Phytochemistry, 1964, Vol. 3, pp. 603 to 607. Pergamon Press Ltd. Printed in England

METABOLISM OF 6-AZAURACIL AND ITS INCORPORATION INTO RNA IN THE COCKLEBUR

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(*Received* 9 *August* 1963)

Abstract—Leaves or shoot tips of the cocklebur, *Xanthium pennsylvanicum* Wall., catabolized 6-azauracil and converted it to the nucleotide level and into RNA. Both catabolic and anabolic reactions were weak when compared to orotic acid, presumably a normal intermediate in pyrimidine nucleotide synthesis in plants.

INTRODUCTION

METABOLIC effects of the uracil analog, 6-azauracil, or its ribonucleoside, 6-azauridine, have been studied in bacteria,[1,2,3] protozoa,[4] and various animal tissues.[5,6] Azauracil has been used with partial success in certain anti-cancer experiments.[7] The primary inhibiting action of the compound, following its conversion to 6-azauridine-5′-phosphate, was on the enzyme orotidylic acid decarboxylase,[8] thus interfering with ribonucleic acid synthesis. Inhibition of polynucleotide phosphorylase[9] and the attachment of amino acids to soluble RNA[10] by nucleotide derivatives of azauracil has also been reported. Azauracil (or azauridine) was found to be incorporated into RNA of *Streptococcus faecalis*,[1] *Trypanosoma equiperdum*,[4] and of cat brain,[5] but not by mouse tissues,[6] or *Escherichia coli*.[2]

No studies on its metabolism by higher plants appear to have been reported. We have found it to inhibit induction of flowering in the short-day cocklebur plant, *Xanthium pennsylvanicum* Wall.[11] As contrasted to the inhibition caused by 5-fluorouracil where the effect was overcome by a pyrimidine nucleotide precursor orotic acid,[12] the latter completely failed to reverse the azauracil inhibition of flowering. If azauracil blocked the orotidylic acid decarboxylation step, orotic acid would not be expected to over come inhibition, except perhaps by a mass action effect. Since as mentioned above, however, only 6-azauridine-5′-phosphate is reported to be an effective inhibitor of orotidylate decarboxylation,[8,13] inter-

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[2] J. SKODA, in *Protein Biosynthesis* (Edited by R. J. HARRIS), p. 185, Academic Press (1961).
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[6] C. PASTERNAK and R. HANDSCHUMACHER, *J. Biol. Chem.* **234**, 2992 (1959).
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[9] J. SKODA, J. KARA and Z. SOROMONA, *Coll. Czech. Chem. Comm.* **24**, 3783 (1959).
[10] F. KALOVSEK, I. RYCHLIK and F. SORM, *Biochim. Biophys. Acta* **61**, 368 (1962).
[11] W. COLLINS, F. SALISBURY and C. ROSS, *Planta* **60**, 131 (1963).
[12] F. SALISBURY and J. BONNER, *Plant Physiol.* **35**, 173 (1960).
[13] R. HANDSCHUMACHER, *J. Biol. Chem.* **235**, 2917 (1960).

ference with flower induction implies conversion of azauracil to the effective nucleotide by the plants. This paper reports the ability of the cocklebur to absorb, catabolize, and to convert 6-azauracil to azauridine, azauridine-5'-phosphate, and into RNA.

RESULTS AND DISCUSSION

In the first experiments conversion of azauracil-2-^{14}C to RNA of shoot tips was compared with orotic acid and with 5-fluorouracil, previously shown to be incorporated into RNA of this plant.[14] Specific activities of the RNA after addition of azauracil, 5-fluorouracil, and orotic acid (all labeled in the 2 position) are shown in Table 1. After correcting for differences in absorption by the plants, azauracil was incorporated only about one-third as efficiently as fluorouracil and one-twentieth as efficiently as orotic acid.

To be certain that the ^{14}C in the RNA from plants fed azauracil-2-^{14}C had remained present in the azauracil molecule, the nucleotides of RNA were chromatographed on paper in two solvent systems: with the isopropanol–HCl solvent, a radioactive spot identical to that of 6-azauridine-5'-phosphate (R_f 0·72) and separate from azauracil (R_f 0·66) was detected upon scanning the strips with a recording windowless flow counter. (This spot probably

