Biochemical Pharmacology, 1966, Vol. 15, pp. 761–763. Pergamon Press Ltd., Printed in Great Britain.

Enzymatic formation of uracil and azauriail nucleotides in *Streptococcus faecalis*

(*Received* 30 *November* 1965; *accepted* 13 *January* 1966)

PREVIOUS studies of the formation of uridine nucleotides from preformed uracil (U) have indicated that in animals there occurs a primary condensation of uracil with ribose-1-phosphate to form uridine and a subsequent phosphorylation by ATP to form uridine 5′-phosphate[UMP(5′)]; this latter reaction is catalyzed by uridine kinase. In bacteria a direct conversion of U to UMP(5′), via condensation with 1-pyrophosphorylribose-5-phosphate (PRPP), has been reported for *Lactobacillus bulgaricus*;[1] a similar mechanism has been observed in *Trypanosoma equiperdum*.[2] The only route for the conversion of orotic acid to the nucleotide form also involves condensation with PRPP.

Earlier studies had shown that conversion of the antimetabolite 6-azauracil (AzU) to 6-azauridine 5′-P (AzUMP), an inhibitor of orotidylic acid decarboxylase,[3] was essential for the inhibition of growth of several species of bacteria by AzU. Substrains selected for resistance to AzU lacked the ability in growing cultures to form AzUMP from AzU and, concomitantly, UMP from U.[4] In the present study, this difference was employed to define at the enzymatic level the mechanism of resistance to AzU, and to study the enzymatic nature of the anabolic pathways for U and orotic acid in *Streptoccoccus faecalis*, with particular attention to the relationship between and the possible identity of the enzymes uridylic acid pyrophosphorylase and orotidylic acid pyrophosphorylase.

METHOD

A strain of *S. faecalis* (ATCC 8043) resistant to AzU (10^{-2} M) was isolated in a basal medium upplemented with folic acid ($2{\cdot}5 \times 10^{-6}$ M), but containing no U. The results of growth assays with the parent sensitive strain and with the resistant subline in the presence of either AzU or azauridine (AzUR) were comparable to those obtained previously with similar organisms.[4] For enzymatic studies, 1-liter cultures were grown in the absence of AzU, and cells were harvested by centrifugation in the logarithmic phase of growth (after incubation for 12 to 16 hr) by centrifugation. To prepare cell-free extracts, 1 g washed cells was suspended in 8·5 ml phosphate buffer (pH 6·8, 0·05 M) and disrupted in a Branson L575 sonicator at 0° to 4° for 4 min; a particle-free fraction was obtained by centrifugation at 106,000 *g* for 1 hr at 4°.

TABLE 1. THE CONVERSION OF PYRIMIDINE DERIVATIVES TO RIBONUCLEOTIDES BY CELL-FREE EXTRACTS OF *S. faecalis*

All incubations contained 0·2 ml of cell-free extract, radioactive substrate ($2{\cdot}0 \times 10^{-4}$ M; $2–10 \times 10^5$ counts/min/ml), and either 1-pyrophosphoryl-5-phosphate (PRPP) ($2{\cdot}50 \times 10^{-3}$ M) or 0·1 ml of a PRPP-generating mixture[2] in a final volume of 0·5 ml. Each incubation was shaken at 37° for the indicated time. 6-Azauridine (2×10^{-3} M) was added in experiment 4.

Experiment	Substrate	Flask supplements	Extract	Conversion to nucleotide (%)		
				0 min	30 min	60 min
1	AzU-2-^{14}C	None	Sensitive			<1·0
			Resistant			<1·0
2	AzU-2-^{14}C	PRPP-generator	Sensitive	2·2	17·3	30·1
			Resistant	1·0	1·0	1·0
3	AzU-2-^{14}C	PRPP	Sensitive	1·8	33·3	42·0
			Resistant	0·2	0·3	0·3
4	AzU-2-^{14}C	PRPP + AzUR	Sensitive	1·3	32·3	37·9
			Resistant	1·0	1·0	1·0
5	Uracil-2-^{14}C	PRPP	Sensitive			20·0
			Resistant			<1·0
6	Orotic acid-4-^{14}C	PRPP	Sensitive			17·4
			Resistant			16·7

The reaction mixtures for the study of nucleotide formation are described in Table 1. After incubation at 37°, aliquots of 0·05 ml were removed and spotted directly on Whatman 1 paper, dried with heat to stop enzyme activity, and the paper developed with butanol:acetic acid:water (50:10:25). The appropriate nucleotide areas were cut out, suspended in a scintillation mixture, and counted in a Packard Tri-Carb liquid scintillation counter; the R_f values of radioactive areas were compared with those of appropriate standard.

RESULTS AND DISCUSSION

As indicated in Table 1 (experiments 1, 2, and 3) extracts of sensitive cells converted AzU to the nucleotide when PRPP was available, but not in its absence, while extracts of resistant cells lacked the capacity to form the nucleotide. These data indicate that the mechanism of resistance of this particular strain of *S. faecalis* to AzU is a reflection of a marked reduction of the uridylic acid pyrophosphorylase responsible for the conversion of AzU to AzUMP.

With active extracts, some radioactivity always was found on the paper chromatograms in an area corresponding to AzUR. To eliminate the possibility that free AzUR was an intermediate in the formation of ribonucleotides of AzU, a dilution experiment was performed (Table 1, experiment 4). Unlabeled AzUR was added to the incubation at 10 times the concentration of radioactive AzU and, after 60 min, the radioactivity in AzUMP still indicated conversion of AzU to its nucleotide to an extent equivalent to experiments in which no pool of AzUR was added. Thus, the reaction is a direct condensation to form the nucleotide. The radioactivity present as AzUR probably is the result of the action of phosphatases on AzUMP.

