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PYRIMIDINE METABOLISM AND SECONDARY PRODUCT FORMATION; BIOGENESIS OF ALBIZZIINE, 4-HYDROXYHOMOARGININE AND 2,3-DIAMINOPROPANOIC ACID

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Key Word Index—*Albizia julibrissin*; *Glycine max*; *Lathyrus tingitanus*; *Phaseolus aureus*; *Pisum sativum*; Leguminosae; pyrimidine metabolism; non-protein amino acids; willardiine; isowillardiine; lathyrine; 4-hydroxyhomoarginine; albizziine; 2,3-diaminopropanoic acid

Abstract—In relation to the biogenesis of pyrimidine-derived secondary products, the relative activities and substrate specificities of the enzymes of pyrimidine metabolism were determined in extracts of seedlings from a number of leguminous species. Carbamoyl phosphate synthetase (CPSase) and aspartate transcarbamoylase (ATCase) were 4 to 6 × more active, and 5′-nucleotidase and uridine hydrolase were 2 to 3 × more active in the secondary metabolite producing species examined. As it was also confirmed that UMP is the most effective nucleotide inhibitor of ATCase activity, these relatively high rates of hydrolysis of UMP to uracil would lessen effective negative feedback control of pyrimidine synthesis in the secondary metabolite producers and further increase the availability of uracil for secondary metabolism. The key enzyme of pyrimidine catabolism, NADPH-dependent dihydrouracil dehydrogenase was shown to use uracil, thymine, lathyrine and 5-aminouracil as substrates and its activity was 2 to 3 × higher in those species not yielding pyrimidine secondary products. This observation is consistent with the diversion, in the secondary product-forming plants examined, of significant amounts of uracil away from catabolism and into secondary product synthesis. The dihydrouracil dehydrogenase of *Albizia julibrissin* exhibited higher activity with 5-aminouracil as substrate than with uracil or thymine. Dihydropyrimidinase, the next enzyme in the catabolic pathway, was extracted from the various species and shown to use 5,6-dihydrouracil, 5,6-dihydrothymine and 5-amino-5,6-dihydrouracil as substrates. Using a NADPH-regenerating system, the combined action of the two enzymes, dihydrouracil dehydrogenase and dihydropyrimidinase, was shown to produce 4-hydroxyhomoarginine from lathyrine. This was identified by co-chromatography and electrophoresis with authentic samples of 4-hydroxyhomoarginine. Preparations of β-ureidopropionase, the third enzyme in the catabolic pathway, were obtained from seedlings of *Pisum sativum* and shown to hydrolyse *N*-carbamoyl-β-alanine to 3-aminopropanoic acid, and *N*-carbamoyl-2-methyl-3-aminopropanoic acid to 3-amino-2-methylpropanoic acid. Additionally, the preparations from *P. sativum* and *A. julibrissin* hydrolysed albizziine to 2,3-diaminopropanoic acid, identified by co-chromatography and electrophoresis with authentic samples. It is concluded that there is a greater production and diversion of uracil into secondary product formation in the plants producing these compounds than in those that do not. In the non-producing plants, there is a more active catabolism of uracil. With *A. julibrissin*, degradation of 5-aminouracil by the pyrimidine catabolic process was shown to yield the non-protein amino acids albizziine and 2,3-diaminopropanoic acid. In *Lathyrus tingitanus*, this process demonstrably produces 4-hydroxyhomoarginine from lathyrine. The observations described are consistent with the view that production of pyrimidine-derived secondary products is attributable to the operation of detoxication processes for bioactive pyrimidines.

INTRODUCTION

In recent years, the biosynthesis of a number of pyrimidine secondary products has been investigated. The isomeric pyrimidinyl amino acids willardiine [β-(2,4-dihydroxypyrimidin-1-yl)alanine] and isowillardiine [β-(2,4-dihydroxypyrimidin-3-yl)alanine] have been shown to originate via the orotate pathway with uracil as their immediate precursor [1–3]. The pyrimidine nucleus of vicine and convicine, which are the 5-*O*-glucosides of 2,6-diamino-4,5-dihydroxypyrimidine and 6-amino-2,4,5-trihydroxypyrimidine, respectively, arises in a similar way [4]. The third known pyrimidine amino acid, lathyrine [β-(2-aminopyrimidin-4-yl)alanine], likewise derives from uracil produced by the orotate pathway [5–7]. The immediate precursor of lathyrine is, however, not uracil itself, but a uracil metabolite, 2-amino-4-carboxypyrimidine [7].

In addition to these pyrimidine derivatives, it has been postulated that the 'non-protein' amino acids 4-hydroxyhomoarginine [5] and albizziine [8] may also originate as secondary products of pyrimidine catabolism. 4-Hydroxyhomoarginine is a constituent of various *Lathyrus* species [9] and was originally suggested to be a precursor of lathyrine [9]. Subsequent biosynthetic studies [5–7] showed, however, that lathyrine originates from the orotate pathway and that the reported small incorporation of radioactivity from [^{14}C]homoarginine into lathyrine [9] was more likely due to a partial experimental reversal of the catabolism of lathyrine via 4-hydroxyhomoarginine. This possibility was examined further in the present study.

