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ACCUMULATION OF 5-RIBOSYLURACIL (PSEUDOURIDINE) WITHIN THE TISSUES OF *PHASEOLUS VULGARIS*

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Key Word Index—*Phaseolus vulgaris*; Leguminosae; 5-ribosyluracil; pseudouridine; nucleosides.

Abstract—Free 5-ribosyluracil (pseudouridine) accumulates within the acid-soluble fraction of germinating seeds and seedlings of *Phaseolus vulgaris*. Accumulation is significantly increased by exogenous uridine. Experiments with [^{14}C]-labelled precursors indicate that UTP is an intermediate in the formation of this free 5-ribosyluracil.

INTRODUCTION

5-Ribosyluracil (pseudouridine), a constituent *C*-nucleoside of tRNA from diverse sources, does not commonly occur in a free state in nature. It is, however, detectable in mammalian urine, especially in that from animals with neoplasms, and some lower organisms produce significant concentrations of the free nucleoside, e.g. *Agrobacterium tumefaciens* [1], *Saccharomyces cerevisiae* [2] and *Streptoverticillium ladakanus* [3]. This present report describes the accumulation of 5-ribosyluracil in a free state within the tissues of a higher plant and considers its metabolic origin in these tissues.

RESULTS AND DISCUSSION

During work with the acid-soluble constituents of *Phaseolus vulgaris*, an unusual pyrimidine riboside was isolated. The electrophoretic behaviour of the compound and its UV spectrophotometric properties at various pH values were identical to those of 5-ribosyluracil. Identification was confirmed by cochromatography with an authentic sample in solvent systems 1–6 (see Experimental). Although occurrence of this nucleoside in hydrolysates of RNA from plant sources is to be expected and has been reported (e.g. ref. [4]), accumulation of the free compound in higher plants has not previously been described. That the free compound is not an artefact resulting from hydrolysis of RNA during the extraction procedure was shown by parallel extraction of a yeast RNA solution.

The concentration of 5-ribosyluracil in germinating seeds and seedlings of *Phaseolus vulgaris* was determined at various stages. The results (Fig. 1) show that over a 14 day period, commencing with imbibition on day 0, there was a three-fold increase in the 5-ribosyluracil content. In seeds treated with uridine (N^1-ribosyluracil), there was a significantly greater concentration of 5-ribosyluracil. Uracil, however, had no significant effect. The increasing concentration of 5-ribosyluracil with age and the effect of uridine were reproducible in several experiments.

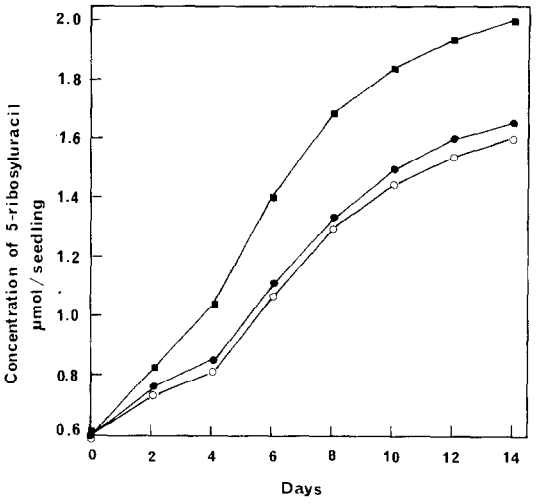

Fig. 1. Free 5-ribosyluracil (pseudouridine) in seeds and seedlings of *Phaseolus vulgaris* during germination and growth. Seeds were put to imbibe at zero time, in water (O); in 30 mM uracil (pH 5.5) (●); in 30 mM uridine (pH 5.5) (■). Batches of 25 seeds or seedlings were used for easy assay.

