CATABOLIC DEGRADATION OF 6-AZAURACIL AND 6-AZAURIDINE BY *Pseudomonas putida*

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Received February 11th, 1977

In washed suspensions and cell-free extracts of *Pseudomonas putida* strain 17 glyoxylic acid semicarbazone, hydrazinoacetic acid and glycine were identified as products of 6-azauracil-[4,5-^{14}C] catabolism. These compounds as well as glyoxylic acid hydrazone accumulated also in the synthetic medium during *Ps. putida* cultivation with 6-azauracil as the main nitrogen source in the presence of low level of uracil. Cultivation with 6-azauridine yielded the same compounds in addition to 6-azauracil. The scheme of 6-azauridine breakdown and the biological activity of the newly formed products of 6-azauracil degradation are presented.

6-Azauracil and 6-azauridine belong to extensively studied pyrimidine analogues due to their antibacterial and cytostatic activity[1-4]. While investigating their inhibitory mechanism it has been shown that both compounds are metabolized to 6-azauridine 5'-monophosphate[5-7] which blocks the decarboxylation of orotidine 5'-phosphate and thus pyrimidine synthesis *de novo*. 6-Azauridine competes with uridine for uridine kinase and is relatively stable against the action of enzymes splitting pyrimidine nucleosides[8,9].

While the inhibitory mechanism of 6-azauridine was studied in detail[10,11] its catabolic degradation was not known. In microorganisms 6-azauridine is degraded to 6-azauracil and in eukaryotic cells in culture the formation of glyoxylic acid semicarbazone from 6-azauracil was described[12]. Recently we attempted to clarify the pathway of 6-azauracil degradation in bacteria[13], and the present paper describes the products of 6-azauridine degradation taking place in *Ps. putida* strain 17.

EXPERIMENTAL

Materials and Conditions of Cultivation

6-Azauracil and 6-azauridine were products of Chemapol, Prague. 6-Azauracil-[4,5-^{14}C] (80 mCi/mmol) was prepared in the Institute for Research, Production and Uses of Radioisotopes, Prague. Glyoxylic acid semicarbazone was prepared in the Institute of Organic Chemistry and Biochemistry. Glyoxylic acid hydrazone was synthesized according to Bailey and Read[14] and

hydrazinoacetic acid by the procedure of Darabsky and Prabhakar[15]. All compounds were purified by repeated chromatography on Filtrak No 18 paper in the solvent system composed of isopropyl alcohol–ammonium hydroxide–water (14 : 1 : 5) (System I).

Ps. putida strain 17 was isolated from freshwater basins of Latvian SSR. The strain was grown in the synthetic medium containing 2·7 g K_2HPO_4, 1·3 g KH_2PO_4, 1·0 g NaCl, 0·1 g $MgSO_4$, 1·0 g succinate, 1·0 g citrate, 1·0 g ammonium sulfate, 3 ml of glycerol and necessary micro-elements per 1 litre. In studies of 6-azauracil degradation the analogue (0·05%) substituted ammonium sulfate as the main nitrogen source. No growth of *Ps. putida* was observed in the presence of 6-azauracil or 6-azauridine unless 0·005% of uracil were added into medium. Initial pH of the medium was adjusted to 7·2 with 1M-NaOH. Cultivation proceeded on laboratory shaker at 28°C in 1 litre Erlenmeyer flasks containing 100 ml of the medium. The growth of culture was followed by photocolorimeter FEK-52 at 560 nm.

The biological activity of products of 6-azauridine catabolism was detected using *Ps. putida* strain 17 and *Ps. fluorescens* growing in a synthetic medium containing inorganic nitrogen. The growth was measured after 5 days of incubation at 28°C in a laboratory shaker in 250 ml Erlenmeyer flasks containing 50 ml of medium.

Isolation and Identification of Products of 6-Azauracil Catabolism

The changes in the level of 6-azauracil and uracil taking place during cultivation were measured in aliquots of media (0·2 ml) separated chromatographically on Filtrak No 18 paper in the solvent system I. 2·8 ml aliquots of the media were evaporated to dryness and used for the qualitative analysis of the decomposition products of 6-azauracil on Filtrak No 11 paper in the following solvent systems: isobutyric acid–ammonium hydroxide–water (44 : 1 : 22) (System II), ethanol–1M ammonium acetate, pH 8·0 (7 : 3) (System III), 1-butanol saturated with water (System IV) and ethyl acetate–acetic acid–water (3 : 1 : 1) (System V).