Albizziine (2-amino-3-ureidopropanoic acid) is a characteristic amino acid constituent of the Mimosaceae [8, 10–14]. All known species of *Acacia*, *Albizia*, *Mimosa*, and seven of the eight other genera contain this compound; the exception is *Prosopis* [15]. The occurrence of albizziine in a plant species not belonging to the Mimosaceae, namely *Dialium*, has been reported [16], and it has also been found to be produced by the hyphae of a wood-rotting basidiomycete, *Coniophora puteana* [17]. Little information is available concerning the biosynthesis of albizziine although it has been suggested that it could arise either by ring-fission of a pyrimidine [8] or by transcarbamoylation of 2,3-diaminopropanoic acid in a reaction analogous to that in the urea cycle by which ornithine is carbamoylated to citrulline [13]. This latter suggestion appeared to be supported by the observation that 2,3-diaminopropanoic acid is also a common constituent of the amino acid pools of seeds from various species of *Mimosa* and *Acacia* [13, 15, 18]. In a preliminary biosynthetic study, Reinbothe reported some small incorporation of radioactivity from [3-^{14}C]serine into albizziine [13, 15].

The present work aimed (a) to study the interface between primary and secondary pyrimidine metabolism, especially in relation to control mechanisms, and (b) to examine the hypothesis that the biogenetic origin of albizziine and 4-hydroxyhomoarginine lies in pyrimidine catabolism.

RESULTS AND DISCUSSION

Pyrimidine production

So that a comparison could be made between the pyrimidine metabolism of plants yielding pyrimidine-derived secondary products and that of related plant species not producing compounds of this type, the relative activities of two key enzymes of the orotate pathway were determined in groups of seedlings of similar age and stage of developement. The enzymes assayed were carbamoyl phosphate synthetase (CPSase; EC 2.7.2.5) and asparate carbamoyl transferase (aspartate transcarbamoylase; ATCase; EC 2.1.3.2). The secondary product producers were *Pisum sativum* (willardiine and isowillardiine) and *Lathyrus tingitanus* (lathyrine). *Albizia julibrissin* was also examined in this category since it has been suggested that albizziine maybe product of pyrimidine metabolism [8]. The non-producers examined for comparison were *Phaseolus aureus* and *Glycine max*.

The results (Table 1) show that both key enzymes, CPSase and ATCase, are more active by a factor of 4 to 6 in the secondary product producers than in the non-producers. *Albizia julibrissin* was clearly in the high enzymic activity group. Previous reports [19, 20] that in plants both ATCase and CPSase are inhibited by uridine nucleotides were confirmed in the present study (Table 2). The results also show that UDP is a more effective inhibitor of CPSase than in UMP, and that UMP and UDP are potent inhibitors of ATCase activity. Inhibitions of 88 to 94% resulted with all the plant ATCases examined.

The implication of these findings is that, subject to availability of precursors, there is a more rapid synthesis of pyrimidines in the plants producing pyrimidine-derived secondary products and that this is further elevated by depletion of UMP and UDP, the negative feedback controllers of ATCase activity. Such depletion would be expected to occur because UMP is not only the source of all the primary products of pyrimidine metabolism, but is also the source of uracil from which the secondary products willardiine, isowillardiine, lathyrine, vicine and convicine are synthesized [1, 4, 5, 7]. Furthermore, as uracil is the limiting factor in the biosynthesis of willardiine and isowillardiine [1] and the biosynthetic mechanisms for these compounds can immediately handle high concentrations of exogenously supplied uracil, up to saturated solutions [1], there is a large metabolic sink for uracil and hence UMP. Because of its high free energy of hydrolysis, UDP is metabolically unstable, undergoing facile hydrolysis to UMP. In the secondary pyrimidine producers, UMP and UDP are, therefore, unlikely to accumulate sufficiently to suppress pyrimidine synthesis.

The release of uracil from UMP, for catabolism or secondary product formation, is catalysed sequentially by 5′-nucleotidase (EC 3.1.3.5), which hydrolyses UMP to uridine, and uridine hydrolase (EC 3.2.2.3), which hydrolyses uridine to uracil and ribose. Determination of the relative activities of these enzymes in the tissues of plants forming pyrimidine-derived secondary products and in those of comparable plants not producing such compounds, revealed significant differences (Table 1). The producer group showed 5′-nucleotidase activities double those of the latter group, and the activity of uridine hydrolase was also two to three times higher in the producer group. These observations also point to the increased availability of uracil for further metabolism in the plants yielding pyrimidine-derived secondary products.

Pyrimidine catabolism

In plants, animals and microorganisms, the reductive catabolism of pyrimidines is channelled through the bases uracil and thymine (5-methyluracil). These are en-

Table 1. Relative activities of the enzymes of pyrimidine metabolism in some pyrimidine secondary metabolite producers and non-producers

