METABOLISM OF PURINE NUCLEOTIDES TO FORM UREIDES IN NITROGEN-FIXING NODULES OF COWPEA (*VIGNA UNGUICULATA* L. WALP.)

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Received 30 December 1980

1. Introduction

Allantoin and allantoic acid are the principal transported forms of nitrogen in the xylem of a wide range of nodulated tropical legumes [1]. Formed in nodules as the major products of nitrogen fixation [2,3], ureides appear to be derived from oxidation of purines [4,5]. Enzymic studies have demonstrated significant activity of allantoinase (EC 3.5.2.5), urate oxidase (EC 1.7.3.3), NAD:xanthine oxidoreductase (EC 1.2.1.37) and guanine aminohydrolase (EC 3.5.4.3) in cell-free extracts of the bacteroid-containing host cells of cowpea and nodules [3,4,6]. Furthermore, in both intact soybean and cowpea plants allopurinol, applied to the root system, abolishes ureide synthesis and export in xylem [4,7]. The metabolic routes for incorporation of fixed nitrogen through de novo purine synthesis with subsequent oxidation of purines to form allantoin have not been elucidated.

This study reports the direct synthesis of ureides from purine nucleotides in cell free extracts of nodules of cowpea at rates commensurate with those of nitrogen fixation and identifies the nature of intermediate reactions between the first nucleotide product of de novo purine synthesis, IMP, and allantoin.

2. Materials and methods

2.1. *Plant material*

Cowpea (*Vigna unguiculata* (L.) Walp. cv. Caloona) plants, effectively nodulated with *Rhizobium* strain CB 756, were grown in N-free sand culture [8].

Abbreviations: IMP, inosine 5'-monophosphate; DTT, dithiothreitol; PVP, soluble polyvinyl-pyrrolidone; HPLC, high performance liquid chromatography; XMP, xanthosine 5'-monophosphate; GMP, guanosine 5'-monophosphate; AMP, adenosine 5'-monophosphate; PRPP, 5-phosphoribosyl-1-pyrophosphate; NAD, nicotinamide adenine dinucleotide, oxidised; ATP, adenosine 5'-triphosphate

2.2. *Preparation of cell-free extracts*

Freshly harvested nodules from 4–6-week-old plants were extracted with 0.1 M Tris–HCl buffer (pH 8.3) containing 5 mM DTT, 0.5% (w/v) PVP, 10 mM $MgCl_2$ and 20 mM KCl so that the central bacteroid-containing tissues were homogenised while the outer cortex was not significantly disrupted [4]. Following filtration through 100 μm mesh to remove cortical material the homogenate was centrifuged (10 000 × *g*, 20 min, 4°C) and the supernatant desalted by passage through a Sephadex G-25 column equilibrated with the breaking buffer mixture. Isolated bacteroids were lysed by osmotic shock following incubation with lysozyme [9] and the bacteroid cell ghosts separated from the solubilised protein by centrifugation (10 000 × *g*, 20 min, 4°C).

2.3. *Assays of purine metabolism*

Routinely 0.1 ml aliquots of cell free extract (containing 0.2–0.3 mg protein and equivalent to 60–100 mg fresh wt nodule) were incubated at 30°C with 0.4–0.8 μmol substrate (IMP, XMP, GMP, AMP or [8-^{14}C]IMP) in 1 ml containing 10 μmol Tris–HCl (pH 7.4), μmol DTT, 5 μmol $MgCl_2$, 50 μmol KCl and 2 μmol NAD. Other additions in some cases were 40–50 μmol allopurinol, 1 μmol L-glutamine or 1 μmol ATP.

