Plant Physiol. (1980) 66, 735–739
0032-0889/80/66/0735/05$00.50/0

Biosynthesis of Ureides from Purines in a Cell-free System from Nodule Extracts of Cowpea [*Vigna unguiculata* (L) Walp.][1]

Received for publication December 28, 1979 and in revised form May 5, 1980

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ABSTRACT

The synthesis of ^{14}C-labeled xanthine/hypoxanthine, uric acid, allantoin, allantoic acid, and urea from [8-^{14}C]guanine or [8-^{14}C]hypoxanthine, but not from [8-^{14}C]adenine, was demonstrated in a cell-free extract from N_2-fixing nodules of cowpea (Walp.). The ^{14}C recovered in the acid/neutral fraction was present predominantly in uric acid and allantoin (88–97%), with less than 10% of the ^{14}C in allantoic acid and urea. Time courses of labeling in the cell-free system suggested the sequence of synthesis from guanine to be uric acid, allantoin, and allantoic acid. Ureide synthesis was confined to soluble extracts from the bacteroid-containing tissue, was stimulated by pyridine nucleotides and intermediates of the pathways of aerobic oxidation of ureides, but was completely inhibited by allopurinol, a potent inhibitor of xanthine dehydrogenase (EC 1.2.1.37). The data indicated a purine-based pathway for ureide synthesis by cowpea nodules, and this suggestion is discussed.

Ureides are important nitrogenous solutes in the metabolism of a wide range of plant species (4, 8, 16, 22, 28). They are especially prominent in certain tropical legumes, in which they act as major products of N_2 fixation (9, 14, 19), as the principal compounds in which fixed N is transported from nodules to shoots in the xylem (15, 20) and as a primary source of N for protein synthesis in shoots of nodulated plants relying on N_2 fixation (9).

Pathways suggested for ureide synthesis in plants involve either the condensation of 2 molecules urea and 1 molecule glyoxylate (5, 24) or the aerobic oxidation of purines (3, 6, 22). With the latter pathway (Fig. 1), deamination of guanine and adenine to xanthine and hypoxanthine, respectively (reactions 1 and 2), and the subsequent oxidation of hypoxanthine and xanthine (reaction 3) are envisaged as donor reactions to the formation of allantoin and allantoic acid from uric acid. Circumstantial evidence for this pathway in legume nodules comes from the demonstration of activity of enzymes of purine oxidation, namely xanthine dehydrogenase (EC 1.2.1.37), xanthine oxidase (EC 1.2.3.2), uricase (EC 1.7.3.3), and allantoinase (EC 3.5.2.5) (25, 26), and *in vivo* inhibition of ureide synthesis in N-fixing plants by allopurinol (2, 7), a potent inhibitor of both xanthine dehydrogenase and xanthine oxidase.

This study reports the direct synthesis of allantoic acid, allantoin, uric acid, and urea from guanine and hypoxanthine in a cell-free system from extracts of nodules of cowpea [*Vigna unguiculata* (L.) Walp.], thus providing further support for a purine-based pathway for ureide synthesis in symbiotically active plants of this species.

MATERIALS AND METHODS

Cowpea [*Vigna unguiculata* (L.) Walp. cv. Caloona] plants, effectively nodulated with *Rhizobium* CB 756, were grown as described previously (9). Freshly harvested nodules from 4- to 6-week old plants were ground with a mortar and pestle in a breaking buffer (1 ml/g nodules) containing 100 mM K-phosphate (pH 8.0), 50 mM KCl, 5 mM $MgCl_2$, 5 mM DTT, 1 mM EDTA, and 0.5% (w/v) PVP. The filtered extracts were centrifuged (40,000*g* for 20 min) and the supernatants were passed through Sephadex G-25 before use in the experiments. All the above procedures were carried out at 4 C.

