod. The clear melt was kept at 180° for 15 min. The mixture was dissolved in 2 ml of 0.25 N NaOH with slight warming. Acidification with HCl to pH 6 produced a precipitate of 6-thioxanthine. The compound was dissolved in 2 ml of warm dimethylformamide and the solution was placed on Whatman No. 3MM. Development of the chromatogram with ethanol–dimethylformamide–water (3:1:1, v/v) gave a single spot of 6-thioxanthine which was extracted with dilute ammonia. After evaporation of the solvent, there remained 37 mg of 6-thioxanthine, λ_{max} (pH 11) 340 mμ (log ε 4.33). The yield was 65%, on the basis of the thiouracil used. The radioactivity was 4.1×10^8 dpm (18.6%) of labeled formate used. Since the latter was 28% of the total formate employed, the radioactive yield was 66%.

Preparation of [2-^{14}C]-8-Azaxanthine by Enzymatic Deamination of 8-Azaguanine. P. aeruginosa was grown in the synthetic medium described below, centrifuged, and washed twice with Tris buffer. The bacteria were resuspended in water to give a density of 3×10^8 cells/ml and the pH was brought to 7.8 by means of bicarbonate. After addition of [2-^{14}C]-8-azaguanine, mixed with carrier 8-azaguanine (50 μg/ml), the suspension was shaken at 30° for 18 hr, and centrifuged. The supernatant was concentrated in a Rotavapor and the extinction coefficient was determined at 265 mμ (pH 8); log ϵ_{mol} of 8-azaguanine is 4.81. The purity of the deamination product was checked by paper chromatography, using 95% ethanol–water–acetic (17:2:1, v/v) as solvent (R_F of 8-azaguanine 0.36, of 8-azaxanthine, 0.26). The spots were located by their fluorescence under ultraviolet light of 255 mμ.

Culture of P. aeruginosa. In all experiments, the strain of *P. aeruginosa* that has been described earlier (Dikstein *et al.*, 1957) was used. The synthetic medium had the following composition: KH_2PO_4, 3 g; K_2HPO_4, 7 g; sodium citrate dihydrate, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; and $(NH_4)_2SO_4$, 1 g/l. The salt mixture was sterilized at 15 psi for 20 min, and sterile glucose was added just before inoculation to give a final concentration of 5 g/l. Cells were grown by shaking at 30°. At the end of the growth period, they were collected in a Lourdes refrigerated centrifuge at +5° and 10,000 rpm and washed twice with a volume of 0.03 M Tris buffer of pH 7.8–8.0, equal to the original volume of the culture, until in the range of 250–300 mμ the optical density of the supernatant was less than 0.1.

Radioactivity of Whole Cells. Cells from 100 ml of culture medium containing the labeled purine were washed twice with cold Tris buffer, using each time 200 ml, and were resuspended in 10 ml of Tris buffer; 0.1–1 ml of the suspension was collected on a Millipore filter (0.45-μ pore diameter). The filter was dried under an infrared lamp and 10 ml of scintillation fluild (3 g of 2,5-diphenyloxazole and 100 mg of 2-(*p*-biphenylyl)-5-phenyloxazole in 1000 ml of toluene) was added. The sample was then counted in a Packard Tricarb liquid scintillation counter.

Radioactivity of the Culture Medium. The culture (1 ml) was withdrawn at the start and at the end of the experiment and centrifuged. To 0.2 ml of the supernatant, 7 ml of scintillation fluid and 2.8 ml of absolute ethanol were added, and the mixture was counted as before.

Separation of RNA. The cells were treated in a Raytheon sonic oscillator for 5 min. The RNA was then extracted by a modification of the phenol method of Kirby (1956). An equal volume of phenol, saturated with water, was added to the sonicate and the mixture was stirred for 1 hr in the cold. In order to separate the two phases, the mixture was centrifuged at 12,000*g* in a Lourdes refrigerated centrifuge for 20 min. The upper phase was pipetted and the lower phase was reextracted with an equal volume of Tris buffer. The two upper phases were then combined, two volumes of a 1% solution of potassium acetate in cold absolute ethanol was added, and the mixture was left overnight in a cold room. The precipitate was collected by centrifugation and dissolved in a minimal volume of water. The purity of the dissolved material was determined by measuring the ratio of the optical densities at 260 and 280 mμ, which should not exceed 0.5.

The RNA isolated was dissolved in 0.15–3 ml of water and an aliquot of 0.2 ml was counted, as described for the culture medium. For quantitative determination of RNA the orcinol reaction was used (Meybaum, 1939).

