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FORMATION OF ALBIZZIINE AND 2,3-DIAMINOPROPANOIC ACID FROM URACIL IN *ALBIZIA* SEEDLINGS

ERIC G. BROWN and YUSUF TURAN

Biochemistry Research Group, School of Biological Sciences, University of Wales Swansea, Swansea SA2 8PP, U.K.

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Key Word Index—*Albizia julibrissin*; *Pisum sativum*; Leguminosae; secondary product biosynthesis; pyrimidines; non-protein amino acids; albizziine; 2,3-diaminopropanoic acid; isobarbituric acid; 5-aminouracil.

Abstract—Albizziine (2-amino-3-ureidopropanoic acid) was identified as a major radioactive metabolite of [2-^{14}C] uracil in tissues and enzymic extracts of seedlings of *Albizia julibrissin*. Consistent with the biogenesis of albizziine via uracil catabolism was the observation that [2-^{14}C]uracil is incorporated into isobarbituric acid (2,4,5-trihydroxypyrimidine) by crude enzymic extracts and microsomal preparations of seedlings of *A. julibrissin* and *Pisum sativum* in a tetrahydropterin-dependent hydroxylation of uracil. Substantial stimulations, more than 500% above the control value with *A. julibrissin*, were seen on addition of 6-methyl-5,6,7,8-tetrahydropterin. That there is a direct precursor relationship between uracil and albizziine was confirmed by the specific incorporation of the ^{14}C-label from [2-^{14}C]uracil into the ureido-carbonyl of albizziine. This is as would be expected for the ring opening of a dihydropyrimidine, catalysed by dihydropyrimidinase. As 5-aminouracil had previously been identified as a precursor of albizziine, it was concluded from the present work that the biogenetic sequence from uracil involves first, hydroxylation to isobarbituric acid, then amination to 5-aminouracil, followed by hydrogenation and ring-opening, to yield albizziine, from which 2,3-diaminopropanoic acid is formed by the action of β-ureidopropionase. The formation of albizziine and 2,3-diaminopropanoic acid represents a further example of the interfacing of pyrimidine primary and secondary metabolism through uracil.

INTRODUCTION

In a recent study of pyrimidine secondary metabolism in plants, it was shown that seedlings of *Albizia julibrissin* and *Pisum sativum* catabolize 5-aminouracil to yield the non-protein amino acids albizziine (2-amino-3-ureidopropanoic acid) and 2,3-diaminopropanic acid [1]. This was demonstrated both in intact tissue and in enzymic extracts. The enzymes involved were shown to be those of the pyrimidine degradative pathway by which uracil and thymine are catabolized. The toxicity of 5-aminouracil to plant, animal and microbial systems has been well documented, and it is both a bacteriostat and a mutagen [2, 3]. Since neither albizziine nor its further degradation product 2,3-diaminopropanoic acid [1] have any significant toxic activity in the tissues of higher plants, enzymic degradation of 5-aminouracil by the pyrimidine catabolic pathway must be regarded as a detoxication mechanism [4]. There is increasing evidence, reviewed in ref. [4], that several other non-protein amino acids (e.g. β-pyrazol-1-ylalanine, willardiine, isowillardiine, lathyrine and lupinic acid) also arise as a result of the action of plant detoxication processes.

That albizziine production and accumulation is almost entirely confined to the Mimosoideae [5, 6–10], whereas seedlings of *P. sativum* readily produce this compound if supplied with 5-aminouracil [1], suggests that the biochemical peculiarity of the Mimosoideae is not so much albizziine production as formation of 5-aminouracil. The question then arises as to the source of 5-aminouracil in plants such as *A. julibrissin*. All known pyrimidine and pyrimidine-derived plant secondary products originate from uracil or its precursor, uracil 6-carboxylic acid (orotic acid). These products include the isomeric non-protein amino acids willardiine and isowillardiine [11, 12], the pyrimidine glucosides vicine and convicine [13], lathyrine [14, 15] and 5-ribosyluracil [16]. It appeared likely, therefore, that uracil is the precursor of 5-aminouracil: the present study aimed to examine this hypothesis in relation to the biosynthesis of albizziine and 2,3-diaminopropanoic acid.