Assays (1 ml total volume) in duplicates were carried out at 25 C using cell-free extract (0.5–1.0 mg protein, equivalent to 30–60 mg fresh weight of nodules) in a reaction medium containing 60 mM Tris-HCl (pH 7.4), 50 mM KCl, 10 mM K_2HPO_4/KH_2PO_4, 5 mM $MgCl_2$, 1 mM DTT, 1 mM NAD^+, 1 mM ATP, 0.5 mM uric acid, and 0.5 mM allantoin. This assay mixture was preincubated for 10 min before the addition of the radiosubstrates (0.3 to 0.6 μCi/μmol) 1 mM [8-^{14}C]guanine, [8-^{14}C]hypoxanthine, or [8-^{14}C] adenine. Assays were stopped after 10, 20, 40, or 60 min with 50 μl 1 M Zn-acetate. The precipitate was removed by centrifugation and the supernatant was separated into basic and acid/neutral components with Dowex 50-X8-H^+ ion-exchange resin.

The basic fraction was assayed for purines by reverse-phase, high-performance liquid chromatography by using a Micropak MCH-10 column (Varian) and a linear gradient of water/methanol (0–50%) containing 5 mM 1-heptanesulfonic acid. Xanthine and hypoxanthine were detected by absorbance at 254 nm and collected from the chromatograph eluate for ^{14}C assay.

Uric acid and allantoic acid in the acid/neutral fractions were separated on a Dowex 1-X10 formate column (45 × 0.6 cm) eluted with a linear gradient of 3 N formic acid. Unlabeled uric acid and allantoic acid were added to the samples prior to separation. Uric acid in the fractions was identified spectrophotometrically at 293 nm, whereas allantoic acid was identified as the phenylhydrazone of glyoxylate at 525 nm (29). In all cases, major peaks of radioactivity in the column eluates were found to co-chromatograph with the elution profiles of uric acid and allantoic acid (Fig. 2). Allantoin and urea were recovered in the neutral fraction. Allantoin was converted to allantoic acid by hydrolysis in base and recovered for ^{14}C assay by column chromatography as above. [^{14}C]Urea was converted to $^{14}CO_2$ with urease (Sigma Type IX) and the $^{14}CO_2$ was recovered in ethanolamine. The radioactivity in all the above compounds was determined by liquid scintillation counting. It was assumed that all labeled products were formed from [8-^{14}C]guanine as outlined in Figure 1. The amounts of ^{14}C-labeled compounds formed were determined on the basis of the

[1] This work was supported by funds from the Australian Research Grants Committee and the Wheat Industry Research Council of Australia.

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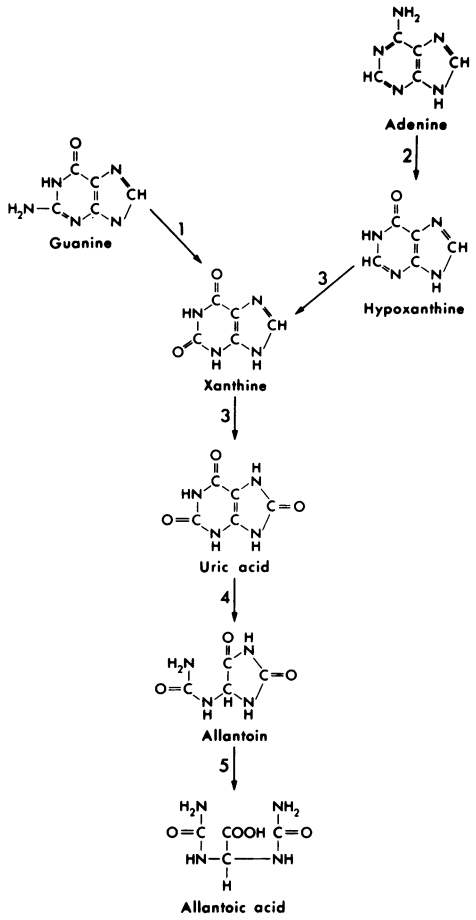

FIG. 1. Pathway of aerobic purine oxidation for ureide synthesis. Enzymes involved: 1: guanine deaminase; 2: adenine deaminase; 3: xanthine oxidase or xanthine dehydrogenase; 4: uricase; 5: allantoinase.

specific activity of the [8-^{14}C]guanine used in the assays.