Representative examples of analysis are given in Table I. Relative specific activity is defined as

$$\frac{\text{cpm/mg of product isolated}}{\text{cpm/mg of substrate used}} \times 100$$

Results

Incorporation of 8-Azaxanthine by P. aeruginosa. 8-Azaguanine is incorporated by many bacteria into RNA and in this way interferes with protein synthesis and

TABLE I: Analysis of Cells of *P. aeruginosa*, after Exposure to Labeled Purines.[a]

Purine Added	cpm/mg ($\times 10^{-6}$)	Strain Used	No. of Cells Collected ($\times 10^{-10}$)	cpm of Total Cell Yield ($\times 10^{-4}$)	% of Radio-activity Added	Amt of Purine Absorbed by Cells (μg)	mg of RNA Isolated	cpm of Total RNA ($\times 10^{-4}$)	Ratio[a]	Sp Act. of RNA ($\times 10^{-3}$)	Rel Sp Act. of RNA (%)
8-Azaxanthine	1.0	Wild type	2.2	3.7	0.74	37	4.9	2.4	65	4.8	0.48
Xanthine	1.4	Wild type	2.5	30.0	4.3	214	6.2	27.0[b]	99	44.0	3.1
Xanthine	1.4	Azaxanthine resistant	2.1	4.8	0.69	34	4.1	4.8[b]	110[c]	11.7	0.8

[a] Ratio = 100 × total radioactivity of RNA/total radioactivity of cells. [b] Efficiency of counting in these experiments was 90%. [c] The excess of radioactivity in the RNA over that of the cells reflects the error of the method (±10%). [d] The culture medium (100 ml) contained 50 μg/ml of labeled purine. Inoculum: 2.7×10^7 cells/ml for 8-azaxanthine and 6.5×10^6 cells/ml for xanthine. The cells were collected after about 5-hr incubation at 30°, washed two times, and counted directly. Subsequently, the RNA was isolated and counted.

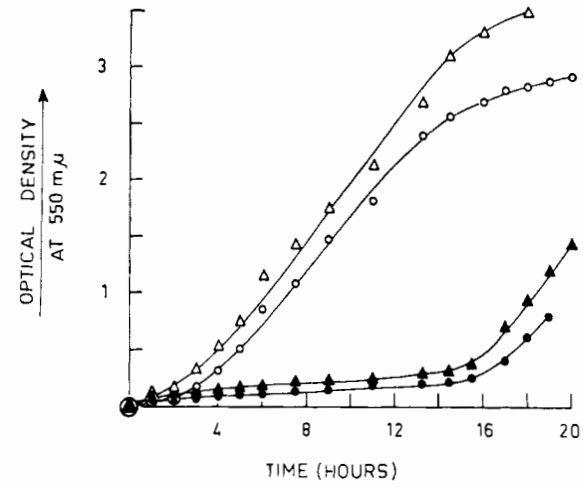


FIGURE 1: Growth curves of *P. aeruginosa* in the presence (black symbols) or absence (open symbols) of 8-azaxanthine (100 μg/ml). Triangles: inoculum of 2.5×10^7 cells/ml; circles: 0.7×10^7 cells/ml.

consequently with growth (Roodyn and Mandel, 1960; Chantrenne and Devreux, 1960). It appeared therefore probable that *P. aeruginosa* would absorb 8-azaxanthine, convert it into azaxanthylic acid, and subsequently into 8-azaguanylic acid, and incorporate the latter into the RNA; this, however, may not take place in the azaxanthine-resistant strain. Because a single exposure to 8-azaxanthine is sufficient for the emergence of a resistant strain, incorporation of the antimetabolite by the wild type may be detected with the greatest probability during the inhibitory phase (see Figure 1). Therefore, wild-type cells, exposed to labeled 8-azaxanthine, were collected after about 5 hr. Since up to this stage the population increased only moderately, it was necessary to start with a heavy inoculum in order to harvest sufficient material for nucleic acid analysis. It was found that cultures starting with 2.5×10^7 cells/ml were inhibited by the same concentration of 8-azaxanthine as those with three to four times smaller inocula (Figure 1).

Incubation with labeled 8-azaxanthine produced a relative specific activity of about 0.5% in the RNA, when the cells were collected during the period of growth retardation, *i.e.*, about 5 hr after inoculation (Table II). On the other hand, a growing culture of wild-type cells that had been exposed to the antimetabolite for 24 hr did not show any significant uptake. Likewise, the azaxanthine-resistant strain did not incorporate measurable amounts of the antimetabolite.

After incubation of wild-type cells with labeled 8-azaguanine no uptake could be detected, in accordance with the observation that this antimetabolite does not retard growth and that its deamination is too slow to produce, during the log phase, concentrations of 8-azaxanthine sufficient to influence the rate of growth (Bergmann *et al.*, 1964).

