'REPARATION OF 6-AZAURACIL-5-^{3}H AND 6-AZAURIDINE-5-^{3}H OF HIGH IOLAR ACTIVITY.

Jiri Filip *, Jan Skoda and Hynek Hradec *.
Institute for Research, Production and Uses of Radioisotopes;
Institute of Organic Chemistry and Biochemistry, Czechslovak
Academy of Sciences, Prague-Czechoslovakia.
* Institute for Research, Production and Uses of Radio-
Isotopes, Pristavni 24, Prague 7, Czechoslovakia.
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SUMMARY

Catalytic reductive dehalogenation of 5-bromo-6-azauracil with carrier-free tritium led to 6-azauracil-5-^{3}H of a molar activity of 19.0 Ci/mmole. The reaction conditions for the catalytic reductive dehalogenation were examined in tracer experiments. Microbial transformation was used for the preparation of 6-azauridine-5-^{3}H from 6-azauracil-5-^{3}H. 6-Azauridine-5-^{3}H was prepared at a molar activity of 18.8 Ci/mmole. In both compounds the stability of tritium was investigated in an aqueous medium at 100°C.

INTRODUCTION.

6-Azauridine was discovered as an intermediate anabolite in a study of the mechanism of inhibitory effects of 6-azauracil in Escherichia coli[1] and Streptococcus faecalis[2]. In biological systems, 6-azauridine is enzymically transformed to 6-azauridine 5'-monophosphate which inhibits orotidine 5'-phosphate decarboxylase and thus also the biosynthesis of pyrimidine precursors de novo. 6-Azauridine is produced on the large scale by fermentation from 6-azauracil[3]. The mechanism of action of 6-azauridine and its application have been reviewed recently[4].

An exceptionally important role in the solution of the mechanism of 6-azauridine action and in the study of pharmacological properties of this anomalous ribonucleoside belonged to labelled 6-azauracil and 6-azauridine. In 1957, Morávek[5] described the preparation of 6-azauracil-4,5-^{14}C, Chang and Ulbricht then published[6] the synthesis of 6-azauracil-2-^{14}C; a fermentation preparation of 6-azauridine-4,5-^{14}C has been described later[7].

At present, labelled 6-azauridine finds application in the estimation of activity of uridine kinase[8] and in connection with the specific problems of clinical application of 6-azauridine in the therapy of psoriasis and of some special neoplastic diseases[4]. It was thus considered useful to prepare a tritium-labelled 6-azauridine; this form is less costly than ^{14}C-labelled 6-azauridine. We were aware that 6-azauridine-^{3}H can be successfully employed only at a very high specific radioactivity.

6-Azauracil-5-^{3}H at a molar activity of 19.0 Ci/mmole was prepared by reductive catalytic dehalogenation of 5-bromo-6-azauracil. An attempt to prepare 6-azauracil-5-^{3}H by the action of n-butyllithium on 5-bromo-6-azauracil and by decomposition of the derivative with tritiated water was not successful[6]. It was concluded that the hydrogen atom in position 5 is too labile. However, the stability of tritium in 6-azauracil-5-^{3}H was examined and it was found to be greater than in uracil-5-^{3}H.

Different methods can be employed for labelling 6-azauracil with tritium[9]. However, to obtain a high molar activity of the product one has to use carrier-free tritium and to restrict its isotopic exchange with the solvent during the reductive catalytic dehalogenation. The isotopic exchange of hydrogen was examined earlier[10] in the system hydrogen-^{3}H - water under

conditions analogous to those in experiments with catalytic reductive dehalogenation of 5-bromo-6-azauracil and the reaction rate was found to increase with increasing concentration of hydrogen ions. In tracer experiments we followed the catalytic reductive dehalogenation of 5-bromo-6-azauracil in an aqueous medium. The reaction conditions were adapted to work on micro-scale conditions. At the relatively high amount of catalyst (100-200% referred to the starting bromo derivative) the dehalogenation of the halogen derivatives proceeded rapidly even at a reduced pressure of hydrogen-^{3}H. During catalytic dehalogenation of halogen derivatives in pyrimidine no significant isotopic effect could be observed[10,11]. The results of tracer experiments permitted to assess the molar activities that could be attained on using carrier-free tritium. This assumption was verified experimentally. From the reaction mixture, 6-azauracil-5-^{3}H was isolated by preparative paper chromatography. Tritium was removed from the "labile bonds" before chromatography by dissolving 6-azauracil-5-^{3}H in water and by evaporation. This procedure was repeated to constant activity in the solid phase. Chemical and radiochemical purity was checked by UV spectrometry and chromatography in three different chromatographic systems. Radiochemical purity of 6-azauracil-5-^{3}H was greater than 96%, specific activity was 19.0 Ci/mmole.

