Biochemical Pharmacology, 1963, Vol. 12, pp. 13–18. Pergamon Press Ltd., Printed in Great Britain.

TWO DIFFERENT METABOLIC FATES OF 5-ETHYL-6-AZAURACIL IN MICE*

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(*Received* 24 *July* 1962; *accepted* 21 *September* 1962)

Abstract—After the injection into mice of a hypnotic dose of 5-ethyl-6-azauracil, two metabolites and the unchanged drug were detected in the urine. The major metabolite, 5-(1-hydroxyethyl)-6-azauracil, accounted for about 70 per cent of the recovered material, and the minor metabolite, 5-ethyl-6-azauracil-1-ribofuranoside, accounted for approximately 1 to 3 per cent. These findings indicate that this compound is metabolized in an anabolic fashion similar to that of a natural component of nucleic acids, uracil, and is oxidized in a manner similar to the catabolism of barbiturates.

IN THE course of investigating the hypnotic effects of certain homologs of 6-azauracil, it was noted that the hypnotic potency increased with the length of the side chain; however, the duration of anesthesia was reduced.[1] To determine whether metabolic alteration was responsible for the shortened duration of action of one of these homologs, 5-ethyl-6-azauracil (5-ethyl-*as*-triazine-3,5-dione; EtAzU), the compounds excreted in the urine of mice given a hypnotic dose of this drug were determined.

MATERIALS

Ethylazauracil was prepared by Dr. P. K. Chang[2] in this department, and samples of azathymine (5-methyl-6-azauracil) and azathymine ribonucleoside were kindly provided by Dr. W. H. Prusoff.

RESULTS AND DISCUSSION

Metabolites derived from EtAzU were obtained after the intraperitoneal injection of 1 ml of a neutral aqueous solution of EtAzU (20 mg/ml) into each of five Swiss Webster male mice (20–35 g). The mice were kept for 24 hr in plastic cages and urine collected on sheets of blotter paper which were separated from fecal matter and the animals by a layer of fine and a layer of coarse screening. During the collection period the animals were allowed water *ad lib.*, but no food was provided.

The urine was eluted from the filter paper by homogenizing with a suitable amount of water in a Waring Blendor, and the eluate was evaporated under reduced pressure below 45° to a final volume of 10 ml. The recovery of EtAzU and its metabolites was judged to be approximately 70–90 per cent by comparing the UV-absorption at 260 mμ

* This research was supported by Grant CY-2817 from the United States Public Health Service.

† Predoctoral Trainee of the United States Public Health Service (CRTY-5012).

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of the urine concentrate obtained from experimental animals with that obtained from five control animals. This estimate depends upon the similarity in the extinction coefficients (see below) of EtAzU and its metabolites.

A comparison of the UV-absorption of chromatograms developed in solvent system no. 1 (Table 1) revealed the presence of three unique components in the urine

TABLE 1. THE R_F VALUES OF DERIVATIVES OF AZAURACIL, AZATHYMINE, AND ETHYLAZAURACIL

Compound	$R_f \times 100$ Solvent systems 1	2	3	4	5	6
6-Azauracil	55	51	51	28	46	10
6-Azathymine	66	60	61	30	63	15
5-Ethyl-6-azauracil	81	80	72	45	78	28
6-Azauracil-1-ribonucleoside	17	5	41	25	18	4
6-Azathymine-1-ribonucleoside	28	9	50	29	32	8
5-Ethyl-6-azauracil-1-ribonucleoside	40	19	58	33	45	12
5-(1-Hydroxyethyl)-6-azauracil	47	30	52	31	49	12

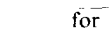
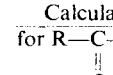
The composition by volume of solvent systems is as follows: solvent 1, upper phase of ethyl acetate:formic acid:water, 65:5:25; solvent 2, upper phase of ethyl acetate:0·05 M phosphate buffer, pH 5·4, 25:10; solvent 3, isobutyric acid-conc. NH_4OH:water, 66:1:33; solvent 4, isopropanol:conc. NH_4OH:water, 85:1·3:15; solvent 5, *n*-butanol:water, 86:14; solvent 6, *n*-butanol: water:conc. NH_4OH, 86:14:5 (conc. NH_4OH in bottom of tank). Descending technique was used in all systems except solvent 3, for which ascending technique was employed. The R_f values in this table were determined on Whatman No. 1 paper.

of animals treated with EtAzU. The amounts of these compounds were determined by elution of the spots from the paper and comparison with the absorbance at 260 mμ of corresponding areas from the chromatogram of the concentrate of the control urine. By this method it was determined that 15–30 per cent of the excreted UV-absorbing material was unchanged EtAzU (R_f 0·81); the remainder of the material was associated with a major metabolite (70–85 per cent, R_f 0·47) and a minor metabolite (1–3 per cent, R_f 0·40).