Considering the losses incurred during the isolation of RNA, it is probable that most or all of the labeled 8-azaxanthine, absorbed by wild-type cells, represents

TABLE II: Incorporation of Labeled Purines by *P. aeruginosa*.[a]

Purine	Concn (μg/ml)	Strain Used	Ratio[b]	Rel Spec Act. of RNA (%)	No. of Expt
I. Hypoxanthine group					
[8-^{14}C]Hypoxanthine	5	Wild type	103	4.8 ± 1.1	4
	50	Wild type	93	9.6 ± 1.4	9
	100	Wild type	81	10.3 ± 0.4	2
	50	Azaxanthine resistant	75	8.8 ± 3.0	2
	100	Azaxanthine resistant	80	9.6 ± 1.0	5
[8-^{14}C]-6-Mercapto-purine	50	Wild type	88	4.1 ± 0.3	2
	100	Wild type	77	4.0 ± 0.7	3
	50	Azaxanthine resistant	86	4.3	1
	100	Azaxanthine resistant	78	4.1 ± 3.7	4
[^{35}S]-6-Mercaptopurine	100	Wild type		(<0.01)[c]	2
II. Xanthine group					
[2-^{14}C]-8-Azaxanthine	50	Wild type[d]	66	0.48 ± 0.01	2
	50	Wild type[e]		<0.003	3
	50	Azaxanthine resistant		<0.003	2
[8-^{14}C]Xanthine	5	Wild type	75	3.1 ± 0.7	5
	50	Wild type	74	3.0 ± 0.2	6
	50	Azaxanthine resistant	100	0.7 ± 0.2	3
[8-^{14}C]-6-Thioxanthine	50	Wild type	*f*	1.3 ± 0.1	2
	100	Wild type	97	1.8 ± 0.4	3
	100	Azaxanthine resistant	67	0.4 ± 0.004	2
[2-^{14}C]-8-Azaguanine	50	Wild type		<0.03	2

[a] Inoculum in all experiments 6.5 × 10^6 cells/ml; for 8-azaxanthine, 2.7 × 10^7 cells/ml. Cells were collected after 5-hr incubation, unless otherwise stated. [b] Ratio = 100 × total radioactivity of RNA/total radioactivity of cells. [c] Radioactive sulfur was found in the cell proteins and also in the cell fluid, after its deproteinization. [d] Collected after 5 hr, *i.e.*, during the phase of growth retardation (see Figure 1). [e] Collected after 25 hr, *i.e.*, at the end of the log phase of growth, following the inhibitory phase. [f] In this experiment, part of the RNA was lost during purification.

8-azaguanylic acid incorporated into the polynucleotide chain (see Table I). No other cell component (*e.g.*, DNA) contained even traces of the label and no small molecules with radioactivity could be detected.

Uptake of Hypoxanthine and Xanthine. P. aeruginosa cannot utilize adenine and guanine directly, but deaminates them first to hypoxanthine and xanthine, respectively (Bergmann *et al.*, 1960; Clarke and Meadow, 1966). When the latter purines are added to the medium, they do not influence growth measurably (Bergmann *et al.*, 1964), but are rapidly oxidized to uric acid, which is in turn degraded by the bacterial urate oxidase (Dikstein *et al.*, 1957). Nevertheless in view of the incorporation of 8-azaxanthine into the RNA of the wild type, it appeared possible that part of the added hypoxanthine or xanthine may enter the cell and serve as substrate for nucleic acid synthesis. Therefore, the uptake of labeled hypoxanthine or xanthine by wild type or azaxanthine-resistant cells of *P. aeruginosa* was studied next.

Table II shows that either purine, when added to the medium, appears in the RNA. However, marked differences between hypoxanthine and xanthine become apparent. (a) Hypoxanthine is utilized to a greater extent than xanthine for the synthesis of RNA; (b) while hypoxanthine is equally incorporated by both strains, the uptake of xanthine by the azaxanthine-resistant strain attains only about one-fourth of the incorporation by the wild type. It should be noted that resting cells do not incorporate these or any of the other purines tested.

The observation that hypoxanthine is taken up more extensively than xanthine indicates that the former must enter the cell as such and not, or only to a minor degree, after its oxidation to xanthine. This fact and the discrepancy in the handling of the two purines by the resistant strain suggest that separate metabolic pathways are used for incorporation of hypoxanthine and xanthine in *P. aeruginosa*.

Incorporation of Thiopurines by P. aeruginosa. In order to test this hypothesis, the incorporation of the thio derivatives of both hypoxanthine and xanthine was studied. It has been observed previously (Bergmann *et al.*, 1962) that 6-mercaptopurine does not affect the growth of *P. aeruginosa*, but is oxidized *via* 6-thioxanthine to 6-thiouric acid. In spite of these results, it appeared possible that a certain portion of 6-mercaptopurine may enter the cells but may subsequently be deprived of its inhibitory properties by metabolic changes. For these experiments, two forms of radioactive 6-mercaptopurine were used: [^{35}S]6-mercaptopurine did not produce any significant labeling of RNA (relative specific activity < 0.01%), but the radioactive sulfur was detected in the protein and in the protein-free cell sap, indicating that

at a certain step of its metabolism the purine had undergone desulfuration. A similar behavior has been found by Carey and Mandel (1960) for *Bacillus cereus*. On the other hand, [8-^{14}C]-6-mercaptopurine was incorporated into RNA, and here again no difference could be detected between the two strains of *P. aeruginosa* (Table II).