TABLE 1. INCORPORATION OF AZAURACIL, 5-FLUOROURACIL, AND OROTIC ACID INTO RNA OF COCKLEBUR SHOOT TIPS*

Compound	^{14}C absorbed/ ^{14}C added	Sp. act. of RNA (cpm/μg)	RNA ^{14}C/ absorbed ^{14}C $\times 10^3$
6-Azauracil-2-^{14}C	0·68	1·29	2
5-Fluorouracil-2-^{14}C	0·60	4·07	6·9
Orotic acid-2-^{14}C	0·50	20·58	40·4

* All values are means of two experiments, each with 15 plants per precursor.

contained a mixture of the 2' and 3' phosphates of azauridine, since the R_f values of the 2', 3' and 5' phosphates of other nucleotides are identical in this solvent.) After chromatography in the isopropanol–formic acid solvent the radioactivity was at R_f 0·29, compared to R_f of 0·28 for 6-azauridine-5'-phosphate and 0·64 for 6-azauracil. Finally, the RNA-nucleotides from the buds fed 6-azauracil-2-^{14}C were hydrolyzed with perchloric acid, neutralized with KOH, and the resulting solution chromatographed in *n*-butanol–acetic acid–H_2O. All of the ^{14}C was detected at R_f 0·58, exactly corresponding to the azauracil spot co-chromatographed. These results are good evidence azauracil was present in the RNA, even though sufficient amounts were not present to allow rigorous chemical proof of identity.

To determine whether the limited incorporation of azauracil was due to its rapid catabolism, experiments with leaf discs were performed in Warburg flasks were the $^{14}CO_2$ was collected for later counting. In Table 2 are listed the percentages of absorbed radioactivity recovered in the various tissue fractions. In leaf discs incubated with either orotic acid or azauracil most of the radioactivity absorbed was found in the alcohol fractions. Only 0·15% was recovered in RNA in the case of azauracil-^{14}C, compared to 6·2% with labeled orotic acid. The relatively weak ability of azauracil to label the RNA was, however, apparently not

[14] J. BONNER and J. A. D. ZEEVAART, *Plant Physiol.* 37, 43 (1962).

due to rapid conversion to carbon dioxide, since the carbon dioxide also contained only little radioactivity. Instead, much of the absorbed azauracil was recovered unmetabolized in the alcohol extracts. Scanning the chromatograms of these alcohol extracts revealed three labeled spots separated by each solvent. Areas of the peaks on the recorder were measured to determine the relative radioactivity in each. The most highly labeled compound was unmetabolized azauracil, the other two spots being 6-azauridine-5′-phosphate and 6-azauridine, respectively. The average percentage of radioactivity present on the chromatograms was 70·5 for azauracil, 6·9 for azauridine, and 22·6 for the nucleotide. No catabolic products were detected.

In the extracts from leaves given orotic acid-^{14}C three major labeled spots were separated using the *t*-butanol solvent.* The fastest moving contained only orotic acid, while the second ran with uridine-5′-phosphate in the three solvent systems used. The slow-moving area chromatographed with uridine-diphosphate and uridine-diphosphoglucose, which were not well resolved by these solvents. The percentages of radioactivity present were 20, 16, and 64, respectively. This shows a much greater conversion of orotic acid than azauracil to the nucleotide level.

TABLE 2. RECOVERY OF RADIOACTIVITY FROM ^{14}C-LABELLED AZAURACIL AND OROTIC ACID ADDED TO COCKLEBUR LEAF DISCS

Compound	cpm added $\times 10^{-3}$	% absorbed	Recovery* (% of absorbed ^{14}C)					
			CO_2	Ethanol soluble	$HClO_4$ soluble	KOH insoluble	DNA	RNA
6-Azauracil	939	12·00	0·62	99·0	0·11	0·01	0·00	0·15
Orotic acid	964	10·55	4·51	83·1	6·10	0·05	0·06	6·20

* Each value is the average of three determinations each from three separate experiments.

Thus while azauracil was absorbed by the tissue as readily as was orotic acid, more of it remained unmetabolized. The presence of azauridine suggests that azauracil is converted to the nucleotide via a uridine phosphorylase enzyme, as in mammalian tissues, rather than a pyrophosphorylase as in the protozoan.[4] That it is converted into RNA, perhaps leading to "ineffective" RNA molecules, may be one reason for its ability to inhibit flowering of cocklebur, since there is good evidence that normal RNA synthesis is essential for induction of flowering in this plant.[14] However, the formation of 6-azauridine-5′-phosphate makes it also very likely that an inhibition of orotidylate decarboxylase may be equally implicated. This possibility is being investigated.

EXPERIMENTAL

Shoot-Tip Experiments

Tips were prepared for use according to the method of Bonner and Zeevaart.[14] Leaves were removed from two-month-old plants until the longest leaf blade remaining did not

* With later two-dimensional chromatograms (using radioautographs) we have detected as many as seven metabolites from orotic acid.