RESULTS AND DISCUSSION

Batches of 40 excised shoots of seedlings of *A. julibrissin* were supplied with [2-^{14}C]uracil (2 μCi; specific radioactivity 54 mCi mmol^{-1}) and after 24 hr the labelled metabolites were examined. Using the chromatographic and electrophoretic techniques previously described [1],

co-migration with authentic samples identified albizziine as a major radioactive product. Subsequent enzymic experiments with buffered extracts of seedlings of *A. julibrissin* showed that these, too, incorporated radioactivity from [2-^{14}C]uracil into albizziine. A typical set of data from these preliminary incorporation studies is shown in Table 1.

To seek confirmation that there is a direct precursor relationship between uracil and albizziine, the position of the ^{14}C-label incorporated from [^{14}C]uracil into the albizziine molecule was investigated. The recently elucidated biosynthetic scheme for albizziine [1] means that *C*-2 of 5-aminouracil would become the ureido-carbon of albizziine (Fig. 1). Thus, if 5-aminouracil arises from the amination of uracil, then [2-^{14}C]uracil should specifically label the carbon of the ureido-carbonyl of albizziine. The ^{14}C-albizziine from the experiment was therefore subjected to vigorous hydrolysis with HBr, a procedure [10] which specifically removes the terminal carbamoyl group and releases 2,3-diaminopropanoic acid (Fig. 1). A typical set of results from this experiment (Table 2) shows that 75% of the original radioactivity in the biosynthesized albizziine was lost when the ureido-carbonyl was removed and that the remaining 25% was in the residual, unhydrolysed, albizziine. The chromatographic procedure used in the recovery of ^{14}C-albizziine was quantitative (> 95%). These results are consistent with uracil being converted into albizziine via 5-aminouracil in the way indicated in Fig. 1 and they confirm the earlier finding [1] that albizziine is a product of pyrimidine catabolism.

Table 1. Total radioactivity incorporated from [2-^{14}C]uracil into albizziine by excised shoots and by enzymic extracts of seedlings of *Albizia julibrissin*

Albizia preparation	Total radioactivity in albizziine (dpm)
Excised shoots	34100
Enzymic extract	2080

Batches of 40 excised shoots were supplied with 2 μCi of [2-^{14}C]uracil (sp. radioactivity 54 mCi mmol^{-1}). Enzymic extract, prepared as described in Experimental, was incubated with 2 μCi of the same ^{14}C-uracil solution. The result shown is for an extract equivalent to 40 seedlings.

Attempts to identify 5-aminouracil as a natural constituent of *A. julibrissin* seedlings were inconclusive. Although a trace compound could be detected behaving chromatographically and electrophoretically in a similar way to an authentic sample of 5-aminouracil, there was insufficient to check further. It was considered probable that the failure to detect accumulation of this toxic compound follows from the efficiency of the detoxication mechanism, described earlier [1], i.e. its catabolism to albizziine. An analogous situation exists with pyrazole formation by cucumber seedlings in which the rapidity of the enzymic removal of pyrazole, by condensation with *O*-acetylserine, means that the enzyme effectively scavenges for this toxic base [17, 18] with the result that it cannot normally be detected in seedling tissues.

The observed direct incorporation of uracil into albizziine (Fig. 1; Table 1) and the earlier identification of 5-aminouracil as a precursor of albizziine [1] mean that uracil undergoes amination before being reduced and ring-opened to albizziine. This raises the question of the nature of the amination process, which has not previously been described for any biological system. Both chemical and biochemical considerations suggest that it is unlikely to be a direct amination of uracil at the 5-position. A more feasible biochemical sequence would involve preliminary hydroxylation of uracil at the 5-position to form 2,4,5-trihydroxypyrimidine (isobarbituric acid) and amination of that to 5-aminouracil. Although isobarbituric acid has not been reported to be a natural product, its riboside 5-hydroxyuridine is a known constituent of yeast RNA [19].