In the case of 6-thioxanthine, the discrepancy between the results with the wild-type and the 8-azaxanthine-resistant strain was similar to the observations with xanthine (Table II). This supports the hypothesis that *P. aeruginosa* employs two different mechanisms for the utilization of hypoxanthine and xanthine in RNA synthesis.

It is noteworthy that 6-thioxanthine is incorporated by wild-type cells to a lesser degree than 6-mercaptopurine. Thus, as in the case of hypoxanthine, it is clear that 6-mercaptopurine is essentially utilized as such and not after its oxidation to 6-thioxanthine.

Experiments with 8-Azahypoxanthine. The results obtained so far show that 8-azaxanthine causes an alteration only in the pathway leading to the incorporation of xanthine, but leaves unchanged the mechanism for the utilization of hypoxanthine in RNA synthesis. On this basis it appeared possible that 8-azahypoxanthine might produce a new type of resistant cells with selective alteration of the hypoxanthine mechanism. Experiments with this antimetabolite gave, however, negative results, no retardation of growth being observed with concentrations up to 1000 μg/ml. It should be recalled that Roblin *et al.* (1945) found 8-azahypoxanthine effective against *Escherichia coli* and *Staphylococcus aureus*.

Competition Experiments. The results reported so far are in accord with the hypothesis that two independent paths are used for uptake and incorporation of hypoxanthine and xanthine. Purines with a 2-carbonyl group employ the xanthine pathway, while those with an unsubstituted 2 position are taken up by the hypoxanthine mechanism. If this is correct, one should also observe selective competition between the two types of purines.

In Table III, we show first the interaction between hypoxanthine and 6-mercaptopurine. The incorporation of [8-^{14}C]hypoxanthine was not affected measurably by an equal or ten times higher concentration of 6-mercaptopurine. On the other hand, hypoxanthine was highly effective in suppressing the incorporation of [8-^{14}C]-6-mercaptopurine. In contrast, the uptake of 6-mercaptopurine was influenced only slightly or not at all by the presence of equal concentrations of xanthine.

Similar relationships were observed for the pair xanthine–6-thioxanthine. The latter, whether present in equal or ten times higher concentrations, did not influence the incorporation of labeled xanthine, but xanthine strongly depressed the uptake of [8-^{14}C]-6-thioxanthine (see Table III).

The competition between 6-oxo- and 6-thiopurines makes it probable that the thio derivatives are transported as such into the cytoplasma, without previous desulfuration. This assumption is also in accord with our previous observation that 6-mercaptopurine and 6-thioxanthine are oxidized to 6-thiouric acid (Bergmann *et al.*, 1962).

TABLE III: Competition between Purines for Uptake into the RNA of *P. aeruginosa*.[a]

Labeled Purine	Concn (μg/ml)	Unlabeled Competitor	Concn (μg/ml)	Strain Used	Ratio[b]	Rel Spec Act. of RNA (%)	No. of Expt
1. Hypoxanthine group							
Hypoxanthine	5	6-Mercaptopurine	50	Wild type	90	4.3 ± 0.2	2
Hypoxanthine	50	6-Mercaptopurine	50	Wild type	55	10.3 ± 0.2	2
6-Mercaptopurine	50	Hypoxanthine	50	Wild type	86	1.1 ± 0.2	2
6-Mercaptopurine	50	Xanthine	50	Wild type	72	3.3 ± 0.3	2
2. Xanthine group							
Xanthine	5	6-Thioxanthine	50	Wild type	72	3.2 ± 0.1	3
6-Thioxanthine	50	Xanthine	50	Wild type	50	0.16	1
6-Thioxanthine	100	Xanthine	100	Wild type	62	0.25	1
6-Thioxanthine	50	Hypoxanthine	50	Wild type	57	0.9	1
6-Thioxanthine	100	Hypoxanthine	100	Wild type	60	1.2	1
Xanthine	50	8-Azaxanthine	50	Wild type	51	3.0 ± 0.3	2
Xanthine	50	8-Azaxanthine	50	AX-r	65	0.7 ± 0.2	2
8-Azaxanthine	50	Xanthine	50	Wild type		<0.01	2

[a] In all experiments cells were collected after a growth period of 5 hr. [b] Ratio = 100 × total radioactivity of RNA/total radioactivity of cells.