The separated compounds were detected in UV-light and by means of the color reaction with Ehrlich's reagent[16]. Glycine was detected with ninhydrin reagent[17]. The concentration of uracil, 6-azauracil and 6-azauridine in culture fluid was assayed after chromatographic separation. The absorbance of both analogues was measured at 260 nm and that of uracil at 280 nm using spectrophotometer SF-16.

To obtain large amounts of products of 6-azauracil degradation the preparative isolation on Filtrak No 18 paper was made use of. Bacterial cells in the stationary phase of growth from 2 litres of the medium were harvested by centrifugation, the supernatant evaporated at 40°C and used for the chromatographic separation. Electrophoretic analysis of products of 6-azauracil degradation was carried out in two buffer systems (400 V, 3 h) using Filtrak No 11 paper. Compounds for the proton NMR spectroscopy were eluted from the paper with deuterium oxide. The measurement was carried out on Perkin Elmer model R 12A (frequency 60 MHz, inner standard hexamethyldisiloxane).

Degradation of 6-Azauracil in Cell Suspension and Cell-Free Extract of *Ps. putida*

Bacterial cells harvested by centrifugation (16000*g*, 30 min, 4°C) were washed twice with 40 mM Tris-HCl buffer (pH 7·4) containing 0·15M-KCl. The cell suspension was desintegrated (MSE sonicator) and cell debris removed by centrifugation (30000 g, 45 min, 4°C). The protein content was determined according to Lowry and coworkers[18]. Incubation mixture contained in 1 ml 40 mM Tris-HCl buffer (pH 7·4), 0·15M-KCl, 10 mM $MgCl_2$, 0·1 mM 6-azauracil-[4,5-^{14}C] and 0·2 ml of bacterial suspension or 0·6 ml of the cell-free extract (0·55 mg protein). At various time intervals of incubation at 28°C 0·2 ml aliquots were removed and extracted with 0·2 ml

of cold 0·4M-$HClO_4$. Extracts were neutralized with 2M-KOH, cooled, centrifuged and separated on Whatman No 3 paper in the solvent system II. Using cell-free extracts the samples after incubation were directly separated chromatographically. The radioactive spots on chromatograms were measured using automatic scanner Frieseke–Hoepfner.

RESULTS

Ps. putida strain 17 was cultivated with 6-azauracil as the main nitrogen source in preparative scale experiment to accumulate the intermediate products of its catabolism in sufficient quantities. The logarithmic phase of culture growth corresponded to the utilization of uracil added to medium in 0·005% concentration (Fig. 1) and its duration was approximately the same as in culture growing with NH_4^+-ions as nitrogen source. Under these conditions the inhibitory effect of 6-azauracil was pronounced. The degradation of the analogue started in the stationary phase of bacterial growth, this phase being considerably prolonged in the presence of the drug. The growth of the culture and the utilization of 6-azauridine as the main nitrogen source were the same as in the case of 6-azauracil.

After 7 days of cultivation with 6-azauridine or 6-azauracil bacterial cells were centrifuged and supernatant fluid was analyzed. During the cultivation with 6-azauridine two main compounds (A1 and A2) and during the cultivation with 6-azauracil five compounds (A3, A4, A5, A6 and A7) were isolated from the media. Chromatographic mobilities and some physico-chemical properties of these compounds were compared with corresponding standards (Table I). According to given data the newly formed compounds were identified as 6-azauridine (A1), 6-azauracil

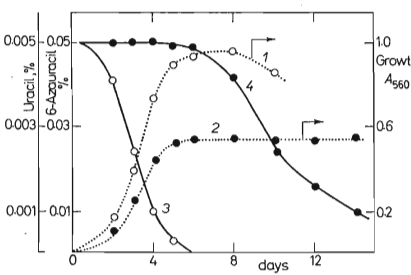

FIG. 1

Growth and Utilization of 6-Azauracil and Uracil in *Ps. putida*

1 Growth of control culture; *2* growth in the presence of 0·05% 6-azauracil and 0·005% uracil; *3* concentration of uracil in the medium; *4* concentration of 6-azauracil in the medium.

(A2 and A3), glyoxylic acid semicarbazone (A4), glyoxylic acid hydrazone (A5), hydrazinoacetic acid (A6) and glycine (A7). None of these compounds accumulated when *Ps. putida* was growing with ammonium ions as nitrogen source.

Analysis of the zone A5 corresponding to glyoxylic acid hydrazone revealed one additional spot with R_F value of hydrazinoacetic acid. The UV-absorbance of the spot of glyoxylic acid hydrazone on paper chromatograms gradually decreased and using Ehrlich's reagent the color of the spot changed from orange to red. These facts led us to suppose that the transformation of glyoxylic acid hydrazone to hydrazinoacetic acid is not an enzyme reaction.