For the preparation of 6-azauridine-5-^{3}H and 6-azauracil-5-^{3}H we used successfully microbial transformation. The high molar activities made it possible to isolate 6-azauridine-5-^{3}H from the reaction mixture by preparative paper chromatography. 6-Azauridine-5-^{3}H of molar activity of 18.8 Ci/mmole and radiochemical purity greater than 97% was thus obtained.

EXPERIMENTAL.

The samples were analyzed by paper chromatography (Table 1) on Whatman 3 in a descending direction at 20°C. The compounds were detected on the chromatograms under UV light (Chromatolite).

The radioactivity of the samples was determined in a liquid scintillator by a single-channel counter NE 5503 (Nuclear Entreprises, England). The scintillation liquid SLD 31 (dioxane, naphthalene) was made by Tesla, n.e., Pardubice. The detection efficiency was determined for every sample by the internal standard method (standard EK-1, toluene-^3H; volume activity 1.97 μCi/ml made in the Institute for Research, Production and Uses of Radioisotopes, Czechoslovakia). The radiochemical purity was determined by paper chromatography and counting the chromatograms.

Mass was estimated by measuring the UV spectra in a CF 4 NIR spectrophotometer (Optica Milano, Italy) using quartz cuvettes (5 mm light path) in a double-beam arrangement.

Table 1. Paper chromatography of 6-azauracil and its derivatives.

Compound	R_F in solvent system[a]			
	A	B	C	D
6-Azauracil	0.57	0.47	0.23	0.50
5-Bromo-6-azauracil	0.74	0.66	0.32	0.65
6-Azauridine	0.30	0.17	0.18	0.20

[a] A - 1-butanol-acetic acid-water (4:1:5); B - 1-butanol saturated with water; C - 1-butanol- 1-propanol-ammonium hydroxide-water (7:5:7:2); D - 1-butanol-ethanol-water (4:1:1).

Chemicals and radioactive material.

Nonactive chemicals were supplied by Lachema (Czechoslovakia). 6-Azauracil and 6-azauridine were made by Spofa (Czechoslovakia). Gaseous tritium (containing 2% H_2) and water-^3H were supplied by Tekhsnabeksport (USSR). 6-Azauracil-4,5-^{14}C and 6-azauridine-4,5-^{14}C were made at the Institute for Research, Production and Uses of Radioisotopes (Czechoslovakia), their radiochemical purity being greater than 98%.

Preparation of 5% palladium on barium sulphate.

The palladium catalyst on barium sulphate was prepared according to ref.[12]. A total of 18.5 g catalyst was obtained; it was stored in a desiccator over phosphorus pentoxide.

Preparation of 5-bromo-6-azauracil.

5-Bromo-6-azauracil was prepared according to Change and Ulbricht[5] by bromination of 6-azauracil. A total of 1.5 g chromatographically pure compound was obtained.

Apparatus for tracer and active experiments.

Previously described apparatus[13] was used in tracer and active experiments. The reservoir of the apparatus for tracer experiments was filled with hydrogen-^3H of volume activity of 1.5 mCi/ml. For the preparation of 6-azauracil-5-^3H of molar activity of the order of Ci/mmole a modified apparatus of Wenzel was used. The consumption of tritium was determined after termination of the reaction by transferring the nonreacted tritium to a calibrated reservoir and by measuring the pressure.

Conditions for catalytic reductive dehalogenation of 5-bromo-6-azauracil in an aqueous medium.

5-Bromo-6-azauracil together with the catalyst (5% palladium on barium sulphate) was placed in a reaction vessel of 1.2 ml capacity. Hydrochloric acid (or potassium or ammonium hydroxide) was then added to the flask at the normality shown in Table 2. The reaction vessel was attached to the apparatus for tracer experiments. After freezing the reaction mixture the apparatus with the exception of the reservoir was evacuated and the reaction mixture freed of gases. Hydrogen-^{3}H was then let in and the reaction

Table 2. Reductive dehalogenation of 5-bromo-6-azauracil.