Analogous results were obtained with the pair xanthine–8-azaxanthine. When the antimetabolite and labeled xanthine were offered to the wild-type strain in various molar ratios, the incorporation of xanthine was not diminished. Conversely, xanthine reduced the uptake of an equal concentration of [2-^{14}C]-8-azaxanthine practically to zero (Table III). The strong competitive action of xanthine in this experiment agrees with our previous observation that xanthine completely abolishes also the growth inhibitory action of 8-azaxanthine (Bergmann *et al.*, 1964).

A small inhibitory effect was apparent when hypoxanthine was applied together with labeled 6-thioxanthine (Table III), but this may be ascribed to the formation of xanthine by oxidation of hypoxanthine. It should be recalled that in the conversion of hypoxanthine into uric acid by *P. aeruginosa*, the formation of xanthine as intermediate has not yet been established (Dikstein *et al.*, 1957). The competition between hypoxanthine and 6-thioxanthine lends support to the assumption that xanthine may be the first oxidation product of hypoxanthine. Experiments to clarify this problem will be described elsewhere.

Localization of the Change in Purine Metabolism in the Azaxanthine-Resistant Strain. Incorporation of xanthine into the RNA of *P. aeruginosa* proceeds in at least four steps, which may be formulated schematically as follows:

$$\text{xanthine}_{ext} \xrightarrow{1} \text{xanthine}_{ext} \xrightarrow{2} \text{XMP} \xrightarrow{3} \text{GMP} \xrightarrow{4} \text{RNA}$$

This scheme is based on the observations of Berlin and Stadtman (1966) with resting cells of *Bacillus subtilis*, but has so far not been verified for *P. aeruginosa*. It is possible that in the latter, steps 1 and 2 represent a single reaction. The alteration taking place during the conversion of the wild-type to the azaxanthine-resistant strain may involve one or several of these reactions. In order to localize a specific step for this change, use was made of our previous observation that 3-methylxanthine is oxidized by the *Pseudomonas* to 3-methyluric acid (Dikstein *et al.*, 1957). Since the latter product accumulates quantitatively in the external medium, it was inferred that the reaction takes place on the cell surface and that 3-methylxanthine is not absorbed into the cell interior. Direct evidence for this conclusion has now been obtained by the use of labeled purine. Table IV reveals that [8-^{14}C]-3-methylxanthine is not incorporated to any measurable extent. Therefore if 3-methylxanthine should inhibit the incorporation of xanthine, competition probably takes place in step 1 (or in the combined steps 1 and 2) of the above scheme, *i.e.*, in the process of penetration of xanthine into the cell interior. Table V shows that indeed in the wild type, 3-methylxanthine does compete with xanthine; however, the effect becomes conspicuous only with a large excess of the 3-methyl derivative. 3-Methylxanthine is also very effective as competitor for 6-thioxanthine, reducing the incorporation of an equal concentration of [8-^{14}C]-6-thioxanthine by the wild type to about one-sixth of the control (Table V). In view of this observation, it was also tried, whether other derivatives of 3-methylxanthine may act as inhibitors of the uptake of 6-thioxanthine. However, 3-methyl-2- or 6-thioxanthine proved ineffective, *i.e.*, they did not influence the incorporation of an equal concentration of 6-thioxanthine.

3-Methylxanthine was next tested on the azaxanthine-resistant strain. Here it proved to be a stronger competitor of xanthine than in the wild type. Thus, 3-methylxanthine reduced the incorporation of an equal con-

TABLE IV: Incorporation of [8-^{14}C]-3-Methylxanthine by *P. aeruginosa*.

Concn of 3-Methylxanthine (μg/ml)	Sp Act. of 3-Methylxanthine (cpm/mg $\times$ 10^{-6})	cpm of Total Cell Yield ($\times$ 10^{-3})[a]	% of Total Radioactivity Added	RNA Isolated (mg)	cpm in Total RNA[b]	Relative Specific Activity of RNA (%)	No. of Expt
A. Wild-type strain							
50	1.35	4.3	0.07	2.9	225	<0.006	2
150	1.42	6.4	0.03	2.0	250	<0.009	2
200	0.34	1.5	0.02	0.9	160	~0.05	2
B. Azaxanthine-resistant strain							
50	1.4	2.6	0.04	5.1	215	<0.003	2
150	1.4	5.5	0.03	3.0	265	<0.006	2
200	0.2	0.7	0.02	6.5	170	~0.01	2

[a] Note that the radioactivity of the cells is one to two orders of magnitude lower than that found in Table I and may be due to material adhering to the cells. [b] The radioactivity of the RNA represents only a small percentage (usually 5–10%) of that of the cells. The actual readings of the scintillation counter were only slightly above the background.

centration of xanthine to about 10% of the control, while a tenfold excess of 3-methylxanthine depressed the uptake of xanthine by 99%. The different behavior of the wild-type and azaxanthine-resistant strain toward this pair of purines suggests that an essential change has occurred in step 1 (or in the combined steps 1 and 2) of the process leading to xanthine incorporation. Naturally these results do not exclude the possibility that subsequent reactions in the xanthine pathway have also undergone alterations.