Experiments in which excised shoots from 8-day-old *Phaseolus* seedlings were supplied with solutions of [^{14}C]-labelled putative precursors (Table 1) showed incorporation into 5-ribosyluracil of radioactivity from [6-^{14}C]orotate, [2-^{14}C]uracil and [4-^{14}C]UTP. Incorporation from [4-^{14}C]UTP was almost twice that from [6-^{14}C]orotate and × 9 that from [2-^{14}C]uracil. In the presence of a pool of exogenously supplied UTP (Table 2), incorporation of radioactivity from [6-^{14}C]orotate was reduced to less than half that in the controls which were not supplied with UTP. Introduction of a pool of unlabelled uracil had no significant effect on incorporation of [6-^{14}C]orotate. Incorporation of radioactivity from [4-^{14}C]UTP into 5-ribosyluracil was not significantly affected by concomitant introduction of relatively large pools of unlabelled uracil, uridine, or

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Table 1. Incorporation of radioactivity from [^{14}C]-labelled putative precursors into 5-ribosyluracil by excised *Phaseolus* shoots

Precursor	Sp. act. of precursor (mCi/mmol)	Total radioactivity in 5-ribosyluracil (dpm)	Sp. act. of 5-ribosyluracil (dpm/μmol)
[6-^{14}C]Orotic acid	61	3885	206
[2-^{14}C]Uracil	59	833	44
[2-^{14}C]UTP	59	7222	378

Batches of 25 excised shoots from 8-day-old seedlings were used for each treatment. The results shown are for 10 μCi of each radioactive precursor supplied.

Table 2. Effect of uracil and UTP on incorporation of [6-^{14}C]orotic acid into 5-ribosyluracil by *Phaseolus vulgaris*

Compounds supplied	Sp. act. of 5-ribosyluracil (dpm/μmol)
6-[^{14}C]Orotic acid	206
6-[^{14}C]Orotic acid + uracil	195
6-[^{14}C]Orotic acid + UTP	95

Results are for 10 μCi orotic acid (sp. act. 61 mCi/μmol). For each treatment, batches of 25 excised shoots were used and 75 μmol uracil or UTP were supplied as indicated. Further details are given in the Experimental.

UMP (Table 3). In this context, it should be pointed out that there is not normally a detectable pool of uracil in seeds and seedlings of *Phaseolus vulgaris* [5] and that uridine, UMP and UTP are only present at concentrations of a nanomolar order [5]. Thus, providing there are no problems of permeability, the artificially induced pools of uracil, uridine and UMP should dilute the extent of radioactive incorporation of any intermediate passing through these pools. The results, therefore, indicate that UTP is not degraded to UMP, uridine or uracil before incorporation into 5-ribosyluracil.

Collectively, the experiments described indicate that UTP is a more immediate precursor of the free 5-ribosyluracil found in the tissues of *Phaseolus* seedlings than is UMP, uridine or uracil. The observation that incorporation of ^{14}C from UTP into 5-ribosyluracil is twice that from orotic acid and × 9 that from uracil is not likely to be a reflection of permeability since the highly charged UTP anion would pass through membranes with much greater difficulty than the relatively weakly cationic uracil and the neutral uridine molecules. The substantial decrease in incorporation of [6-^{14}C]orotate into 5-ribosyluracil in the presence of an introduced pool of UTP also points to UTP being an intermediate in the formation of 5-ribosyluracil from orotate. Uracil had no such effect. These preliminary findings, therefore, indicate that free 5-ribosyluracil is produced via the orotate pathway and that UTP is an intermediate in its formation.

As UTP is the immediate precursor of the uridine residues of RNA, the present findings are compatible with formation of 5-ribosyluracil by post-transcriptional modifications of the uridine residues of tRNA and subsequent hydrolytic release during RNA catabolism. Evidence for such formation of 5-ribosyluracil by intramolecular rearrangement of uridine (N^1-ribosyluracil) residues to pseudouridine (5-ribosyluracil) residues has come from a number of sources [6–11]. Present results do not, however, exclude the possibility of a more direct conversion of UTP to 5-ribosyluracil, i.e. without prior incorporation into tRNA. If such an alternative route does exist in *Phaseolus*, it would not appear to involve uracil, uridine or UMP as an intermediate between UTP and 5-ribosyluracil.