After varying periods duplicate reactions were terminated by addition of 0.05 ml 4.2 M ice-cold $HClO_4$. Excess $HClO_4$ was neutralised with KOH and the precipitates removed by centrifugation (5000 × *g*, 10 min). The supernatant was sampled and assayed for allantoin + allantoic acid colorimetrically [10] and for purines by direct HPLC analysis. Separation

of purine nucleotides, nucleosides, bases and uric acid was achieved by ion suppression chromotography on a reverse phase, C_{18} column (Varian Micropak MCH-10) with a linear gradient of 20 mM (pH 7.5) ammonium phosphate:60% (v/v) methanol in water (0–58% in 23 min) at a flow rate of 1 ml/min. Eluted compounds were detected by absorbance at 254 nm and quantitated by calibration of peak height with mass of authentic compound. A paired-ion reverse phase HPLC separation of purines [6] was also used to confirm the identity of metabolites and to quantitate hypoxanthine which was not well resolved from NAD in the ion-suppression system. Metabolic products of [8-^{14}C]IMP metabolism were recovered and analysed as above. The elutant of the HPLC column was collected (0.2 ml fractions) for ^{14}C assay for liquid scintillation. Labelled alantoin and allantoic acid were recovered by ion exchange chromatography [6].

2.4. *Assay of NAD reduction*

The same reaction mixtures as in section 2.3 were incubated at 30°C and NAD reduction assayed continuously at 340 nm.

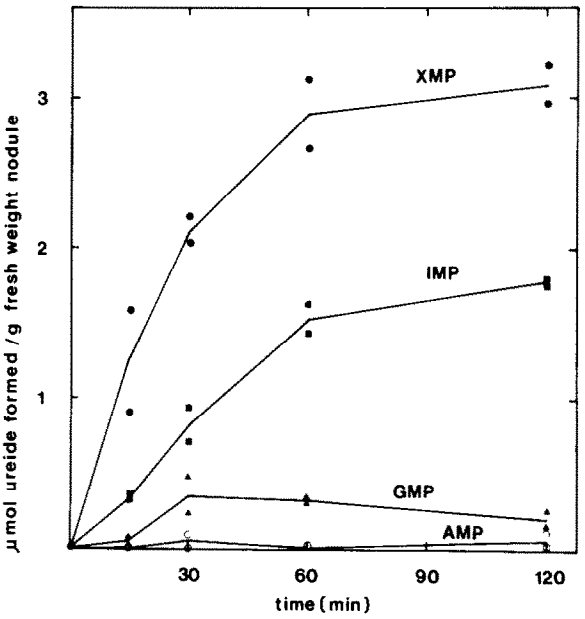

Fig.1. Formation of ureides (allantoin and allantoic acid) from purine nucleotides by a cell-free preparation isolated from the cytosol of the bacteroid-containing host cells of cowpea nodules.

3. Results

Both XMP and IMP were readily metabolised to ureides whereas GMP was only slowly converted and AMP not at all (fig.1). However the low yield of ureides from GMP and AMP was not due to lack of metabolism of the nucleotides (fig.2). Together with the progressive disappearance of the nucleotide in each case the corresponding nucleosides (inosine, xanthosine, guanosine and adenosine) and free purine bases (hypoxanthine, xanthine, guanine and adenine) were formed (fig.2). Only in the case of IMP metabolism was there sufficient accumulation of uric acid to allow detection (fig.2A), although a study with cowpea nodule extracts had shown that metabolism of [^{14}C]purine bases to form [^{14}C]allantoin proceeded through uric acid oxidation [6].

The synthesis of ureides from both IMP and XMP was accompanied by NAD reduction (fig.3). With IMP there was a pronounced lag with constant rates reached 15 min after the addition of substrate. This lag, which was less pronounced with XMP and inosine and absent from reactions with xanthine and hypoxanthine, corresponded to the time required for significant accumulation of the substrates for xanthine oxidoreductase (see fig.2A,B). In all cases addition of 40 μM allopurinol caused complete inhibition of NAD reduction (shown for IMP, fig.3). Consistent with the apparent lack of adenylate aminohydrolase activities in cell free extracts (fig.2D) NAD reduction was not supported by AMP, adenosine or adenine.

In the absence of NAD, IMP was metabolised to inosine and hypoxanthine, XMP to xanthosine and xanthine. The kinetics of XMP conversion under these conditions were unaffected by the addition of 1 mM glutamine and 1 mM ATP and synthesis of GMP, guanosine or guanine from XMP could not be detected.