Using Whatman 3 MM paper in solvent system No. 1, the metabolites were isolated from 20 ml of urine concentrate. The bands containing the EtAzU derivatives were eluted with water and concentrated to a small volume. Purification of the major metabolite was accomplished by adsorption at pH 9·0 on a 1 × 25 cm column of Dowex-1 × 4 (formate, 100–200 mesh) and elution with a gradient of 0·01 N formic acid (500 ml) into water (500 ml). The major UV-absorbing peak was evaporated to dryness under reduced pressure, and the residue was dissolved in absolute ethanol and evaporated to dryness; this latter process was repeated three times to remove water. The residue was crystallized twice from ethyl acetate, and a final yield of 50 mg was obtained. The product was in the form of white needles, mp 153° to 154°, and was homogeneous in the six solvent systems tested (Table 1). The UV-absorbtion spectra resembled those of EtAzU and displayed a hyposochromic shift above pH 7 and a

bathochromic shift at pH 14, which suggests that the triazine ring was intact and that the pKa was similar to that reported for EtAzU (7·47).[2] This absorption coefficient of the metabolite (E^{1cm}_{max} = 5,280)was similar to that of unchanged EtAzU (E^{1cm}_{max} = 5,580). There were no changes in the spectrum at pH 4–5, and the metabolite had an affinity for the anionic Dowex resin approximately equal to that of unchanged ethylazauracil. It therefore appeared improbable that oxidation or ring opening had occurred to form a carboxylic acid. Since oxidation of the side chain was suspected, an iodoform test was performed to determine whether the oxidation had taken place on the carbon α or β to the triazine ring.[3] EtAzU gave a negative test, and a positive test was obtained with the metabolite, a finding which suggests that either

must be present. Elemental analysis indicates that the product is the secondary alcohol 5-(hydroxyethyl)-*as*-triazine-3,5-dione.

	Calculated for 	Calculated for 	Found
C	38·23	38·71	38·45
H	4·49	3·25	4·52
N	26·75	27·09	26·68

The infrared spectra of EtAzU and the major metabolite in KBr disks have the anticipated NH absorption bands at 3·1–3·4 μ and carbonyl bands at 5·75–5·9 μ. In addition, the metabolite displayed an absorption at 2·85 μ that is characteristic of a hydroxyl group and is similar to that found with the hydroxylation product of pentobarbital.[4] Although this metabolite might have been expected to be optically active if a stereospecific oxidation of the side chain had occurred, optical rotation measurements indicated that the α^{D}_{20} was less than 1°.

Isolation of the second metabolite was facilitated by the reaction of this material with the periodate-Schiff spray on paper;[5] a blue color, suggestive of a glycol or related group in the molecule, was obtained. The concentrates of this metabolite obtained by preliminary chromatography in solvent system No. 1 on Whatman 3 MM paper contained almost equivalent amounts of the major metabolite, and the resolution achieved by ion exchange chromatography with the gradient mixture used above was inadequate to separate these two compounds. However, chromatography of the minor metabolite in the form of a borate complex on Dowex-1 resin provided excellent resolution. The crude concentrate of this compound was diluted to 50 ml with a concentrated buffer so that the final concentration was 0·03 M with respect to ammonium formate and 0·02 M with respect to $K_2B_4O_7$. This solution was passed through a 1 × 25 column of Dowex-1 × 4 (formate, 200–400 mesh); the column was first eluted with 0·1 M ammonium formate and 0·007 M $K_2B_4O_7$, adjusted to pH 7·0 with formic acid. This eluate contained the contaminating major metabolite in the fraction between 165 and 210 ml. Elution with this buffer was continued for a total of 700 ml. The minor metabolite was then eluted as a sharp peak with 0·1 N formic acid after 30 ml of this solution had been collected. This fraction was used for further

characterization of this minor metabolite, which was suspected to be 5-ethyl-6-azauracil-1-ribofuranoside.

The UV-absorbtion spectrum of this material (Table 2) differed from that of 5-(1-hydroxyethyl)-6-azauracil in that a hypsochromic shift was noted beyond pH 7, but a bathochromic shift did not occur even at a concentration of 0·5 M NaOH. This difference, which is consistent with replacement of the hydrogen in the 1-position of EtAzU with ribose, has been observed with the ribonucleosides of 6-azauracil[6] and 6-azathymine.[7]

TABLE 2. THE EFFECT OF pH ON THE UV-ABSORPTION OF 5-ETHYL-6-AZAURACIL AND ITS METABOLITES

	Wavelength of maximal absorbance in mμ		
	0·1 N HCl	pH 9	0·5 N NaOH
5-Ethyl-6-azauracil	260	250	288
5-Ethyl-6-azauracil-1-ribonucleoside	264	251	251
5-(1-Hydroxyethyl)-6-azauracil	261	252	288