To ensure the presence of glyoxylic acid hydrazone in cultivation medium the zones corresponding to A5 and A6 were further eluted in deuterium oxide to carry out the proton NMR spectroscopy. The parameters of isolated compounds A5 and A6 and of chemically prepared glyoxylic acid semicarbazone, glyoxylic acid hydrazone and hydrazinoacetic acid are compared in Table II. Zone A5 containes a mixture of two compounds: one with the resonance peak of glyoxylic acid hydrazone and one resembling hydrazinoacetic acid. The resonance peak of the latter corresponds to that in the zone A6. The presence of glyoxylic acid semicarbazone in zones A5 and A6 was excluded due to their different R_F values.

The studies following the degradation of 6-azauracil-[4,5-^{14}C] in washed suspensions of *Ps. putida* also showed the formation of new radioactive compounds (Fig. 3). Both the newly formed substances were prepared in pure state by repeated paper chromatography. Peak 2 was identified as glyoxylic acid semicarbazone and the next one was identical with hydrazinoacetic acid. Beside 6-azauracil-[4,5-^{14}C] the rechromatography of the peak *1* in the solvent system I revealed still another radio-

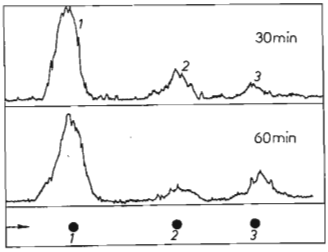

FIG. 2

Products of Catabolic Degradation of 6-Azauracil-[4,5-^{14}C] in Cell Suspension of *Ps. putida*
The samples after 30 or 60 min of incubation at 28°C were analyzed chromatographically on Whatman No 3 paper using the solvent system composed of isobutyric acid–ammonium hydroxide–water. *1* 6-Azauracil; *2* glyoxylic acid semicarbazone; *3* hydrazinoacetic acid.

TABLE I

Chromatographic and Electrophoretic Mobilities and Some Physico-Chemical Properties of 6-Azauridine and Its Degradation Products

Compound	R_F-Values in the system				
	I	II	III	IV	V
6-Azauridine	0·58	0·48	0·66	0·66	0·51
6-Azauracil	0·56	0·62	0·62	0·70	0·66
Glyoxylic acid semicarbazone	0·54	0·49	0·47	0·77	0·70
Glyoxylic acid hydrazone	0·13	0·42	0·35	0·15	0·55
Hydrazinoacetic acid	0·14	0·25	0·37	0·11	0·38
Glycine	0·42	0·41	0·39	0·42	0·37
A1	0·57	0·48	0·67	0·59	0·51
A2	0·56	0·62	0·62	0·69	0·65
A3	0·57	0·62	0·61	0·70	0·66
A4	0·53	0·48	0·46	0·77	0·71
A5	0·13	0·40	0·37	0·10	0·40
A6	0·15	0·30	0·42	0·12	0·38
A7	0·40	0·40	0·40	0·40	0·38

[a] 0·05M succinate-borax buffer, pH 5·0; [b] 0·05M Tris-HCl buffer, pH 8·0; [c] reaction with ninhydrin

TABLE II

^{1}H-NMR Spectra Parameters of Products of 6-Azauracil Degradation
Deuterium oxide was used as solvent system.

Compound	Chemical shift δ, ppm	Coupling constant J, Hz	Chemical shift δ, ppm	Coupling constant J, Hz
	—CH_2—		—CH=	
Hydrazinoacetic acid	2·1	8·11	—[a]	—[a]
Glyoxylic acid hydrazone	—[a]	—[a]	2·6	7·63
Glyoxylic acid semicarbazone	—[a]	—[a]	2·1	8·04
A6	2·1	8·12	—[a]	—[a]
A5	2·1	8·09	2·6	7·66

[a] No corresponding group was found in the compound.

TABLE I
(*continued*)

Relative elfo mobility 10^5 cm^2 V^{-1} s^{-1}		UV-Absorbance, max. λ, nm			Reaction with Ehrlich's reagent
I[a]	II[b]	0·1M-HCl	pH 9·0	0·2M-NaOH	
−0·1	+4·8	261	253	264	none
−0·1	+4·6	258	251	286	none
+7·2	+6·8	255	250	287	yellow
+0·1	+8·2	256	250	248	orange
+0·1	+8·3	268	263	256	red
—	—	—	—	—	none[c]
−0·1	+4·9	160	253	253	none
−0·1	+4·7	258	252	284	none
−0·1	+4·6	258	253	286	none
+7·2	+6·8	255	250	287	yellow
+0·1	+8·2	256	250	247	orange
+0·1	+8·3	266	260	253	red
—	—	—	—	—	none[c]

reagent.