Enzyme	Substrate	Enzyme source	Specific activity (nkat × 10^2 mg^{-1} protein)		
CPSase	ATP + $NaHCO_3$ +glutamine	*P. sativum*	13.3		
		L. tingitanus	8.6		
		A. julibrissin	10.0		
		P. aureus	2.1		
		G. max	2.3		
ATCase	Carbamoyl phosphate +aspartate	*P. sativum*	9.3		
		L. tingitanus	10.4		
		A. julibrissin	8.4		
		P. aureus	2.5		
		G. max	3.3		
5′-Nucleotidase	UMP	*P. sativum*	8.3		
		L. tingitanus	8.8		
		A. julibrissin	7.6		
		P. aureus	5.1		
		G. max	4.0		
Uridine hydrolase	Uridine	*P. sativum*	17.2		
		L. tingitanus	16.1		
		A. julibrissin	nd		
		P. aureus	5.3		
		G. max	8.2		
			(a)	(b)	(c)
Dihydrouracil dehydrogenase	(a) Uracil	*P. sativum*	12.5	9.1	8.3
	(b) Thymine	*L. tingitanus*	6.0	3.8	5.8
	(c) 5-Aminouracil	*A. julibrissin*	20.1	15.3	21.4
		P. aureus	21.6	16.6	19.1
		G. max	16.1	12.6	14.5
			(d)	(e)	(f)
Dihydro-pyrimidinase	(d) Dihydrouracil	*P. sativum*	21.9	10.5	17.6
	(e) Dihydrothymine	*L. tingitanus*	21.9	10.2	17.5
	(f) 5-Aminodihydrouracil	*A. julibrissin*	18.8	9.5	21.3
		P. aureus	14.1	5.7	7.8
		G. max	13.1	5.2	9.4

Details of substrate concentrations and incubation conditions are given in Experimental.
nd not determined.
Data are a typical set from replicate experiments showing similar trends.

Table 2. Effect of nucleotides on carbamoyl phosphate synthase and apartate carbamoyl transferase activity

	Relative activity (%)							
	CPSase				ATCase			
Addition	*P. sativum*	*L. tingitanus*	*P. aureus*	*G. max*	*P. sativum*	*L. tingitanus*	*P. aureus*	*G. max*
None	100	100	100	100	100	100	100	100
UMP	93.7	86.8	92.8	93.8	6.2	8.6	6.6	6.8
UDP	58.3	66.0	71.4	69.2	8.7	11.6	11.5	9.6
UTP	75.0	90.0	85.7	79.2	14.3	17.7	24.2	15.1
CMP	89.0	90.0	89.2	84.6	98.1	100	103	98.6
CDP	67.7	77.3	85.7	80.7	100.6	101.6	104	99.2
CTP	83.8	84.9	87.5	83.0	102.5	103.3	107.2	100.6

All nucleotide additions were at a final concentration of 3 mM.
The absolute values (100%) for the controls (no addition) are given in Table 1.
Data are a typical set from replicate experiments showing similar trends.

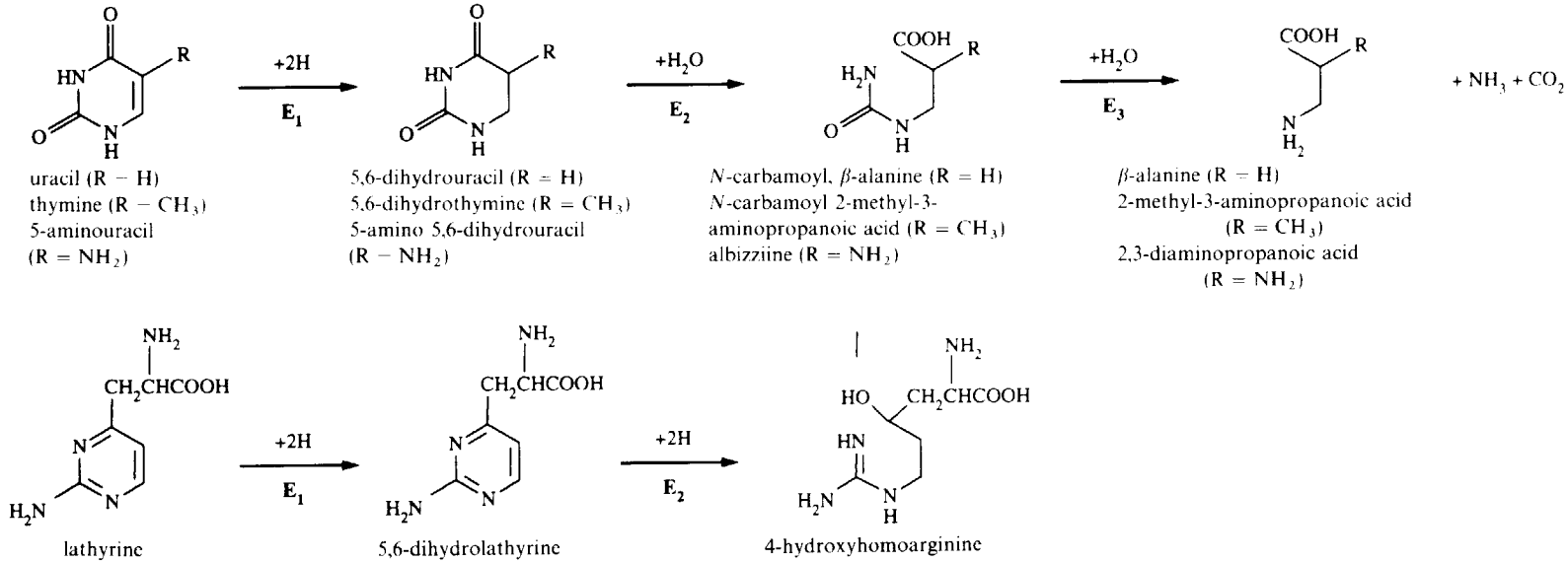

Fig. 1. Group specificity of the enzymes of the pyrimidine catabolic pathway results in the biogenesis of albizziine and 2,3-diaminopropanoic acid from 5-aminouracil, and of 4-hydroxyhomoarginine from lathyrine. E_1, dihydrouracil dehydrogenase; E_2, dihydropyrimidinase; E_3, β-ureidopropionase.