All results expressed were the average of duplicate experiments.

RESULTS

The cell-free system synthesized ^{14}C-labeled uric acid, allantoin, allantoic acid, and urea from [8-^{14}C]guanine and [8-^{14}C]hypoxanthine and not from [8-^{14}C]adenine (Table I). This suggested the presence and activity of guanine deaminase (reaction 1, Fig. 1) and the activity of enzymes associated with reactions 3, 4, and 5 in Figure 1, but the absence, inactivity or inactivation of adenine deaminase (reaction 2, Fig. 1).

The data of Table II illustrate the effect of addition of the supposed intermediates of the pathway of aerobic purine oxidation (Fig. 1) on the biosynthesis of ureides from [8-^{14}C]guanine by the cell-free system. Incorporation of ^{14}C into ureides was increased most by allantoic acid and allantoin and to a lesser extent by uric acid + allantoin or uric acid alone. The increased incorporation of ^{14}C was recovered almost entirely as allantoin, with the ^{14}C of uric acid, allantoic acid, and urea being essentially unaffected. No radioactivity was detected in glyoxylate and the ^{14}C label in allantoic acid and urea constituted a small proportion ($\leq$10%) of the total ^{14}C products. Additions of hypoxanthine and xanthine decreased total ^{14}C incorporation by about 20% without affecting the distribution of ^{14}C incorporation among the products. In contrast, the addition of uric acid (and allantoin) did not lead to a decrease in ^{14}C incorporation. This could be attributed partly to, at least, two factors.

First, the nodule extracts used contained high uricase activity

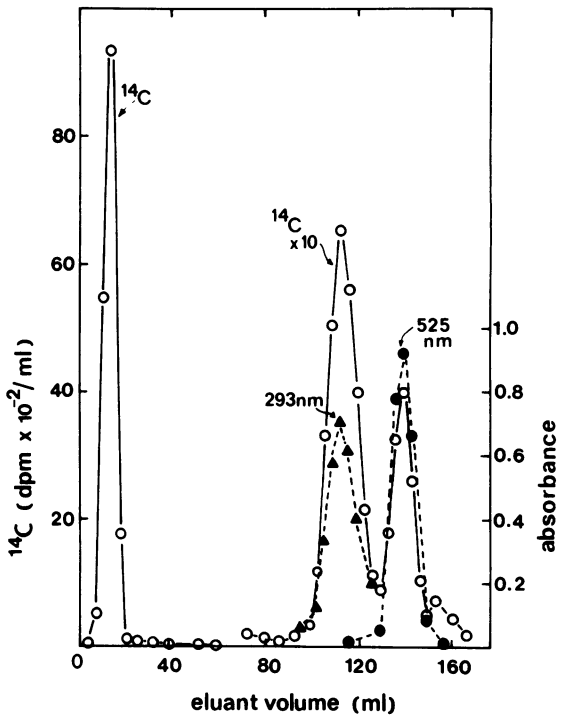

FIG. 2. Elution profile of the acid/neutral fraction in a cell-free system for ureide synthesis from [8-^{14}C]guanine (0.64 μCi/μmol), taken from the experiment with the crude nodule extract in Table III.

Table I. *Biosynthesis of Ureides from 8-^{14}C-labeled Purine Bases in a Cell-free System from Cowpea Nodules*

8-^{14}C-labeled Substrate	Uric Acid	Allantoin	Allantoic Acid + Urea	Total
	nmol synthesized/g fresh wt·h			
Adenine	—[a]	—[a]	12	12
Hypoxanthine	91	192	9	292
Guanine	107	173	21	301

[a] —, not detectable.

Table II. *Effect of Metabolic Intermediates on Biosynthesis of Ureides from [8-^{14}C]Guanine in a Cell-free System from Cowpea Nodules*

Assays were stopped after 60 min and the ^{14}C products were analyzed as described. Nodule extracts used in each assay were equivalent to 50 mg fresh weight of nodules. Numbers in parentheses represent per cent of control.