Using a crude enzymic extract of pea seedlings in Tris–HCl buffer (50 mM; pH 7.3) containing 2 mM dithioerythritol, evidence was sought for the possible direct 5-hydroxylation of uracil. As enzymic hydroxylation of aromatic ring systems, e.g. phenylalanine and tyrosine [20–22], requires tetrahydropteridines such as tetrahydrobiopterin or 6-methyl-5,6,7,8-tetrahydropterin, the latter compound was added to reaction mixtures, as appropriate. The standard reaction mixture comprised Tris–HCl buffer (50 mM; pH 7.3), dithioerythritol (2 mM), NAD^+ (0.2 mM), $NADP^+$ (0.2 mM), glucose 6-phosphate (20 mM), glucose 6-phosphate dehydrogenase (100 units), $MgCl_2$ (1.5 mM), uracil

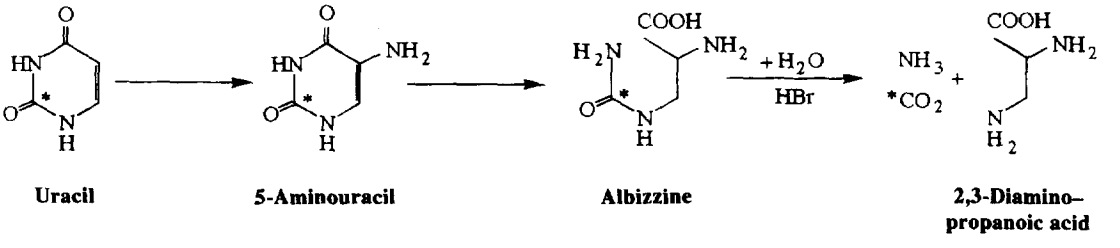

Fig. 1. Expected location of ^{14}C incorporated from [2-^{14}C]uracil, via 5-aminouracil, into albizziine [1], and its release as $^{14}CO_2$ by HBr hydrolysis of the latter compound [10].

(1 mM), [2-^{14}C]uracil (2 μCi; specific radioactivity 54 mCi mmol^{-1}) and 4 ml of the enzyme preparation. The final volume was 5 ml. Incubation was at 35° for 3 hr and reactions were stopped with trichloroacetic acid (final concentration, 5% w/v). Precipitated protein was sedimented by centrifuging and trichloroacetic acid was removed from the supernatant by extraction into diethyl ether. The supernatant was then concentrated *in vacuo* and ^{14}C-isobarbituric acid was isolated by sequential chromatography in systems (i), (ii) and (iii) and high-voltage electrophoresis (see Experimental). In each system, the radioactivity co-migrated with an authentic sample of isobarbituric acid. After elution, the radioactivity of the individual samples of [^{14}C]isobarbituric acid was determined; the results are given in Table 3.

Table 2. Specific hydrolysis of ^{14}C-albizziine, biosynthesized from [2-^{14}C]uracil, to remove the terminal carbamoyl group and yield 2,3-diaminopropanoic acid

Compound	Before hydrolysis (dpm)	After hydrolysis (dpm)	Radioactivity recovered (%)
Albizziine	6000	1500	25
2,3-Diamino-propanoic acid	—	39	0.6

The data obtained from this experiment show that the crude extract was active in hydroxylating [2-^{14}C]uracil to ^{14}C-5-hydroxyuracil (isobarbituric acid) and that a 138% stimulation of this process occurs in the presence of the known hydroxylation cofactor 6-methyl-5,6,7,8-tetrahydropterin.

The hydroxylation experiments were repeated with microsomal preparations in place of the crude enzymic extract, and also both with crude extracts and microsomal preparations from seedlings of *A. julibrissin* (Table 4). The results of these experiments (Tables 3 and 4) show that hydroxylation of uracil to isobarbituric acid occurs in all the preparations examined and that it is stimulated substantially in the presence of 6-methyl-5,6,7,8-tetrahydropterin. With microsomal preparations of *A. julibrissin* a stimulation of 544% above the control value was seen. It is, therefore, concluded that the non-protein amino acid albizziine arises from uracil via hydroxylation to isobarbituric acid, followed by amination to 5-aminouracil, and, as shown previously [1], catabolism of the latter by the pyrimidine degrading enzymes dihydrouracil dehydrogenase and dihydropyrimidinase. The