Formation of [^{14}C]allantoin and allantoic acid from [8-^{14}C]IMP (fig.4) was similar in kinetic pattern to the metabolism of unlabelled nucleotide (fig.2A) and there was quantitative recovery of ^{14}C in the intermediates shown. In the presence of allopurinol, labelled ureides and xanthine were not formed but an equivalent amount of ^{14}C from IMP accumulated as hypoxanthine (fig.4).

The activity of the cell-free preparation was relatively stable, retaining 80% of the initial activity towards IMP and 100% towards XMP after 24 h at

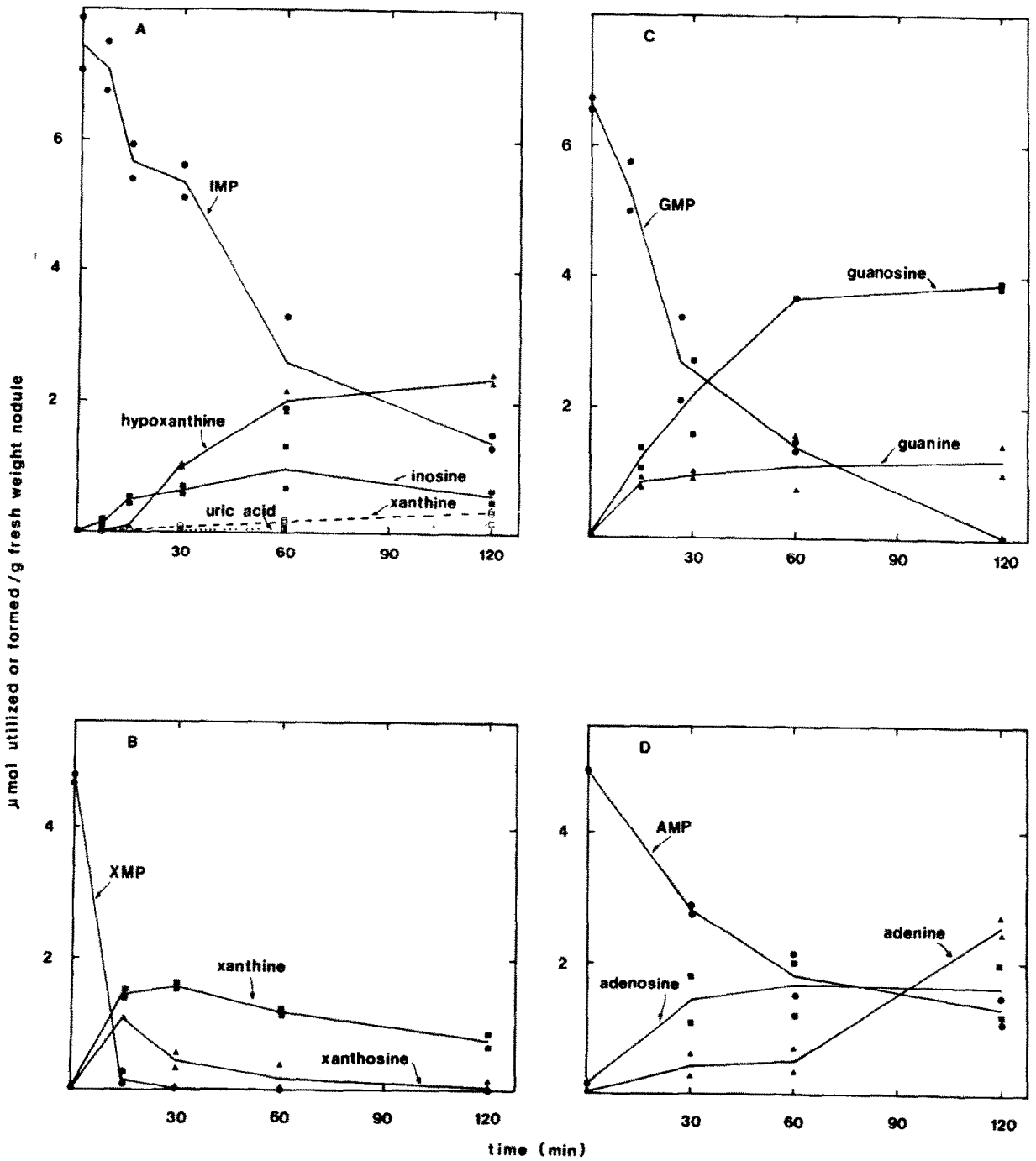