3-Methylxanthine had no influence on the uptake and incorporation of hypoxanthine by wild-type cells of the *Pseudomonas*.

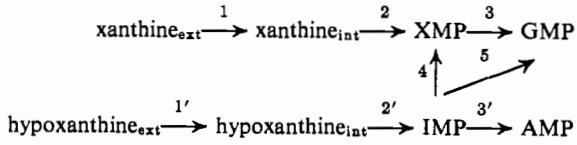
The Structural Specificity of the Xanthine Site. All the data presented indicate that *P. aeruginosa* uses two separate mechanisms for the incorporation of hypoxanthine and xanthine. Purines interact with the xanthine mechanism if they contain, in addition to the substituent at position 6, a 2-oxo group.

It was of interest to determine the possible influence of substituents at position 8 on either mechanism. Table VI shows that 6,8-dihydroxypurine has no influence on the uptake of hypoxanthine, even if the latter is present only at one-tenth the concentration of the competitor tested. Similarly, uric acid or its 3-methyl derivatives do not influence the incorporation of labeled X.

It was furthermore observed that 8-methylxanthine is not a competitor in the uptake of xanthine. Obviously the effect of a methyl group at C-8 is quite different from that of a methyl substituent at N-3.

Discussion

P. aeruginosa does not utilize external sources of adenine or guanine for RNA synthesis. In addition, as shown in the present study, the organism employs two different pathways for the metabolism of hypoxanthine and xanthine. In a separate study, we shall demonstrate that hypoxanthine is incorporated in about equal parts into the AMP and GMP of RNA, while xanthine appears practically only in GMP. These observations suggest that *P. aeruginosa* lacks GMP reductase, the enzyme responsible for conversion of GMP into IMP in *Enterobacteriaceae* (Mager and Magasanik, 1960; Magasanik and Karibian, 1960). We may therefore describe the utilization of external purines by the *Pseudomonas* in the following tentative scheme



Previous investigators have shown that the uptake of purines by bacteria is controlled by specific nucleotide pyrophosphorylases. Berlin and Stadtman (1966) advanced evidence that in *B. subtilis* external adenine, guanine, and xanthine are converted into ribotides by different enzymes, while the situation was less clear for hypoxanthine. Similarly, according to Kalle and Gots (1964) three different nucleotide pyrophosphorylases,

TABLE V: Inhibition of Uptake of Labeled Xanthines by 3-Methylxanthines.[a]

Labeled Purine	cpm/mg (× 10^{-6})	Concn (μg/ml)	Strain Used	cpm of Total Cell Yield (× 10^{-5})	% of Total Radioactivity Added	cpm of Total RNA (× 10^{-5})[b]	Ratio[c]	Relative Specific Activity of RNA (%)	No. of Expt
[8-^{14}C]Xanthine	12	5	Wild type	2.6	4.3	2.2	103	0.74 ± 0.09	3
	1.1	50	Wild type	1.3	2.5	0.9	76	3.4 ± 0.4	3
	12	5	Azaxanthine resistant	0.04	0.07	0.02	(55)[d]	0.004	1
	1.1	50	Azaxanthine resistant	0.04	0.08	0.04	110	0.05 ± 0.002	3
[8-^{14}C]-6-Thioxanthine	1.3	50	Wild type	0.15	0.23	0.09	64	0.25 ± 0.05	2
	1.35[e]	50	Wild type	0.7	1.0	0.39	61	1.7	1
	1.35[f]	50	Wild type	0.7	1.05	0.45	69	1.4	1

[a] In all runs, besides the last two, 50 μg/ml of 3-methylxanthine was added. [b] Counting efficiency of RNA was close to 90%. [c] Ratio = 100 × total radioactivity of RNA/total radioactivity of cells. [d] In this experiment, part of the RNA was lost during isolation. [e] In this experiment, 50 μg/ml of 3-methyl-2-thioxanthine was used as competitor. [f] Here, 50 μg/ml of 3-methyl-6-thioxanthine was added.

TABLE VI: 8-Substituted Purines as Potential Competitors of Hypoxanthine or Xanthine.