Metabolite Added[a]	Uric Acid	Allantoin	Allantoic Acid	Urea	Total
	nmol synthesized/g fresh wt·h				
Control	9.8	134	3.6	7.0	154 (100)
+ Xanthine	20.0	95	—[b]	4.6	119 (78)
+ Hypoxanthine	8.0	108	7.3	3.8	127 (83)
+ Uric acid	88.0	110	4.9	5.4	208 (135)
+ Allantoin	9.2	334	17.0	24.0	384 (249)
+ Allantoic acid	9.7	365	16.0	23.0	415 (269)
+ Uric acid, allantoin	128.0	137.0	17.0	8.8	291 (189)

[a] All additions were 0.5 mM; control treatment did not contain uric acid and allantoin.

[b] —, not detectable.

of about 40 μmol uric acid converted/g fresh weight nodule extract · h. With preincubation, the small amount (0.5 μmol) of uric acid added (Table II) would likely be converted to allantoin in the assays within 15 min, thereby minimizing the expected dilution of ^{14}C label from [8-^{14}C]guanine. Second, the addition of uric acid would probably prime the pathway involved and increase total ^{14}C flow through it. The observed increase in total ^{14}C incorporation resulting from the addition of uric acid indicates that the second factor probably played a significant role in stimulating C flow through the purine oxidation pathway in the cell-free system. Similar factors could also be involved in the observed increase in ^{14}C incorporation resulting from the additions of the other intermediates.

The time course for synthesis of uric acid, allantoin, and allantoic acid from [8-^{14}C]guanine by the cell-free system (Fig. 3) indicated formation of uric acid before allantoin and that of allantoin in advance of allantoic acid (see sequence of Fig. 1). The low rate of recovery of ^{14}C label in allantoic acid was puzzling in view of earlier demonstrations (2) of significant allantoinase activity in the soluble fraction of cowpea nodules.

Cell-free extracts prepared from the bacteroid-containing (leghemoglobin hemoglobin-pigmented) central tissues of nodules were compared with extracts from the peripheral cortical tissues of the nodule and from whole nodules in their ability to form ^{14}C-labeled ureides from labeled guanine (Table III). The data suggested a 7-fold higher activity from the bacteroid-containing tissue, a result consistent with earlier studies (2) showing that enzymes of ureide metabolism were located principally in bacteroid-containing tissues. The low activity of ureide synthesis that was found in cortex extracts was probably due to contamination of the tissue slices with small amounts of bacterial tissue, as suggested in an earlier study (2).

The effect of the addition of NAD^+, $NADP^+$, and ATP on the biosynthesis of ureides was studied using cell-free extracts supplied with [8-^{14}C]guanine. Both NAD^+ and $NADP^+$ stimulated the synthesis of uric acid, allantoin, allantoic acid, and urea (Table IV). The effects of NAD^+ and ATP were additive, although ATP alone had only a small effect on the rate of biosynthesis. The results suggested that xanthine dehydrogenase, rather than xanthine oxidase, could be involved in the oxidation of purines in the cell-free system from cowpea nodules.

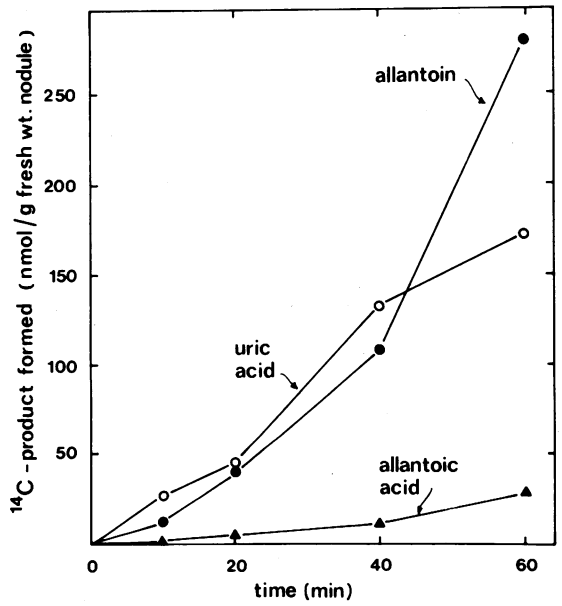

FIG. 3. Time course for the synthesis of uric acid, allantoin, and allantoic acid from [8-^{14}C]guanine in a cell-free system from extracts of cowpea nodules. (○) Uric acid; (●) allantoin; (▲) allantoic acid.