Fig.2. Utilisation of purine nucleotides and the formation of metabolic intermediates by a cell-free preparation from the cytosol of the bacteroid-containing host cells of cowpea nodules: (A) IMP; (B) XMP; (C) GMP; and (D) AMP as substrate.

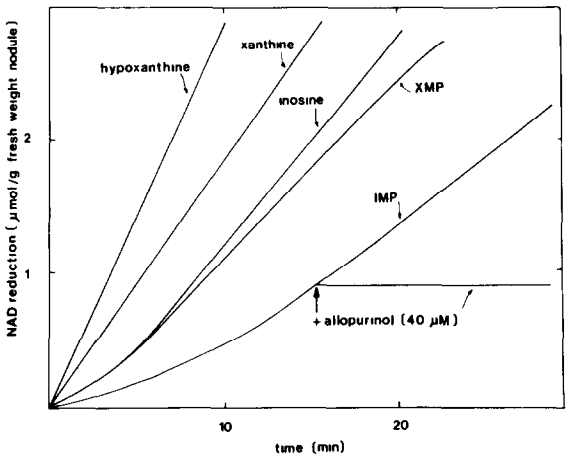

Fig.3. Purine-dependant NAD reduction by a cell-free preparation from the cytosol of the bacteroid-containing host cells of cowpea nodules. Allopurinol completely inhibited NAD reduction with all substrates but is shown only for IMP.

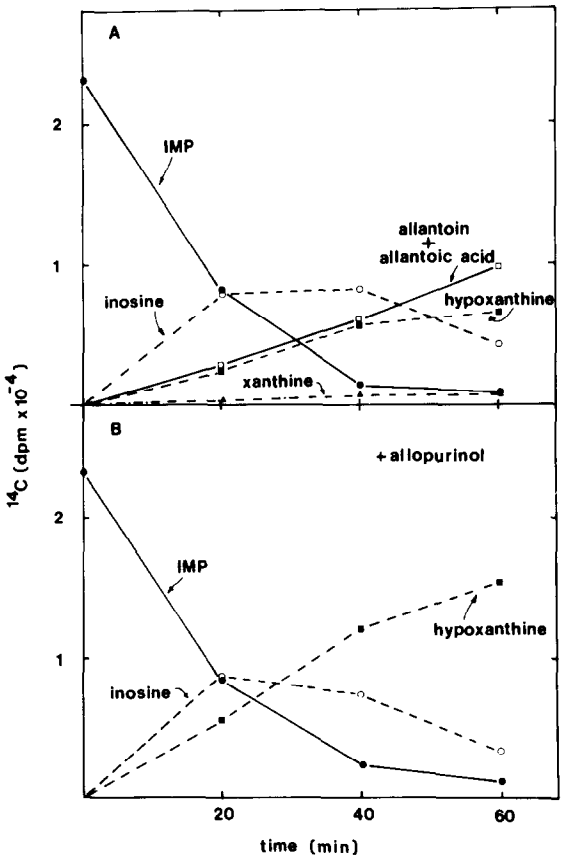

Fig.4. Metabolism of [8-^{14}C]IMP by a cell-free preparation from the cytosol of the bacteroid-containing host cells of cowpea nodules in the absence of allopurinol (A) or with addition of 50 μM allopurinol (B).