Table 3. Reduction of lathyrine and uracil by NADPH-dependent dihydrouracil dehydrogenase from *L. tingitanus* and *P. sativum*

Enzyme source	Substrate (0.15 mM)	Specific activity (nkat × 10^2 mg^{-1} protein)
Lathyrus tingitanus	Lathyrine	3.4
	Uracil	6.0
Pisum sativum	Lathyrine	7.7
	Uracil	12.5

The enzyme preparation is described in Experimental.
Data are a typical set from replicate expirements showing similar trends.

zymically degraded by parallel routes (Fig. 1), the first step in which is reduction of the 5,6-double bond by NADPH-dependent dihydrouracil dehydrogenase (EC 1.3.1.2). This is the rate-limiting step in pyrimidine catabolism in most animal tissues (see, e.g. [21–24]), but relatively little is known of this enzyme in plants, especially concerning its relative activity and specificity. During the present study, the enzyme was extracted from the same two groups of seedlings used in the investigation of pyrimidine production, described in the preceeding section, i.e. producers and non-producers of pyrimidine-derived secondary products. Specific enzymic activities were determined using uracil, thymine, lathyrine and 5-aminouracil as individual substrates. 5-Aminouracil was included because if, as has been suggested [8], albiziine does originate in the catabolism of a pyrimidine, on structural grounds, this would be expected to be the precursor. The results (Table 1) showed that the dihydrouracil dehydrogenase preparations from all the species examined, reduced all three pyrimidine substrates. The non-producers, *P. aureus* and *G. max*, exhibited an activity two to three times higher than that of the producer group of plants. These data are compatible with the diversion of relatively large amounts of uracil away from catabolic ring-opening and into secondary product formation. The scale of this diversion is exemplified by lathyrine production; lathyrine constitutes >2% of the dry weight of seeds of *L. tingitanus* [25] and 20% of the orotate metabolized by this plant finishes up as lathyrine [26]. The results with *A. julibrissin* fall into the same high dehydrogenase activity range as the non-producers of pyrimidine secondary products. This is as would be expected since albizziine is postulated to arise from pyrimidine ring-opening and not by biosynthetic elaboration of an intact pyrimidine ring. In addition to the foregoing results, the dehydrogenases from *L. tingitanus* and *P. sativum* were shown to reduce lathyrine at about half the rate of uracil (Table 3). The other species were not examined in this respect.

The second enzyme in the pyrimidine catabolic sequence (Fig. 1) is dihydropyrimidinase (EC 3.5.2.2), which catalyses hydrolytic ring-cleavage of the 5,6-dihydropyrimidine product of the first enzyme. All the plants examined possessed this enzyme and it was active against all three of the dihydropyrimidine substrates tested, i.e. 5,6-dihydrouracil, 5,6-dihydrothymine and 5-amino-5,6-dihydrouracil (Table 1). With each of the dihydropyrimidinase preparations, except that from *A. julibrissin*, 5,6-dihydrouracil was the preferred substrate. With the enzyme from *A. julibrissin*, however, the preferred substrate was 5-amino-5,6-dihydrouracil, from which the expected product would be albiziine (Fig. 1). To confirm its identity, the compound was extracted from a parallel incubation mixture, kept at 37° for 1 hr. The reaction was stopped by adding trichloroacetic acid (TCA) to a final concentration of 5% (w/v), and the precipitate was removed by centrifuging. TCA was removed from the supernatant by extraction into diethyl ether and the remaining solution was evaporated to dryness *in vacuo* at 40°. After redissolving the residue in 0.5 ml of water, it was chromatographed in solvents (i), (ii) and (iii) and subjected to high-voltage paper electrophoresis at pH 2 and pH 9 in buffer systems (v) and (vi) (see Experimental). The compound gave a purple colour with ninhydrin, and co-chromatographed and migrated electrophoretically with authentic samples of albizziine in the systems indicated. It was concluded that the product of the action of dihydropyrimidinase on 5-amino-5,6-dihydrouracil is albizziine.

To test the hypothesis that the sequential action of 5,6-dihydropyrimidine dehydrogenase and dihydropyrimidinase in *A. julibrissin* produces albizziine from 5-aminouracil, and enzymic extract of *Albizia* seedlings was prepared using 50 mM Tris–HCl buffer (pH 7.4) and containing 7 mM 2-mercaptoethanol. The extract was dialysed overnight against the same buffer. As NADPH, required by the dehydrogenase to reduce the pyrimidine, has a relatively short existence in enzymic reaction mixtures, an NADPH-regenerating system was included. Thus, the enzymic incubation mixture consisted of 0.2 mM NADP, 10 mM glucose 6-phosphate, 1 mM 5-aminouracil, 50 units of glucose 6-phosphate dehydrogenase and 9 ml of enzymic extract, in a total volume of 10 ml. In control incubations, NADP, glucose 6-phosphate and glucose 6-phosphate dehydrogenase were omitted. After incubating for 30 min at 2.5°, the temperature was raised to 33° and incubation continued for a further 2.5 hr. This incubation regime had been found empirically to give the best conditions. The reaction was stopped with TCA (5% final concentration), and the