Routes for the formation of free 5-ribosyluracil, not involving RNA, have been suggested by the work of Uematsu and Suhadolnik [3] working with *Streptoverticillium ladakanus*, which accumulates this nucleoside in its culture medium.

Table 3. Effect of uracil, uridine and UMP on incorporation of [4-^{14}C]UTP into 5-ribosyluracil by *Phaseolus vulgaris*

Compounds supplied	Sp. act. of 5-ribosyluracil (dpm/μmol)
[4-^{14}C]UTP	7611
[4-^{14}C]UTP + uracil	7445
[4-^{14}C]UTP + uridine	7278
[4-^{14}C]UTP + UMP	7111

Batches of 25 excised shoots from 8-day-old seedlings were used for each treatment. Results are for 10 μCi UTP in each case, plus 75 μmol UMP, uridine or uracil as appropriate.

EXPERIMENTAL

Seeds and seedlings. After brief exposure to a 0.1 % (v/v) soln of a mild detergent ('Stergene'), seeds of *Phaseolus vulgaris* cv The Prince (Bees Seeds Ltd., Sealand, U.K.) were surface-sterilized before germination by immersion for 5 min in 0.1 % (w/v) $HgCl_2$. They were then washed in running H_2O and allowed to imbibe for 24 hr in aerated, sterile H_2O. Where appropriate, seeds were imbibed in a test soln in place of H_2O. Subsequent germination and growth was at a constant temp. of 25° in moist vermiculite. A lighting regime of 16 hr light (6 klx)–8 hr dark was employed.

Chemicals. Uracil, uridine, UMP and UTP were purchased from Sigma (London) Chemical Co. Poole, U.K. Radioactive compounds were from Amersham International, Amersham, U.K.

Introduction of compounds into the plant tissue. The cut ends of

freshly excised shoots from 8-day-old seedlings were washed to remove enzyme activity released from damaged cells. The shoots were then placed with the cut ends in either H_2O (controls) or a soln of the compound to be examined. Using dilute HCl or KOH soln as appropriate, all solns for uptake were first adjusted to pH 5.5. The solns were dispensed in 0.1 ml portions into small glass tubes (3 × 0.5 cm diam.) and one cut shoot introduced per tube. To facilitate uptake of these solns in the transpiration stream, a slow current of air was maintained over the shoots; a temp. of 25° and daylight illumination of 6 klx was employed. When the 0.1 ml of soln in each tube had been taken-up, a further 0.2 ml H_2O was introduced to rinse them out. The shoots were allowed to take up this additional 0.2 ml and then supplied with sufficient H_2O for the remainder of the 24 hr period.

Extraction of 5-ribosyluracil. The procedure used for the extraction of 5-ribosyluracil was essentially that described previously [12] for routine extraction of purine and pyrimidine bases, ribosides and ribotides. It involved homogenizing tissues in ice-cold 0.3 M $HClO_4$ (1 ml/g fr. wt of tissue), removing debris by centrifuging at 12 000 *g* for 20 min at 4° and neutralizing the extract with KOH. Tissues were extracted × 3 in this way and the extracts pooled; $KClO_4$ was removed from the chilled soln by centrifuging at 4000 *g* for 15 min at 4°. The supernatant was subjected to preliminary purification by charcoal adsorption and elution [13].

Chromatography and electrophoresis. Descending chromatography on Whatman No. 1 paper was used in conjunction with one of the following solvent systems: (1) butan-1-ol–conc. HOAc–H_2O (60:15:25); (2) propan-2-ol–NH_4OH (sp. gr. 0.88)–H_2O (70:10:20); (3) propan-2-ol–conc. HCl–H_2O (130:33:37); (4) EtOH–NH_4OH (sp. gr. 0.88)–H_2O (80:10:10), (5) H_2O, (6) EtOH–100 mM $Na_2B_4O_7$ (67:33, pH 10). High voltage paper electrophoresis was on Whatman 3 MM paper using HOAc–HCO_2H buffer (pH 2) and $Na_2B_4O_7$ buffer (pH 9.2) [14]. Bands were located in UV light.

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