Experiment 5 demonstrates that the normal anabolism of U in *S. faecalis* required PRPP and is catalyzed by the same enzyme that is responsible for the formation of ribonucleotides of AzUR. Experiment 6, however, indicates that an extract of AzU-resistant cells, in the presence of PRPP, had not lost the ability to convert orotic acid to UMP(5′) via orotidylic acid. Thus, the difference observed between uridylic acid pyrophosphorylase and orotidylic acid pyrophosphorylase in *Escherichia coli*[5] has been confirmed in *S. faecalis* by use of an AzU-resistant subline in which the enzyme uridylic acid pyrophosphorylase is virtually absent.

The dependence of the enzyme uridylic acid pyrophosphorylase on PRPP and its relationship to the mechanism of resistance also is seen in the effects of unlabeled AzU on the conversion of carboxy-labeled orotidylic acid (OMP-^{14}COOH) to UMP(5′) by an extract of sensitive cells in modified Warburg flasks (Table 2). The inhibition of the synthesis of pyrimidines *de novo* through orotic acid, which depends upon effective conversion of AzU to AzUMP,[3] is dependent upon the concentration of AzU and requires PRPP.

TABLE 2. INHIBITION OF OROTIDYLIC ACID DECARBOXYLASE BY AzUMP FORMED IN CELL-FREE EXTRACTS

All flasks contained 0·2 ml of 2 N NaOH in the center well and were preincubated with 0·4 ml of cell-free extract supplemented with PRPP ($6{\cdot}25 \times 10^{-4}$ M) and AzU, as indicated, for 15 min in a volume of 1·95 ml. After this time, 0·05 ml of a solution of orotidylic acid-^{14}COOH was added to give a final concentration of 5×10^{-7} M and 450 counts/min/ml in 2·0 ml. Incubation was continued for 30 min at 37°. The reaction was stopped with 0·3 ml of 6 N perchloric acid, and the evolved $^{14}CO_2$ was measured in the manner described previously.[2] The results are the average of data from two experiments.

Flask	PRPP	AzU $\times 10^{-4}$ M	Conversion (%)	Inhibition (%)
1	−		41·5	
2	+		48·5	
3	+	0·5	44·0	9·0
4	+	5·0	8·2	83·1
5	−	5·0	34·9	16·0

In conclusion, it may be said that in *S. faecalis* the conversion of AzU to AzUMP involves a condensation of AzU with PRPP. The reaction is catalyzed by uridylic acid pyrophosphorylase, the

same enzyme that catalyzes the conversion of U to UMP(5′), but a different enzyme from orotidylic acid pyrophosphorylase. In the resistant subline, the mechanism of resistance to AzU is attributed to a reduction in the level of this enzyme, uridylic acid pyrophosphorylase; consequently, growing cultures are unable to form AzUMP from AzU, and inhibition of the synthesis *de novo* of pyrimidine is not achieved.

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Biochemical Pharmacology, 1966, Vol. 15, pp. 763–768. Pergamon Press Ltd., Printed in Great Britain.

Carcinostatic agents—XVI. Inhibition of purine biosynthesis and of tetrahydrofolic acid formylase by β-4-methoxybenzoyl-β-bromoacrylic acid

(*Received* 7 *October* 1965; *accepted* 22 *December* 1965)

Cis-β-4-methoxybenzoyl-β-bromoacrylic acid (MBBA) has been prepared as a potential anticancer agent.[1] It proved effective against mammary adenocarcinoma, Crookers' sarcoma and SAK sarcoma in mice, and Yoshida sarcoma in rats.[2, 3] Administration of the compound to a number of patients with advanced carcinoma of the uterus resulted in a marked subjective and sometimes objective improvement.[4] Studies of the mode of action of MBBA indicate that this drug interferes with purine biosynthesis. The details of this investigation are presented in this paper. MBBA and its parent compound β-4-methoxybenzoylacrylic acid were used in the form of their sodium salts. Tetrahydrofolic acid was prepared from folic acid (Lepetit) by catalytic hydrogenation of platinum in glacid acetic acid[5] and was dissolved in a 0·76% solution of cysteine, pH 8, a few drops of 1% $NaHCO_3$ being added to accelerate solution.

METHODS

Enzyme preparation. The enzyme preparation used in all experiments was obtained by extraction of the acetone powder of pigeon liver with 10 vol. of 0·05 M barbiturate buffer, pH 7·6, at 0°. After standing for 10 min at 0° the residue was removed by centrifugation at 2000 *g* and the supernatant used for the experiments.

Purine biosynthesis. The biosynthesis of purines was followed by measuring the incorporation of formate-^{14}C into inosinic acid.[6]

Determination of the effect of MBBA on formimino transferase + cyclodeaminase activity. To 3 μmoles tetrahydrofolate, 13·8 μmoles sodium formiminoglutamate, 19·0 μmoles cysteine, and 3·2 ml 0·1 M triethanolamine–HCl buffer (pH 7·0) was added 0·3 ml enzyme extract to give a total volume of 4·4 ml. Incubation was carried out at 37°. Immediately after addition of the enzyme and after 60-min incubation, 1·2-ml samples were withdrawn and deproteinized with 2·4 ml of 8% perchloric acid. The amount of methenyltetrahydrofolate formed was determined spectrophotometrically at 355 mμ.[7] A nonincubated sample was used as a blank.