Labeled Purine	Concn (μg/ml)	Competitor	Concn (μg/ml)	Ratio[a]	Relative Specific Activity of RNA (%)	No. of Expt
Hypoxanthine	50	6,8-Dihydroxypurine	50	87	10.2 ± 0.3	3
Hypoxanthine	50	8-Methylhypoxanthine	50	90	8.1 ± 0.5	2
Xanthine	5	Uric acid	50	53	3.2 ± 0.4	3
Xanthine	5	3-Methyluric acid	50	62	3.0 ± 0.2	2
Xanthine	50	8-Methylxanthine	50	53	2.8 ± 0.3	2

[a] Ratio = 100 × total radioactivity of RNA/total radioactivity of cells.

viz., for adenine, guanine, and xanthine, are present in *Salmonella typhimurium*, but it could not be decided whether the enzyme, responsible for conversion of hypoxanthine to IMP, is identical with guanylic pyrophosphorylase. This problem remained also unsolved for *E. coli* (Coggin *et al.*, 1966).

On the basis of these observations, two alternative explanations can be proposed for the fact that *P. aeruginosa* is unable to utilize an external source of adenine or guanine: (a) the cells lack specific transport systems for purines, bearing an amino group; or (b) the cells lack the specific guanylic and adenylic pyrophosphorylases. Since the *Pseudomonas* utilizes hypoxanthine, it would follow that at least in this case guanylic and inosinic pyrophosphorylases are not identical.

Analogous alternative explanations may be used to rationalize the separate metabolic pathways of hypoxanthine and xanthine. The *Pseudomonas* may have at its disposal a separate transport system to move either purine into the cell interior, or may convert them directly into IMP and XMP, respectively, by enzymes located at the cell surface, and simultaneously absorb these nucleotides.

3-Methylxanthine is not absorbed by *P. aeruginosa*, but reduces the percentage of xanthine that becomes incorporated into RNA. On the other hand, 3-methylxanthine does not affect the metabolism of hypoxanthine. Therefore, inhibition of the uptake of xanthine must take place in the very beginning, *i.e.*, either at step 1 or at the combined steps 1 and 2. A more precise definition of the critical step requires a study of the inhibition of the xanthylic pyrophosphorylase of the *Pseudomonas* by 3-methylxanthine. In this context, the observations of Demain (1964) with a *B. subtilis* mutant should be mentioned. The mutant grew only on media, supplemented with guanine, while adenine or hypoxanthine inhibited the utilization of guanine. It was shown by Demain that the inhibition is due to a block of the permeation step $\text{guanine}_{ext} \rightarrow \text{guanine}_{int}$.

The xanthine mechanism in *P. aeruginosa* is also responsible for the uptake of 8-azaxanthine into wild-type cells. The alteration of the xanthine mechanism, which has taken place in the azaxanthine-resistant strain, makes the cells inaccessible to the antimetabolite. Here again, two alternative explanations are possible. Either a specific transport mechanism for xanthine has been changed; or the xanthylic pyrophosphorylase has been modified in such a way that it cannot use 8-azaxanthine as substrate. In addition, other possible changes of the xanthine metabolism have to be considered, and this problem awaits further experimental clarification.

The finding that, in the wild-type cells xanthine and azaxanthine utilize the same metabolic pathway, may help to explain our previous observations that certain purines are able to counteract the growth inhibition by 8-azaxanthine. Inspection of Table III in the paper of Bergmann *et al.* (1964) reveals that xanthine and thioxanthines had a marked influence; hypoxanthine was nearly as effective as xanthine, but 6-mercaptopurine showed only a minor antagonistic action. Uric acid did not reduce growth retardation by 8-azaxanthine. On the basis of a specific xanthine mechanism, these observations can now be rationalized if we assume that hypoxanthine is partly converted by the bacterial cells into xanthine. The problem of the pathway of hypoxanthine oxidation by *P. aeruginosa* will be discussed elsewhere.

References

Beaman, A. G. (1954), *J. Am. Chem. Soc. 76*, 5633.

Bergmann, F., and Dikstein, S. (1955), *J. Am. Chem. Soc. 77*, 691.

Bergmann, F., Kwietny, H., Levin, G., and Engelberg, H. (1960), *Biochim. Biophys. Acta 37*, 433.

Bergmann, F., Levin, G., and Kalmus, A. (1961), *J. Org. Chem. 26*, 1504.

Bergmann, F., and Tamari, M. (1961), *J. Chem. Soc.*, 4468.

Bergmann, F., Ungar-Waron, H., and Kwietny-Govrin, H. (1964), *Biochem. J. 91*, 270.

Bergmann, F., Ungar-Waron, H., Kwietny-Govrin, H., Goldberg, H., and Leon, S. (1962), *Biochim. Biophys. Acta 55*, 512.

Berlin, R. D., and Stadtman, E. R. (1966), *J. Biol. Chem. 241*, 2679.

Bredereck, H., Schuh, H., and Martini, A. (1950), *Chem. Ber. 83*, 201.