Table III. *Biosynthesis of Ureides from [8-^{14}C]Guanine in Crude Extracts of Outer Cortex and Inner Bacteroid-containing Tissue from Cowpea Nodules*

The cortex and bacteroid-containing tissue were isolated as described (2). The cell-free extracts were not passed through Sephadex G-25 but were used directly for assays. Uric acid and allantoin were not present in the standard assay mixture; otherwise, assay conditions were as described. Numbers in parentheses represent per cent of control.

Tissue	Uric Acid	Allantoin	Allantoic Acid	Urea	Total
	nmol synthesized/g fresh wt·h				
Nodule	86	389	39	8.7	522 (100)
Cortex	7	53	—[a]	1.8	62 (11.8)
Bacteroid	54	356	10	13	433 (82.9)

[a] —, not detectable.

Table IV. *Effects of NAD^+, $NADP^+$, and ATP on Biosynthesis of Ureides from [8-^{14}C]Guanine in a Cell-free System from Cowpea Nodules*

Numbers in parentheses represent per cent of control.

Treatment[a]	Uric Acid	Allantoin	Allantoic Acid + Urea	Total
		nmol synthesized/g fresh wt·h		
Control	56	62	3	121 (100)
+ ATP	54	21	69	144 (120)
+ $NADP^+$	122	120	11	253 (210)
+ NAD^+	112	132	15	259 (216)
+ NAD^+, ATP	107	173	21	301 (252)

[a] All additions were 1 mM.

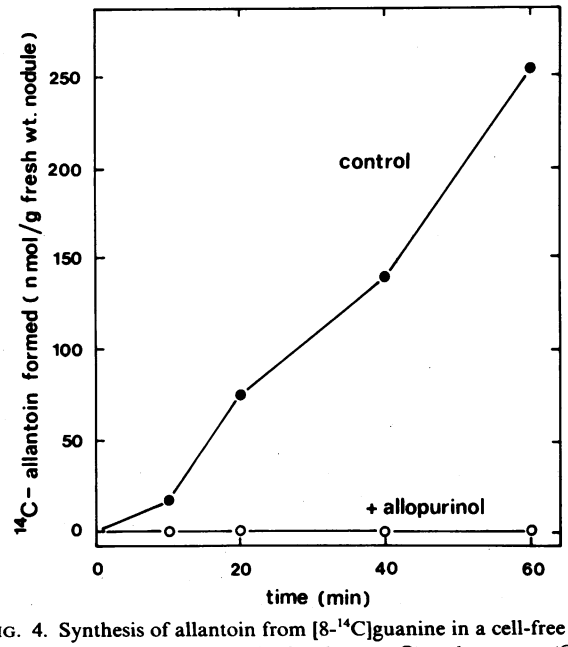

FIG. 4. Synthesis of allantoin from [8-^{14}C]guanine in a cell-free system from extracts of cowpea nodules in the absence (●) and presence (○) of 20 μM allopurinol. Uric acid and allantoin were not present in the assay medium.

Ureide synthesis in the cell-free system was completely inhibited by the hypoxanthine analog allopurinol (Fig. 4). This compound was shown to be a potent inhibitor of xanthine dehydrogenase in cowpea (2) and of xanthine oxidase in soybean (*Glycine max*) (7).

DISCUSSION

This study demonstrated synthesis of ureides from [8-^{14}C]guanine or [8-^{14}C]hypoxanthine by cell-free extracts of the bacteroid-containing tissue of cowpea nodules. Recovery of label in uric acid and urea, a labeling sequence from uric acid to allantoin to allantoic acid, a stimulation of synthesis by pyridine nucleotides and by the recognized intermediates of the aerobic oxidation of purines, and an inhibition of ureide synthesis by allopurinol were all consistent with the involvement of the pathway of aerobic oxidation of purines for ureide production in this tissue.