precipitate and TCA were removed as before. The reaction mixtures were concentrated by evaporation *in vacuo* and subjected to high-voltage electrophoresis in buffers (v) and (vi), and chromatography in solvent systems (i)–(iii). The presence of albizziine was demonstrated in the complete reaction mixtures, but not in the controls. It was apparent that much smaller amounts of albizziine were produced by this procedure than by using the two pyrimidine-catabolizing enzymes separately and sequentially. This is attributable to the difficulty in maintaining the NADPH concentration and also the necessity of using a compromise incubation pH of 7.4 (the optimum for dihydrouracil dehydrogenase is pH 7.4–8.0, that for dihydropyrimidinase is pH 9.5, and for glucose 6-phosphate dehydrogenase it is pH 7.4). Repeating the experiment with enzymic preparations from seedlings of *P. sativum* gave essentially similar results.

A parallel experiment was carried out with a dialysed enzymic preparation from *L. tingitanus* seedlings and with lathyrine as substrate. The reaction mixture was similar in composition to that used in demonstrating the formation of albizziine and included the components of the NADPH-regenerating system. After incubating for 30 min at 27°, followed by 150 min at 37°, the reaction was stopped, as before, with TCA, and the precipitate and TCA, were removed. The extract was concentrated *in vacuo* and examined by high-voltage electrophoresis in buffer (v) followed by sequential paper chromatography in solvent systems (i)–(iv). In addition to some unmetabolized lathyrine, the incubation mixture, but not the controls, were found to contain 4-hydroxyhomoarginine. This supports the theory advanced earlier [5] that 4-hydroxyhomoarginine, a natural constituent of *L. tingitanus* seedlings [9] is essentially a catabolite of lathyrine.

After hydrolytic fission of the pyrimidine ring, catalysed by dihydropyrimidinase, the ensuing step in the catabolic pathway (Fig. 1) is enzymic hydrolysis of the ring-opened product to release the carbamoyl group as CO_2 and NH_3 (Fig. 1). With uracil as the initial catabolic substrate, the product at this stage is 3-aminopropanoic acid (β-alanine), and with thymine it is the corresponding methyl derivative, 3-amino-2-methylpropanoic acid. A preparation of the enzyme responsible for removal of the carbamoyl group, β-ureidoproionase (EC 3.5.1.6) was obtained from seedlings of *P. sativum* and demonstrated to produce 3-aminopropanoic acid from *N*-carbamoyl-β-alanine, and 3-amino-2-methylpropanoic acid from *N*-carbamoyl-β-aminoisobutyric acid. The products were identified by co-chromatography with authentic samples in solvents (i)–(iii), by high-voltage electrophoresis in buffer (v), and by their colour reactions with ninhydrin and Ehrlich's reagent (see Experimental).

Additionally, enzymic extracts of *A. julibrissin* and *P. sativum* seedlings were shown to catalyse the parallel reaction with albizziine, yielding 2,3-diaminopropanoic acid which was identified by similar chromatography and electrophoresis with authentic samples in systems (iii) and (v), and by reaction with ninhydrin and with Ehrlich's reagent (see Experimental). 2,3-Diaminopropanoic acid is a non-protein amino acid found in various species of *Mimosa* and *Acacia* [13, 15, 18]. Its biogenesis had hitherto been obscure, but it had been suggested to be the precursor of albizziine [13]; however, as with the relationship of lathyrine to 4-hydroxyhomoarginine, the present work shows that the connection is catabolism and not biosynthesis (Fig. 1).

It is concluded from the experimental data presented here that seedlings of *A. julibrissin*, *L. tingitanus* and *P. sativum*, which accumulate pyrimidine-derived secondary products, have a greater capacity for uracil production than do seedlings of *G. max* and of *P. aureus* and that this is mainly attributable to greater relative activity of CPSase and especially of ATCase, the rate-limiting enzyme in pyrimidine synthesis [21–24]. Interestingly, UMP is both the source of uracil for secondary product formation and the feedback inhibitor of ATCase. Thus, secondary product synthesis removes the main restraint on uracil production. As uracil is the limiting factor in the synthesis of the pyrimidine amino acids [1, 2, 26], this explains the relatively large accumulations that can occur of these compounds [1, 25].

The present study shows that the enzymes of pyrimidine catabolism in *A. julibrissin* and *P. sativum* (Table 1) degrade 5-aminouracil to albizziine as effectively as they do uracil to β-alanine. Similarly, the pyrimidine catabolic machinery of *L. tingitanus* and of *P. sativum* produces 4-hydroxyhomoarginine from lathyrine. This latter observation confirms earlier evidence that 4-hydroxyhomoarginine is a catabolite rather than a mainstream precursor of lathyrine [5–7]. The findings with albizziine support the theory that this non-protein amino acid also originates from pyrimidine catabolism, and this is further strengthened by the observed production of 2,3-diaminopropanoic acid during catabolism of albizziine by the pathway. In nature, 2,3-diaminopropanoic acid commonly accompanies albizziine, but its biochemical origin was hitherto unknown. The precursor of albizziine and 2,3-diaminopropanoic acid in these experiments, 5-aminouracil, has not been reported to occur in plants, but the riboside of the corresponding hydroxy compound, 5-hydroxyuracil, has [27] and amination of hydroxyl compounds is biochemically facile. There are numerous reports, reviewed in refs [28, 29], of the biological potency of 5-aminouracil; e.g. in plants it blocks the mitotic cycle and inhibits incorporation of guanosine into nucleic acids, and is both a bacteriostat and a mutagen. Production of albizziine and 2,3-diaminopropanoic acid may well therefore be another example of a plant detoxication mechanism yielding non-protein amino acids [26]. The earlier report of some small incorporation of radioactivity from [3-^{14}C]serine into albizziine [13, 15] may be explained by the action of serine dehydratase yielding [3-^{14}C]pyruvate and, via oxaloacetate, this producing [^{14}C]aspartate, a pyrimidine precursor.