Carey, N. H., and Mandel, H. G. (1960), *Biochem. Pharmacol. 5*, 64.

Chantrenne, H., and Devreux, S. (1960), *Biochim. Biophys. Acta 39*, 486.

Clarke, P. H., and Meadow, P. M. (1966), *J. Gen. Microbiol. 44*, 195.

Coggin, J. H., Loosemore, M., and Martin, W. R. (1966), *J. Bacteriol. 92*, 446.
Demain, A. L. (1964), *Arch. Biochem. Biophys. 108*, 403.
Dikstein, S., Bergmann, F., and Henis, Y. (1957), *J. Biol. Chem. 224*, 67.
Kalle, G. P., and Gots, J. S. (1964), *Science 142*, 680.
Kirby, K. S. (1956), *Biochem. J. 64*, 405.
Levin, G., Kalmus, A., and Bergmann, F. (1960), *J. Org. Chem. 25*, 1752.
Magasanik, B., and Karibian, D. (1960), *J. Biol. Chem. 235*, 2672.
Mager, J., and Magasanik, B. (1960), *J. Biol. Chem. 235*, 1474.
Meybaum, W. (1939), *Z. Physiol. Chem. 258*, 117.
Roblin, R. O., Jr., Lampen, J. O., English, J. P., Cole, Q. P., and Vaughan, J. R., Jr. (1945), *J. Am. Chem. Soc. 67*, 290.
Roodyn, D. B., and Mandel, H. G. (1960), *J. Biol. Chem. 235*, 2036.
Traube, W. (1900), *Chem. Ber. 33*, 3035.

Irreversible Thermal Denaturation of *Escherichia coli* Ribosomes*

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ABSTRACT: Irreversible thermal denaturation of high salt-washed *Escherichia coli* (B and Q13) ribosomes has been followed in terms of biological activity, sedimentation properties, and turbidity, as a function of solvent composition. In a buffer composed of 0.01 M Tris–0.01 M $MgAc_2$ (pH 7.4) ribosomes lose 50% of their ability to form polyphenylalanine from polyuridylic acid in 5 min at 57° ($T_{d,5}$). In this temperature range 70S ribosomes are destroyed, presumably by aggregation. The sharpness of this thermal transition is illustrated by the fact that in 0.01 M Tris–0.01 M $MgAc_2$ several hours are required for 50% inactivation at 50°, while at 60° 50% inactivation occurs within 1 min. Reduction of the magnesium ion concentration to 10^{-4} M reduces the $T_{d,5}$ to 53.5° and under these conditions the normal 50S and 30S subunits are converted into slower sedimenting particles (~35–40 and 25S). Monovalent cations ($K \geqslant NH_4 > Na$) in the presence of 0.01 M $MgAc_2$ (but not 10^{-4} M $MgAc_2$) protect ribosomes from thermal inactivation presumably by preventing aggregation. In 0.01 M Tris–0.01 M $MgAc_2$–0.1 M KCl (pH 7.4), ribosomes exhibit a $T_{d,5}$ of 60.5° and the loss in biological activity is closely paralleled by the destruction of the 30S subunit. The effect of heating on ribosome activity is characterized by an initial increase in activity (variable with the preparation) followed by a rapid but decreasing rate of inactivation. No explanation is provided for this activation, but it apparently results from an increase in the number of active ribosomes and is not due to a relaxation of the ribosomal structure which would also lead to translational ambiguity. Evidence is presented which suggests that the observed denaturation does not result from enzymatic degradation. It is also shown that ribosomes can be lyophilized from a variety of buffers with little or no alteration of physical or functional properties.

The ribosomes of *Escherichia coli* are not only structurally complex but they have a complex role in protein synthesis. They are known to contain 3 RNA molecules and probably as many as 40 different protein molecules. Very little information, however, is available on the way in which these individual components interact to produce the structural and biological features of the ribosomal particle.

Magnesium ion is essential to the maintenance of both the structural and functional integrity of the ribosome and several investigators have shown that ribosomes undergo a characteristic and partially reversible unfolding upon the removal of magnesium ion (see Gavrilova *et al.*, 1966; Gesteland, 1966). Kaji *et al.* (1966) have shown that mild proteolysis of ribosomes progressively destroys their biological activity in the order, protein synthesis, aminoacyl-tRNA binding, mRNA binding, and ultimately causes irreversible dissociation into apparently normal 50S and 30S subunits without the release of appreciable soluble material. On the basis of chemical modification of the ribosome, Moore (1966) has concluded that amino groups of rRNA are also involved in the binding of mRNA and in the interaction of the subunits. Protein sulfhydryl groups have also been implicated in ribosomal structure and function (Wang and Matheson, 1966; Tamaoki and Miyazawa, 1967; Traut and Haenni, 1967).

In addition to their role in binding mRNA and amino-

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