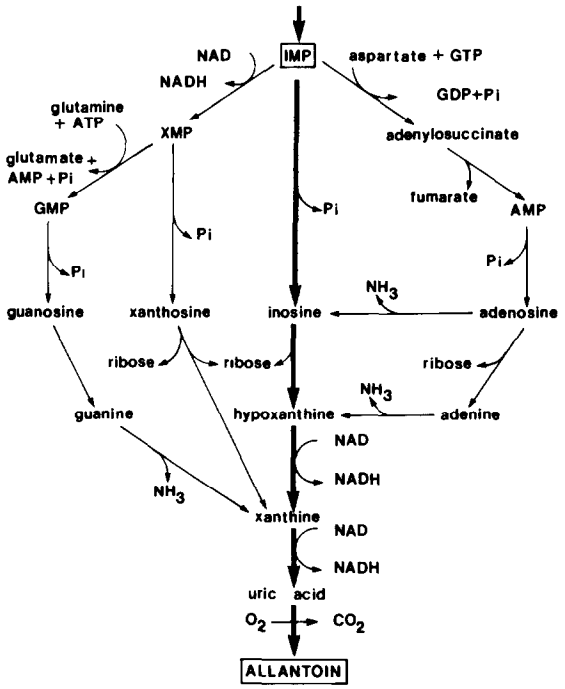

Fig.5. Branched metabolic pathway for the possible utilisation of the product of de novo purine synthesis, IMP, to form allantoin. The probable route found in cell-free preparations of the cytosol of bacteroid-containing cells of cowpea nodules is indicated by the heavy arrows.

4°C. In both cases the maximum activity was found between pH 7–8 with app. K_m values of 30 μM IMP and 50 μM XMP. The metabolism of IMP was unaffected by 1 mM AMP, GMP, PRPP or pyrophosphate but ureide synthesis from both IMP and XMP was inhibited by uric acid (50% at 0.1 mM). Bacteroids or bacteroid extracts assayed for ureide synthesis from IMP failed to show significant activity.

4. Discussion

Rates of nitrogen fixation in cowpea plants grown under similar conditions and at the same stage of development as these were 3–6 μmol N . h^{-1} . g fresh wt^{-1} nodule tissue [11] and would be equivalent to 1.5–3 μmol ureide . h^{-1} . g^{-1} if all fixed nitrogen was channeled through allantoin. The maximum rates of ureide synthesis from purine nucleotides found with cell free extracts were sufficient to account for this rate of fixation, being

5.6 μmol . h^{-1} . g fresh wt^{-1} nodule with XMP and 1.5 μmol . h^{-1} . g^{-1} with IMP (fig.1).

The patterns of metabolism of the nucleotides, the ^{14}C-labelling of ureides from [8-^{14}C]IMP, the kinetics of substrate-dependant NAD reduction and the effects of allopurinol taken together support the scheme shown in fig.5 with the predominant route for ureide synthesis from IMP occurring through inosine and hypoxanthine. While XMP was the most effectively utilised substrate for ureide synthesis the apparent lack of IMP oxidoreductase (EC 1.2.1.14) coupled with the absence of enzymes for the deamination of the adenylates and for synthesis of GMP (assayed as GMP synthetase, EC 6.3.4.1) results in restriction of IMP metabolism to a direct route leading to formation of allantoin. Some of the enzyme activities found to be low or absent in extracts could have been caused by modification or denaturation as a result of tissue disruption. However, direct metabolism of relatively low levels of IMP in this way leads to the effective transfer of nitrogen assimilated in de novo purine nucleotide synthesis and avoids release and reassimilation of ammonia which would result if the branched pathways of purine nucleotide formation (fig.5) also contributed to ureide synthesis.

The ready conversion of purine nucleotides to allantoin and allantoic acid by cowpea nodule cytosol extracts, as well as by those from soybean [5], supports the proposal [2,4,5] that in the nodules of ureide-forming legumes, fixed nitrogen is assimilated through a pathway of de novo purine biosynthesis.

Acknowledgements

The technical assistance of L. Owen, M. Lucks and D. Waldie is gratefully acknowledged. This work was supported by a grant from the Australian Research Grants Committee.

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