In summary, the data indicate that, in the plants examined, the enzymes of pyrimidine catabolism exhibit group specificiy and that uracil, thymine, 5-aminouracil and lathyrine can be processed in a parallel biochemical

fashion (Fig. 1), accounting for the biogenesis of albizziine, 2,3-diaminopropanoic acid, and 4-hydroxyhomoarginine.

EXPERIMENTAL

Materials. Seeds of *P. sativum* cv. Meteor were from Booker Seeds Ltd., Sleaford, Lincs, *Albizia julibrissin* seeds were from Thompson & Morgan, Ipswich, Suffolk. *Phaseolus aureus* and *G. max* seeds were purchased locally and those of *L. tingitanus* were supplied by the University Botanic Garden. In all cases, seeds were well washed and allowed to imbibe for 15 hr 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.

Analytical grade chemicals and Polyclar AT were from BDH. Pyrimidines, coenzymes and enzymes, amino acids, *p*-nitrophenyl phosphate, *p*-methylaminophenol sulphate and *p*-dimethylaminobenzaldehyde were from Sigma. [2-^{14}C]Uracil (sp. radioactivity 54 mCi mmol^{-1}) and $NaH^{14}CO_3$ (sp. radioactivity 0.1 mCi mmol^{-1}) were from Amersham. The Apex II ODS column was supplied by Jones Chromatography.

CPSase; extraction and assay. Seedlings (10–13 days old) were homogenized, using a pre-chilled mortar and pestle, in 0.1 M Tris–HCl buffer (pH 7.6) containing 3 mM glutamine and 10 mM 2-mercaptoethanol, allowing 1 ml buffer g^{-1} tissue. The homogenate was pressed through two layers of cheesecloth and the filtrate centrifuged at 12000 g for 20 min at 4°. The supernatant was used directly as the crude enzymic prepn. Its activity was assayed essentially by the method of ref. [30] in which carbamoyl phosphate is converted into hydroxyurea, but 4 mM ornithine was added to the reaction mixture. [^{14}C]Hydroxyurea was determined by scintillation counting 0.5 ml samples in 5 ml of Optiphase Ria Luma (LKB). Counting efficiency was 86%.

Extraction and assay of ATCase. Seedlings (10–13 days old) were homogenized, using a prechilled mortar and pestle, in Tris–HCl buffer (0.1 M; pH 8.0), allowing 1 ml g^{-1} tissue. After pressing through a double layer of cheesecloth, the filtrate was centrifuged (as above) and the supernatant dialysed overnight at 4° against the same buffer. Enzymic activity was assayed by the procedure of ref. [31] with minor modifications. Incubation mixtures contained 0.1 M Tris–HCl buffer (pH 8.0), 5 mM carbamoyl phosphate, 10 mM aspartic acid (pH 8.0), 0.1 ml enzymic extract, in a final vol. of 1 ml. Incubation was at 30° for 1 hr. The reaction was stopped by adding 3 M $HClO_4$ (0.1 ml) and the ppt. removed by centrifuging. Carbamoyl aspartate was determined by the method of ref. [32].

UMP-5′-nucleotidase and uridine hydrolase; extraction and assay. A nucleotidase preparation was obtained essentially by the procedure described for CPSase, except that a 0.2 M Tris–HCl buffer (pH 8.0) containing 2 mM dithioerythritol was used. The supernatant was dialysed overnight at 4° against the same buffer. For assay of 5′-nucleotidase activity, the reaction mixture comprised 0.1 M Tris–HCl buffer (pH 7.5) containing 2 mM $MgCl_2$, 5 mM $NaMoO_4$ and 10 mM UMP; correction was made for non-specific phosphatase activity, determined in the same preparation by a similar protocol, but in which UMP was replaced by 10 mM *p*-nitrophenyl phosphate. For both assays, 0.1 ml of enzymic extract was used in a final reaction volume of 1 ml; incubation was at 37° for 30 min. Reactions were terminated by adding 0.1 ml 75% TCA and precipitated proteins were removed by centrifuging. P_i released was determined colorimetrically [33]. Uridine hydrolase activity was determined in the same enzymic extract using HPLC. The assay mixture comprised 0.1 M Tris–HCl (pH 7.5), 10 mM uridine, and 0.6 ml extract, in a final vol. of 1 ml. After incubation at 37° for 1 hr, the reaction was terminated by adding 0.1 ml of 3 M $HClO_4$, and the pptd proteins were removed by centrifuging. The supernatant was neutralized at 4° with 3 M KOH soln. and the pptd $KClO_4$ removed by centrifuging. Phenolics were removed by stirring the supernatant for 20 min at 4° with insoluble PVP (Polyclar AT) prepared for use as described in ref. [34] and allowing 500 mg 10 g^{-1} tissue extracted. After clarifying the prepn by centrifuging, the supernatant was filtered through a Millipore membrane (por. 0.45 μM) for HPLC. A reversed-phase column of Apex II ODS (25 cm × 4.6 mm diam.) was used in a linear gradient of 0–100% 3 M KH_2PO_4 containing 15% MeOH in 10 min. The initial soln. was 1.5 M KH_2PO_4 containing 15% MeOH, and the flow rate was 1 ml min^{-1}. The retention times for UMP, uracil and uridine were 4.0, 4.8 and 5.6 min, respectively.