In a separate experiment carried out in the absence of added unlabeled metabolic intermediates of the pathway (data not shown), the total ^{14}C incorporation into xanthine/hypoxanthine and ureides from [8-^{14}C]guanine in the cell-free system was estimated to be about 6% of the rate recorded by Herridge *et al.* (9) for intact nodules. This value was certainly an underestimate of the capacity of the cell-free system for ureide synthesis since the addition of unlabeled metabolic intermediates significantly increased the total transfer of ^{14}C from [8-^{14}C]guanine to ureides (Table II). The decrease in ^{14}C label recovered in ureides from [8-^{14}C]guanine observed in the presence of added unlabeled hypoxanthine and xanthine (Table II) could be explained on a similar basis.

Since ureides account for 50 to 75% of the fixed N exported to shoots from cowpea nodules (9, 20), the pathway suggested is likely to represent a significant flow path for N assimilation by the nodule. Were this the case, nodule metabolism would involve high rates of turnover of purines.

The ready labeling of ureides from [2-^{14}C]glycine, [^{14}C]glyoxylate, [8-^{14}C]adenine, and [8-^{14}C]hypoxanthine by roots, cotyledons, and leaves of various species (3, 6, 11, 16, 17, 21, 22) provides evidence that the involvement of purines in ureide synthesis may be widespread in plant tissues. In particular, the studies of Butler *et al.* (6) on *Symphytum*, demonstrating labeling of carbon atoms 2, 5, and 8 of the allantoin molecule by [2-^{14}C]glycine but no labeling of ureides from [^{14}C]urea, provide particularly convincing evidence of purine involvement, as opposed to a more direct pathway of synthesis involving condensation of glycine (or glyoxylate) with some other compound, such as carbamyl phosphate.

The nucleotide pathway in animals and bacteria is known to be tightly regulated (12). Although there is evidence to suggest that a similar pathway for nucleotide biosynthesis exists in plants (30), little is known about its regulation. There are likely to be differences in the modulation of and flow through the purine pathway between different species and tissues. In cotyledons of germinating seeds, for example, containing large amounts of storage RNA and nucleoprotein, ureide synthesis may be related to the breakdown of these molecules (7) and a role of light in regulating the turnover of adenylic and inosinic compounds in cotyledon tissue of *Pharbitis* has been suggested (17). In roots (22) and root nodules of legumes (9, 13), in which there is a large and continuous synthesis of ureides for export to other nonassimilatory parts, the situation may be very different. Possibly a more direct linkage between IMP and hypoxanthine exists in this system to facilitate flow-through to ureides with minimal accumulation of secondary end products. Our results showing that adenine does not support ureide synthesis in the cell-free system of cowpea nodules are consistent with this suggestion.

A number of studies suggest that considerable variation exists between species and tissues in the intracellular localization of enzymes of purine metabolism. In pea leaves, for example, xanthine dehydrogenase is located in the soluble fraction (18). In soybean nodules, uricase is associated with the bacteroid fraction (26) and xanthine oxidase is associated with the nuclear fraction (25), whereas, in cowpea nodules, these enzymes are located predominantly in the soluble fraction of bacteroid-containing tissue (2). In legume nodules, allantoinase appears to be located in the soluble fraction (2, 25); in potato tubers and castor bean endosperm, this enzyme and uricase is associated with microbodies (1, 10, 23, 27). In soybean nodules, where uricase has been reported to be located in the bacteroid, away from other purine oxidation enzymes (25, 26), it is difficult to see how integrated flow of metabolites through the pathway of ureide synthesis might be achieved.

In legume nodules, current photosynthate provides C for ureide biosynthesis (9). The integration of intermediary C metabolism and the pathway of ureide biosynthesis presents an interesting study. Likewise, the flow of fixed N within the nodule, especially in relation to the possible competition between asparagine and ureide synthesis for recently assimilated N (20), may also be important in understanding the regulation and control of ureide metabolism.

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