Expt. number	Solvent	Reaction period (min)	Consumption of hydrogen-^{3}H (ml)	6-Azauracil-5-^{3}H Activity before chromatography (µCi)	Activity (µCi)	Chemical yield (mg)	%	Specifi activit (mCi/mm
1	1N KOH	65	1.1	340	307	4.2	71.3	8.2
2	0.1N KOH	41	1.4	530	375	4.25	72.2	9.9
3	0.01N KOH	44	1.4	472	370	5.3	90.0	7.9
4	distilled water	44	1.4	340	290	4.8	81.4	6.8
5	0.01N HCl	51	1.4	350	320	5.0	84.8	7.2
6	0.1N HCl	37	1.5	250	220	5.0	84.8	4.9
7	1N HCl	120	1.35	148	125	5.1	86.6	2.7

The reaction conditions were as follows: Volume activity of hydrogen-^{3}H 1.5 mCi/ml; 20°C, 10.0 mg 5-bromo-6-azauracil; 0.5 ml solvent, 20 mg 5% Pd/$BaSO_4$.

mixture was stirred electromagnetically at room temperature. The dependence of consumption of hydrogen-^{3}H on time is shown in Fig. 1 and 2. After termination of the reaction, gases were again removed, the pH was adjusted to 7.1 and the solvent was removed by freeze-drying. Tritium from the labile bonds was removed by dissolving 6-azauracil in water and by freeze-drying of the solution formed. 6-Azauracil-5-^{3}H was isolated from the reaction

Fig. 1. Reductive dehalogenation of 5-bromo-6-azauracil. Dependence of consumption of hydrogen-^{3}H on time at different concentrations of hydrochloric acid. Reaction conditions: 10.0 mg 5-bromo-6-azauracil, 0.5 ml solvent, 20.0 mg 5% Pd/$BaSO_4$. Volume activity of hydrogen-^{3}H 1.5 mCi/ml. 1 - 1N HCl, 2 - 0.1N HCl, 3 - 0.01N HCl, 4 - water.

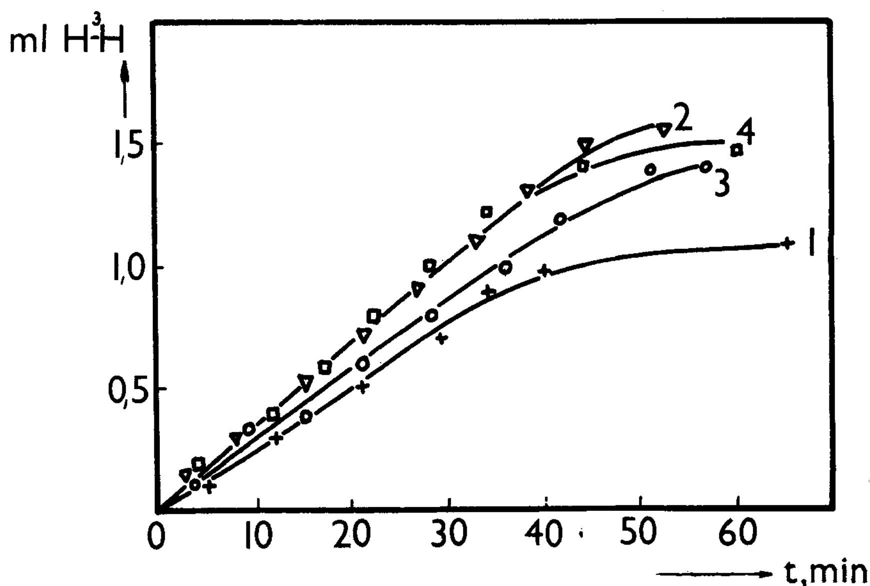
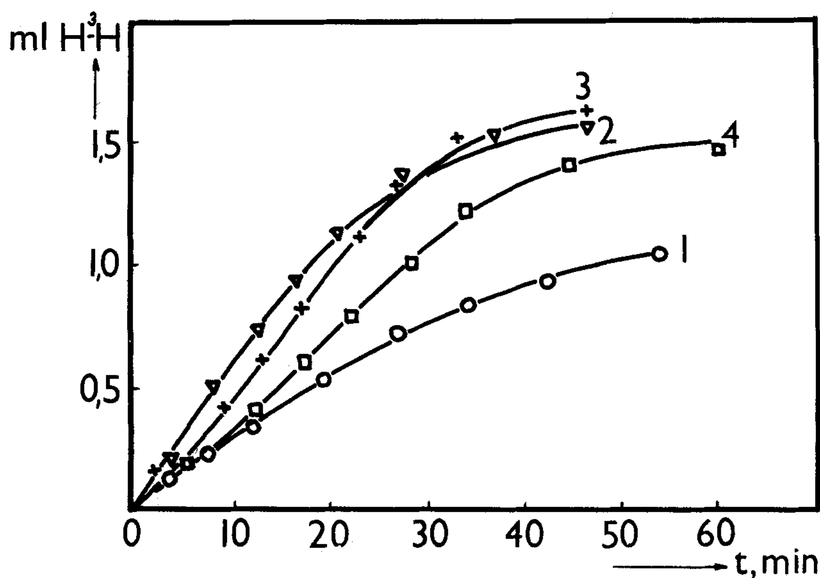