Dihydrouracil dehydrogenase; preparation and assay. This enzymic activity was obtained by homogenizing seedlings at 4°, as described above, in K – P_i buffer (50 mM; pH 7.6) containing 5 mM 2-mercaptoethanol and 5 mM EDTA. After centrifuging, the supernatant was dialysed overnight against the same buffer. Enzymic activity was assayed spectrophotometrically at 37° by monitoring ΔA_{340}. Reaction mixtures contained 50 mM K-P_i buffer (pH 7.6), 5 mM 2-mercaptoethanol, 5 mM EDTA, 0.15 mM NADPH, 0.15 mM substrate (uracil, thymine, 5-aminouracil or lathyrine) and 0.1 ml enzymic prepn, in a final vol. of 0.6 ml. The reaction was initiated by adding NADPH; the reference cuvette contained the same mixture from which substrate had been omitted.

Dihydropyrimidinase; preparation and assay. Seedlings were homogenized, as before, but using a 50 mM Tris–HCl buffer (pH 9.5). After filtration through two layers of cheesecloth and centrifugation at 12000 *g* for 20 min, the supernatant was dialysed overnight at 4° against the same buffer. For enzymic assay, the reaction mixture comprised 50 mM Tris–HCl buffer (pH 9.5), 3 mM substrate (dihydrouracil, dihydrothymine or 5-amino dihydrouracil) and 0.1 ml of enzyme preparation, in a final vol. of 1 ml. After incubating for 1 hr at 37°, the activity was determined colorimetrically by the method of ref. [35] with minor modifications.

β-Ureidopropionase; extraction and incubation. For examination of β-ureidopropionase activity, seedlings were homogenized, as above, but in 50 mM K-P_i buffer (pH 7.3) containing 10 mM $MgCl_2$ and 10 mM 2-mercap-

toethanol. After centrifuging at 12000 *g* for 20 min, the supernatant was dialysed for 12 hr at 4° against the same buffer. The reaction mixtures contained 50 mM K-P_i buffer (pH 7.3), 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 10 mM substrate (*N*-carbamoyl-β-alanine, *N*-carbamoyl-β-aminoisobutyric acid or albizziine) and the enzymic extract, in a final vol. of 5 ml. After incubation at 37° for 3 hr, the reaction was stopped with TCA (final concn 5%, w/v) and the ppt. removed by centrifuging. TCA was removed by extraction with Et_2O and the remaining soln was concd *in vacuo* for chromatography and electrophoresis.

Chromatography and electrophoresis. The PC solvent systems used with Whatman No. 1 or 3 MM paper were (i) 1-BuOH–HOAc–H_2O (60:15:25), (ii) 2-PrOH–aq.NH_3–H_2O (70:10:20), (iii) 2-MePrOH–MeCOEt–H_2O–aq.NH_3 (40:30:20:10), and (iv) H_2O. High-voltage electrophoresis (HVE) was effected on Whatman 3 MM paper. The buffers used were (v) HO_2CH–HOAc at pH 2 prepd by mixing HOAc (50 ml) with HO_2CH (40 ml of 98–100%, v/v) and making up to a final vol. (1 l) with H_2O; and (vi) $Na_2B_4O_7$ (50 mM). The voltage gradient applied was 28 V cm^{-1} for 2 hr.

Compounds were located either by viewing in UV light or with ninhydrin (0.2% w/v in Me_2CO). A modified Ehrlich reagent was also used as appropriate. This comprised 1% (w/v) *p*-dimethylaminobenzaldehyde in Me_2CO–HCl (9.5:0.5) [36]. Before using this, the paper was sprayed with 0.5 M NaOH and allowed to dry. The modified Sakaguchi reagent used in this study was that of ref. [37]; it consisted of 0.1% (w/v) 8-hydroxyquinoline in Me_2CO followed by 0.2% Br_2 in 0.5 M NaOH.

N-Carbamoyl-β-alanine, *N*-carbamoyl-β-aminoisobutyric acid and 5-aminodihydrouracil gave a yellow colour with Ehrlich's reagent and under the same conditions, β-aminoisobutyric acid gave a dark orange colour; albizziine gave a yellow/pink colour. With ninhydrin, all the amino acids except lathyrine and β-alanine gave the usual purple reaction. Lathyrine appeared red and β-alanine blue, gradually turning purple. With the Sakagushi reagent, lathyrine appeared purple and 4-hydroxyhomoarginine gave an orange-red colour.