Table 3. 5-Hydroxylation of [2-^{14}C]uracil by preparations obtained from seedlings of *Pisum sativum* and its stimulation by 6-methyl-5,6,7,8-tetrahydropterin

Preparation	Additions	Total radioactivity in isobarbituric acid (dpm)	Stimulation (% of control)
Crude enzymic extract	None (control)	3190	—
	6-Methyl-5,6,7,8-tetrahydropterin	7580	138
Microsomal preparation	None (control)	1090	—
	6-Methyl-5,6,7,8-tetrahydropterin	4710	332

6-Methyl-5,6,7,8-tetrahydropterin was present at a final concentration of 0.4 mM.

Table 4. 5-Hydroxylation of [2-^{14}C]uracil by preparations obtained from seedlings of *Albizia julibrissin* and its stimulation by 6-methyl-5,6,7,8-tetrahydropterin

Preparation	Additions	Total radioactivity in isobarbituric acid (dpm)	Stimulation (% of control)
Crude enzymic extract	None (control)	3040	—
	6-Methyl-5,6,7,8-tetrahydropterin	10600	249
Microsomal preparation	None (control)	1340	—
	6-Methyl-5,6,7,8-tetrahydropterin	8630	544

6-Methyl-5,6,7,8-tetrahydropterin was present at a final concentration of 0.4 mM.

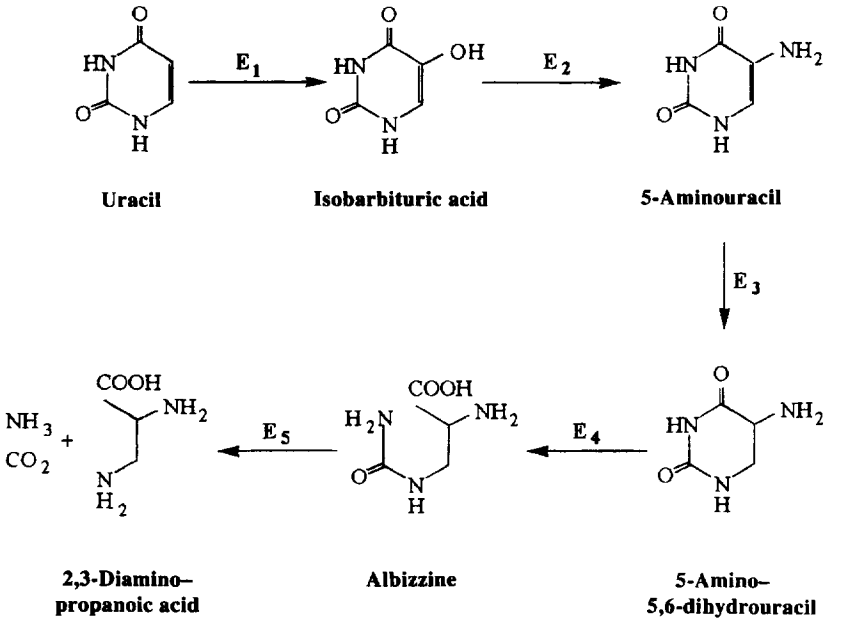

Fig. 2. Biosynthetic route by which albizziine and 2,3-diaminopropanoic acid are produced from uracil by *Albizia julibrissin*. E_1, microsomal hydroxylase; E_2, enzymic amination, E_3, dihydrouracil dehydrogenase (EC 1.3.1.21); E_4, dihydropyrimidinase (EC 3.5.2.2); E_5, β-ureidopropionase (EC 3.5.1.6).

overall biosynthetic scheme for albizziine and 2,3-diaminopropanoic acid is outlined in Fig. 2. 2,3-Diaminopropanoic acid is produced by the action of β-ureidopropionase [EC 3.5.1.6] on albizziine [1].

It is concluded from the foregoing observations that the biosynthesis of albizziine and of 2,3-diaminopropanoic acid represents a further example of the interfacing through uracil of pyrimidine primary and secondary metabolism.