Fig. 2. Reductive dehalogenation of 5-bromo-6-azauracil. Dependence of consumption on hydrogen-^{3}H on time at different concentrations of potassium hydroxide. Reaction conditions: 10.0 mg 5-bromo-6-azauracil, 0.5 ml solvent, 20.0 mg 5% Pd/$BaSO_4$. Volume activity of hydrogen-^{3}H 1.5 mCi/ml. 1 - 1N KOH, 2 - 0.1N KOH, 3 - 0.01N KOH, 4 - water.



mixture by preparative paper chromatography in 1-butanol-acetic acid-water (4:1:5). The results are shown in Table 2.

Preparation of 6-azauracil-5-^{3}H.

a) Tracer experiment.

10.2 mg 5-bromo-6-azauracil (52.9 μmole), 20 mg 5% palladium on barium sulphate and 0.5 ml 0.1N potassium hydroxide was placed

in the reaction vessel. The vessel was attached to the apparatus for tracer experiments. After degassing of the reaction mixture, hydrogen-^{3}H was admitted to the mixture (volume activity of 1.5 mCi/ml). The consumption of hydrogen-^{3}H was followed by the drop of pressure. After 46 min, 1.3 ml hydrogen was consumed and the reaction was terminated. The reaction mixture was then evaporated to dryness in a closed system, the residue was dissolved in 1 ml water and the solution evaporated again. This procedure was twice repeated. 6-Azauracil-5-^{3}H was isolated from the reaction mixture by preparative paper chromatography in 1-butanol-acetic acid-water (4:1:5). The radiochemical purity of 6-azauracil-5-^{3}H was confirmed in three solvent systems (Table 1) to be better than 97%. Then chemical yield of 6-azauracil-5-^{3}H was 4.7 mg (i.e. 78.4% referred to the 5-bromo-6-azauracil used). The total activity of the product was 395 μCi, the molar activity of 6-azauracil-5-^{3}H was 9.5 mCi/mmole.

b) Active experiment.

13.0 mg 5-bromo-6-azauracil (65.4 μmole), 20 mg 5% palladium on barium sulphate and 0.5 ml 0.1N potassium hydroxide was placed in a reaction vessel. After attaching to the apparatus for active experiments, the mixture was degassed and 2.0 Ci tritium admitted from the reservoir to the reaction vessel. After 28 min, a total of 50% of the tritium was consumed, after 2 h 86% tritium was consumed, whereafter the reaction was terminated. The pH value of the reaction mixture was adjusted to 7.0. The total activity of the reaction mixture was 1.36 Ci. The labile activity was removed as described with the tracer experiment. Activity in the solvent and the labile activity amounted to 900 mCi. Preparative paper chromatography in 1-butanol-acetic acid-water (4:1:5)

was used to isolate from the reaction mixture a total of 2.56 mg 6-azauracil 5-^{3}H (i.e. 63.2% referred to the tritium used) of a total activity of 430 mCi. The molar activity of the product was 19.0 Ci/mmole. The radiochemical purity checked in three solvent systems (cf. Table 1) was greater than 97%.