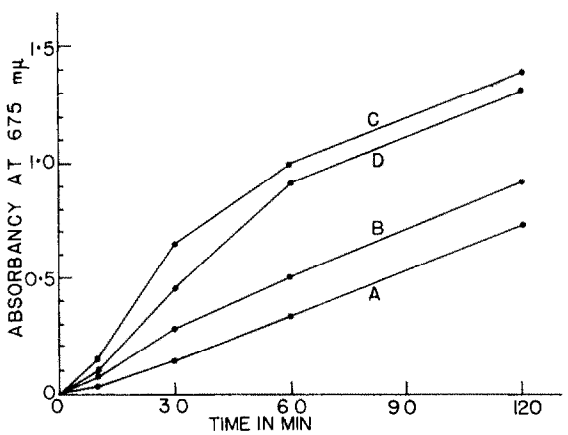


FIG. 1. The rate of development of the orcinol reaction product from the ribonucleosides of uracil, azauracil, azathymine, and ethylazauracil. The reaction mixture contained: 0·25 μmole ribonucleoside in 2 ml water, 2 ml $FeCl_3 \cdot 6H_2O$/HCl (33·6 mg/100 ml conc. HCl) and 0·2 ml 10% orcinol solution. The reaction tubes were heated for the indicated length of time in a boiling-water bath and compared with a blank to which no ribonucleoside had been added. Uridine (A), azauracil ribonucleoside (B), azathymine ribonucleoside (C), and ethylazauracil ribonucleoside (D).

Titration of an aliquot of the metabolite that contained 18·2 absorbance units at 260 mμ with a three-fold excess of sodium metaperiodate[8] in a sodium acetate buffer (pH 4·5, 0·07 M) resulted in the consumption of 3·0 μmole within 15 min. Longer reaction with excess periodate did not result in further oxidation. Since the extinction coefficients of 6-azauracil ribonucleoside and 6-azathymine ribonucleoside at 260 mμ

are between 5 and 6 $\times$ 10^3, the uptake of periodate by the minor metabolite is consistent with the presence of a ribofuranose moiety attached to EtAzU.

The presence of ribose in this derivative was confirmed by the orcinol reaction product,[9] which had an absorption spectrum in the region of 400–700 mμ identical with that obtained from ribose. The possibility that this metabolite was a hexuronic acid derivative which would give a similar absorption spectrum in the orcinol reaction was discounted because of the low affinity of the metabolite for the anion-exchange resin. An indication of the acid-stability of the ribose-triazine linkage can be obtained by comparison of the rate of color development in the orcinol reaction with that observed with other selected ribonucleosides (Fig. 1).

It was shown that the triazine moiety of this metabolite was unchanged EtAzU, by hydrolysis at 100° in 6N HCl for 2 hr. The solution was evaporated to dryness, dissolved in 0·1 ml water, and the residual material was chromatographed on Whatman 1 paper in solvent system No. 1. Only one UV-absorbing spot could be detected, and this was identical with EtAzU.

A compilation of the chromatographic properties of all the compounds involved in this study in six solvent systems is given in Table 1.

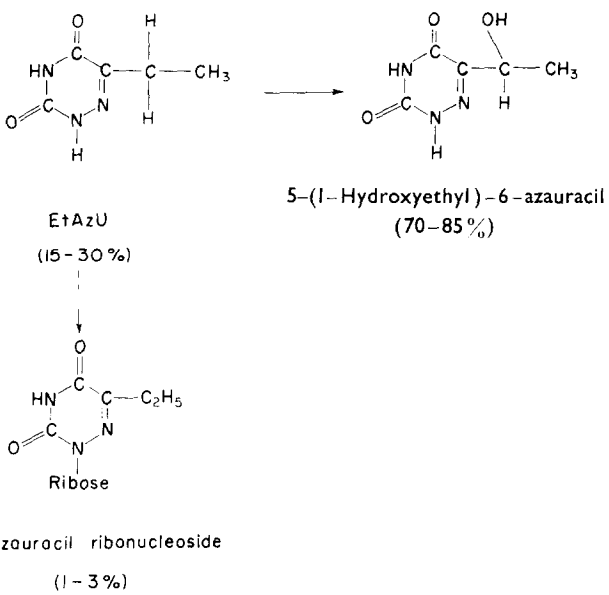


FIG. 2. Ethylazauracil derivatives found in urine of mice within 24 hr after injection. The values within parentheses indicate the form of the recovered 5-ethyl-azauracil derivatives in percentage of the total. Approximately 80 per cent of the injected analog was accounted for in these three compounds.

This investigation indicated that EtAzU undergoes two independent metabolic alterations (Fig. 2); one of these is characteristic of the anabolism of pyrimidines, and the other is typical of that responsible for the oxidation of the 5,5′-side chains of certain barbiturates.[4] Related metabolic alterations of the homologous triazine, 6-azathymine, have been reported.[10]

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