EXPERIMENTAL

Materials. Seeds of *P. sativum* cv. Meteor were from Booker Seeds., Sleaford, Lincs., those of *A. julibrissin* from Thompson & Morgan, Ipswich, Suffolk. Before use, seeds were washed and then soaked in H_2O overnight before sowing. *Albizia* seeds were chipped before imbibition. Seeds were germinated in moist vermiculite and grown at 25° in a light cycle of 16 hr light (6 klx) and 8 hr dark. Analyt. grade chemicals were from BDH. Pyrimidines, enzymes and coenzymes were from Sigma. [2-^{14}C]Uracil (sp. radioactivity 54 mCi mmol^{-1}) was initially from Amersham International, and latterly from ICN, Thame, Oxfordshire.

Uptake of [2-^{14}C]*uracil.* Aliquots (0.1 ml) of the radioactive soln were dispensed into Eppendorf tubes (1.5 ml) and excised shoots of 14-day-old *Albizia* seedlings, one per tube, were allowed to take up this soln under 6 klx illumination at 25°. The cut ends of the shoots were washed beforehand to remove exudates. After the soln in each individual tube had been taken up, 0.1 ml of H_2O was added to rinse and this was allowed to be taken up by the shoot. In this way, each tube was rinsed 5 times, and then the shoots were allowed to stand with the cut ends in H_2O for a total of 24 hr under the same conditions of light and temp. before extraction.

Extraction of metabolites. Tissues were finely ground in ice-cold 0.6 M $HClO_4$ using a prechilled mortar and pestle. The homogenate was centrifuged at 12 000 *g* for 20 min at 4° and the supernatant retained. The residue was re-extracted a further 3 times in the same way and the supernatants pooled with the first. After adjusting to pH 7.4 with KOH soln, the extract was held at 4° for 15 min and the $KClO_4$ ppt. was removed by centrifuging at 5000 *g* for 10 min at 4°. The supernatant was evapd *in vacuo* to dryness and the residue redissolved in 0.5 ml of H_2O for chromatographic and electrophoretic analysis.

Chromatography and electrophoresis. PC on Whatman No. 1 or 3MM paper employed the solvent systems: (i) 1-BuOH–HOAc–H_2O (12:3:5); (ii) 2-PrOH–aq. NH_3–H_2O (7:1:2); (iii) 2-MePrOH–MeCOEt–H_2O–aq. NH_3 (4:3:2:1). High-voltage paper electrophoresis was carried out on Whatman 3MM paper using a HCO_2H–HOAc buffer at pH 2 [14]. A gradient of 28 V cm^{-1} was applied for 2 hr. Pyrimidines were detected by viewing in UV and the amino acids by use of ninhydrin (0.2% w/v in Me_2CO).

Preparation of enzymic extract and microsomal fraction. The enzymic extracts used in these studies were prepd by homogenizing 14-day-old seedlings of *P. sativum* or *A. julibrissin* in an ice-cold 50 mM Tris–HCl buffer (pH 7.3) containing 2 mM dithioerythritol; a prechilled mortar and pestle was used. For each g of tissue 1 ml of buffer was used. The homogenate was filtered through a double layer of cheesecloth to remove coarse debris, and the filtrate centrifuged at 12 000 *g* for

20 min at 4°. The supernatant was used as a crude enzymic prepn. The microsomal fr. used in the hydroxylation experiments was prepd from the crude enzymic extract by centrifuging at 100 000 *g* for 1 hr at 4° and resuspending the pellet in 2 ml initial buffer.

Hydrolysis of albizziine with HBr. The procedure was essentially that described in ref. [10] and involved refluxing the albizzine sample in 5 ml (48% v/v) HBr for 5 hr. The hydrolysed compound was evapd *in vacuo*, 3 ml H_2O added, and evapd again. This process was repeated several times until all traces of HBr had been removed. The product, 2,3-diaminopropanoic acid, was purified by sequential chromatography in solvent systems (i), (ii) and (iii).

Scintillation counting. Radioactivity was determined by counting samples (0.5 ml) in 5-ml portions of Optiphase Ria Luma scintillation cocktail (LKB).

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