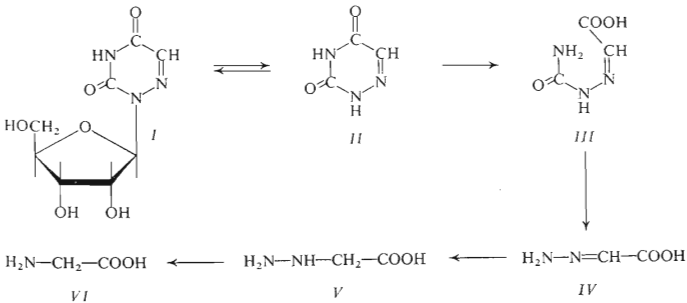

FIG. 3

Scheme of 6-Azauridine Degradation in *Ps. putida*

I 6-Azauridine; II 6-azauracil; III glyoxylic acid semicarbazone; IV glyoxylic acid hydrazone; V hydrazinoacetic acid; VI glycine.

active component with the R_F value of glycine. The incubation of cell-free extracts of *Ps. putida* with the labelled 6-azauracil yielded only one radioactive component corresponding to glyoxylic acid semicarbazone.

DISCUSSION

Cultivation of *Ps.putida* strain 17 with 6-azauridine or 6-azauracil as the main nitrogen source results in their degradation. The studies carried out with 6-azauracil-[4,5-^{14}C] in washed cell suspensions and cell-free extracts confirmed the degradation. The pathway of 6-azauridine catabolism in *Ps. putida* strain 17 can be depicted by the scheme shown in Fig. 3. The observed hydrolysis of 6-azapyrimidine ring differs from the catabolic pathway of natural pyrimidines or their halogen derivatives. The cleavage of the pyrimidine ring is preceded either by the reduction of 5–6 double bond resulting in the formation of corresponding dihydro derivatives[19,20] or by the oxidation yielding derivatives of barbituric acid[21,22] or by the dehalogena-

TABLE III

Bacteriostatic Activity of 6-Azauracil and Products of its Degradation in *Ps. putida* and *Ps. fluorescens* and Its Reversion with Pyrimidine Precursors of Nucleic Acids

Bacterial strains were grown in a synthetic medium containing 0·05% of inhibitors and/or 0·005% of pyrimidine precursors. Growth was measured after 5 days of cultivation (A_{560}).

Added	Growth, A_{560}		Added	Growth, A_{560}	
	Ps. putida	*Ps. fluorescens*		*Ps. putida*	*Ps. fluorescens*
Control			Glyoxylic acid semicarbazone		
Control	0·95	1·20	Control	0·20	0·20
Uracil	0·90	1·25	Uracil	0·80	0·80
Uridine	1·00	1·25	Uridine	0·80	0·95
Cytosine	1·05	1·20	Cytosine	0·75	0·95
Cytidine	1·10	1·15	Cytidine	0·85	0·90
6-Azauracil			Hydrazinoacetic acid		
Control	0·15	0·10	Control	0·15	0·15
Uracil	0·55	0·70	Uracil	0·20	0·15
Uridine	0·45	0·70	Uridine	0·20	0·10
Cytosine	0·35	0·65	Cytosine	0·15	0·15
Cytidine	0·50	0·65	Cytidine	0·20	0·20

tion[23]. The degradation of another azapyrimidine, 5-azauracil, results similarly to 6-azauracil in the formation of 1-formylbiuret due to direct cleavage of the triazine ring[24]. However, the reaction is spontaneous and is not enzyme dependent.

Although the possible consequences of the administration of 6-azauridine were widely revealed[25,26] the effect of its degradation products has not been recognized. The measurement of growth inhibition of *Ps. putida* and *Ps. fluorescens* in the presence of 6-azauracil, glyoxylic acid semicarbazone and hydrazinoacetic acid in a synthetic medium containing inorganic nitrogen, and the competitive effect of natural pyrimidine precursors are summarized in Table III. It is evident that all the tested compounds strongly inhibit the growth of bacteria. However, hydrazinoacetic acid seems to have a different inhibitory mechanism on the growth of *Pseudomonas* than 6-azauracil or glycoxylic acid semicarbazone which cannot be reversed by adding pyrimidine precursors of nucleic acids.

The elucidation of the inhibitory mechanism of biologically active products of 6-azauridine catabolism is the aim of our present study.

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