[15] R. MARKHAM, in *Modern Methods of Plant Analysis* (Edited by K. PAECH and M. TRACY), Vol. IV, p. 296, Springer-Verlag (1955).

exceed 0·8 cm. In addition, one photoperiodically sensitive leaf about 8·0 cm was left on the plants. All RNA precursors were adjusted to a specific activity of 4·2 mc/mmole and 0·08 μc was added to each of 15 shoot tips of plants with a micropipette. The solutions contained 0·1% Tween 20, which allowed uniform spreading. After 16 hr continuous darkness at 24°, tips were excised and rinsed quickly but thoroughly in 100 ml water to wash off unabsorbed metabolites. They were then killed and extracted in 80% ethanol, followed by homogenization in 50% ethanol (adjusted to pH 4 with acetic acid) to remove remaining soluble nucleotides, as suggested by Markham.[15] Following centrifugation the residues were further rinsed with this solvent until no more radioactivity was detectable in the rinses, then twice with cold 0·2 N perchloric acid. RNA extraction with 0·3 M KOH and its measurement were as described previously.[16] Radioactivity was detected with a thin window gas-flow geiger tube. Ribonucleotides of RNA were separated on 55-cm paper strips using either the descending isopropanol-HCl–water system of Wyatt[17] or isopropanol:formic acid:water (7:1:2). 6-Azauridine-5′-phosphate and azauracil were also chromatographed for comparison. Nucleotides were detected with a Mineralight u.v. lamp.

The nucleotides from azauracil-^{14}C-labeled RNA were hydrolyzed into their constituent bases by perchloric acid.[15] After evaporation of water from the nucleotides, 0·40 ml of 70% $HClO_4$ was added. The tubes were stoppered tightly and kept for 2 hr in a boiling water bath. (We found shorter periods of time did not completely hydrolyze 6-azauridine-5′-phosphate to azauracil; some azauridine also remained.)

Absorption of the pyrimidines added to the shoot tips was calculated by subtracting the amount washed off after the experiment from the amount added at the start. Preliminary studies showed that there were large differences among these compounds in catabolism to carbon dioxide, which could not be easily collected in these experiments. Thus, direct measurements of the radioactivity present in the tissues were not reliable estimates of the amount of absorption.

Leaf Disc Experiments

Discs (0·7 cm dia.) were cut from the most rapidly growing leaf of several plants, pooled and 12 placed in each flask (130 mg fresh tissue). The flasks contained 0·01 M phosphate buffer, pH 5·8, and 2·0 μc 6-azauracil-2-^{14}C or orotic acid-2-^{14}C, in a final volume of 3·0 ml.

After incubation at 28° for 6 hr in darkness the discs were removed, rinsed thoroughly, then killed and extracted as described above. After aliquots were taken for radioactivity measurements, the alcohol extracts were evaporated and descendingly chromatographed with suspected metabolites in *n*-butanol:acetic acid:water (2:1:1) (solvent 1) and *t*-butanol:methyl-ethyl ketone:water:NH_4OH (4:3:2:1) (solvent 2). Compounds were then eluted and co-chromatographed in isopropanol–formic acid–water with various metabolites to further indicate their identities. Whatman papers, type 3 MM, 55 cm long were used. Finally, spots thus suspected to contain azauracil, azauridine, and azauridine-5′-phosphate were co-chromatographed two-dimensionally on 47 × 56 cm papers in solvents 1 and 2 with commercial compounds. These were located with the Mineralight u.v. lamp and the shapes of the absorbing areas traced on the chromatograms. Autoradiograms were then made by placing the paper against Kodak Blue Brand X-ray film for two weeks. The shapes and positions of the exposed areas on the film coincided exactly to the u.v. absorbing areas. This was considered sufficient evidence to identify these compounds.

[16] C. ROSS, *Biochim. Biophys. Acta* **55**, 387 (1962).
[17] G. WYATT, *Biochem. J.* **48**, 584 (1951).

In counting radioactivity in all fractions (except alcohol extracts) corrections for self absorption were made. Carbon dioxide was converted to $BaCO_3$ and counted at infinite thickness. The residue insoluble in KOH was wet combusted to CO_2 and also counted as $BaCO_3$. DNA was measured by the method of Bonner and Zeevaart.[14]

Acknowledgements—This work was supported by a grant (GM 06374-05) from the National Institutes of Health. Janet Thomas provided capable technical assistance.