6-Azauridine-5-^{3}H (active experiment).

Inoculum (0.1 ml) of a 15-h culture of Escherichia coli B growing at 37 °C on a mineral medium containing glucose was diluted with 5 ml medium of the same composition. The medium was flushed with sterile air and culture growth was followed nephelometrically at 575 nm. During the logarithmic phase of culture growth (after 6 h of cultivation) a total of 200 mCi 6-azauracil-5-^{3}H of molar activity of 19.0 Ci/mmol in 0.5 ml water was added. Concentration of 6-azauracil-5-^{3}H in the medium was 1.88 x 10^{-3}M. The culture was further flushed with sterile air. After 15 h of cultivation, the process was stopped, the medium was cooled to 2 °C and bacteria were centrifuged. The supernatant was placed on 2 sheets of Whatman no.3 and chromatographed in 1-butanol-acetic acid-water (4:1:5) in a descending direction. A strip corresponding to the standard of 6-azauridine was cut out and eluted with water. A total of 72 mCi 6-azauridine-5-^{3}H (36%) of a molar activity of 18.8 Ci/mmole was obtained. The radiochemical purity checked in systems A, B and D (Table 1) was greater than 96%. Preparative chromatography recovered from the reaction mixture 100.6 mCi 6-azauracil-5-^{3}H (53%).

Isotopic exchange of hydrogen in 6-azauracil-5-^{3}H and 6-azauridine-5-^{3}H in an aqueous medium and dependence on the

concentration of hydrochloric acid and sodium hydroxide.

100 μCi 6-azauracil-5-^{3}H of molar activity of 16.0 mCi/mmole and 10.0 μCi 6-azauracil-4,5-^{14}C of molar activity of 80.0 mCi/ /mmole was dissolved in 0.5 ml water of known pH and sealed in a glass ampoule. The ampoule was heated for 1 h to 100°C. After cooling, the pH was adjusted to 7.0 and water was evaporated from the solution in a closed system. Stability of 6-azauracil-4,5-^{14}C was estimated chromatographically in 1-butanol-acetic acid-water (4:1:5) and by measuring the radioactivity of the chromatogram.

Table 3. Isotopic exchange of hydrogen in 6-azauracil-5-^{3}H in an aqueous medium (100°C, 1 h). Dependence on the concentration of hydrochloric acid and sodium hydroxide.

Expt. no.	Medium	Exchange of ^{3}H (%)	Radiochemical purity of 6-azauracil-4,5-^{14}C (%)
1	1N HCl	2.3	96.0
2	10^{-1}N HCl	3.4	100.0
3	10^{-2}N HCl	4.9	-
4	10^{-4}N HCl	6.1	-
5	10^{-5}N HCl	5.6	-
6	H_2O	3.4	99.9
7	10^{-5}N KOH	3.8	-
8	10^{-4}N KOH	4.8	-
9	10^{-2}N KOH	3.3	-
10	10^{-1}N KOH	3.8	85.0
11	1N KOH	5.6	75.0

Results of isotopic exchange of hydrogen of 6-azauracil-5-^{3}H and of radiochemical stability of 6-azauracil-4,5-^{14}C are shown in Table 3.

100 μCi 6-azauridine-5-^{3}H of molar activity of 12.6 mCi/mmole was dissolved in 0.5 ml water of known pH (see Table 4) and sealed in a glass ampoule. The ampoule was heated for 1 h to 100 °C. After cooling and adjusting the pH to 7.0, water-^{3}H was evaporated from the solution in a closed system.

The results of isotopic exchange of hydrogen in 6-azauridine--5-^{3}H are ahown in Table 4.

Table 4. Isotopic exchange of hydrogen in 6-azauridine-5-^{3}H in an aqueous medium (100°C, 1h). Dependence on the concentration of hydrochloric acid and sodium hydroxide.

Expt. no.	Medium	Exchange of ^{3}H (%)
1	1N KOH	38.0
2	10^{-1}N KOH	8.6
3	10^{-2}N KOH	3.7
4	H_2O	2.8
5	10^{-2}N HCl	2.2
6	10^{-1}N HCl	2.3
7	1N HCl	2.2

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