Prepn of compounds. 5-Amino-5,6-dihydrouracil was prepared by cyclizing albizziine [38]. An albizziine soln. (0.1 g in 4 ml 15% v/v HCl) was refluxed for 2 hr, evapd to dryness, and redissolved in H_2O (0.5 ml) for chromatographic purification.

N-Carbamoyl-β-aminoisobutyric acid was prepd by hydrolysis of 5,6-dihydrothymine (DHT). A soln of DHT (50 mmol) and NaOH (75 mmol) in 10 ml H_2O was heated to boiling, cooled to room temp. and acidified with conc. HCl to pH 2. After standing overnight at 4°, the supernatant was removed by centrifuging and the crystals were recrystallized (× 3) from MeOH [39].

Lathyrine and 4-hydroxyhomoarginine were extracted from *L. tingitanus* seeds by the procedure of ref. [5]. Purification was by sequential PC in solvents (i)–(iv) and HVE in buffer (v).

Protein determination. Protein concentrations were determined by the Bradford method [40].

REFERENCES

1. Ashworth, T. S., Brown, E. G. and Roberts, F. M. (1972) *Biochem. J.* **129**, 897.
2. Al-Baldawi, N. F. and Brown, E. G. (1983) *Phytochemistry* **22**, 1928.
3. Ahmmad, M. A. S., Maskall, C. S. and Brown, E. G. (1984) *Phytochemistry* **23**, 265.
4. Brown, E. G. and Roberts, F. M. (1972) *Phytochemistry* **11**, 3203.
5. Brown, E. G. and Al-Baldawi, N. F. (1977) *Biochem. J.* **164**, 589.
6. Brown, E. G. and Mohamad, J. (1990) *Phytochemistry* **29**, 3117.
7. Brown, E. G. and Mohamad, J. (1994) *Phytochemistry* **36**, 285.
8. Kjaer, A. and Olesen-Larsen, P. (1959) *Acta Chem. Scand.* **13**, 1565.
9. Bell, E. A. (1963) *Nature* **199**, 70.
10. Gmelin, R., Strauss, G. and Hasenmaier, G. (1958) *Z. Naturforsch.* **13b**, 252.
11. Krauss, G. J. and Reinbothe, H. (1973) *Phytochemistry* **12**, 125.
12. Seneviratne, A. S. and Fowden, L. (1968) *Phytochemistry* **7**, 1039.
13. Reinbothe, H. (1962) *Flora* **152**, 545.
14. Gmelin, R., Strauss, G. and Hasenmaier, G. (1959) *Z. Physiol. Chem.* **314**, 28.
15. Reinbothe, H. (1967) in *Physiologie, Ökologie und Biochemie der Keimung* (Borriss, H., ed.) p. 609. Ernst-Moritz-Arndt Universität, Greifswald.
16. Peiris, P. S. and Seneviratne, A. S. (1977) *Phytochemistry* **16**, 1821.
17. Evans, C. S., Burns, P. J., Dutton, M. and Brown, S. (1990) *Phytochemistry* **29**, 2159.
18. Evans, C. S., Qureshi, M. Y. and Bell, E. A. (1977) *Phytochemistry* **16**, 565.
19. O'Neal, D. and Naylor, A. B. (1968). *Biochem. Biophys. Res. Commun.* **31**, 322.
20. Shibata, H., Sawa, Y., Ochiai, H., Kawashima, T. and Yamane, K. (1987) *Plant Sci.* **51**, 129.
21. Canellakis, E. S. (1956) *J. Biol. Chem.* **221**, 315.
22. Fritzson, P. (1962) *J. Biol. Chem.* **237**, 150.
23. Traut, T. W. and Loechel, S. (1983) *Biochemistry* **23**, 2533.
24. Naguib, F. N. M., El-Kouni, M. H. and Cha, S. (1985) *Cancer Res.* **45**, 5405.
25. Nowacki, E. and Przbylska, J. (1961) *Bull. Acad. Pol. Sér. Sci. Biol.* **9**, 279.
26. Brown, E. G. (1995) in *Amino Acids and Their Derivatives in Higher Plants* (Wallsgrove, R. M., ed.), p. 119. SEB Symposium Series, Vol. 56, Cambridge University Press, Cambridge, U.K.
27. Lis, A. W. and Passarge, W. E. (1966) *Arch. Biochem. Biophys.* **114**, 593.
28. Cheng, C. C. (1969) *Prog. Med. Chem.* **6**, 67.
29. Cheng, C. C. and Roth, B. (1970) *Prog. Med. Chem.* **7**, 285.
30. Shibata, H., Ochiai, H., Sawa, Y. and Miyoshi, S. (1986) *Plant. Phyiol.* **80**, 126.

31. Achar, B. S., Savithri, H. S., Vaidyanathan, C. S. and Rao, N. A. (1974) *Eur. J. Biochem.* **47**, 15.
32. Prescott, L. M. and Jones, M. E. (1969) *Analyt. Biochem.* **32**, 408.
33. LeBel, D., Poirier, G. G. and Beaudoin, A. R. (1978) *Analyt. Biochem.* **85**, 86.
34. Brown, E. G. and Davies, D. (1989) *Analyt. Biochem.* **183**, 312.
35. West, T. P., Shanley, M. S. and O'Donovan, G. A. (1982) *Analyt. Biochem.* **122**, 345.
36. Smith, I. (1953) *Nature* **171**, 43.
37. Jepson, J. B. and Smith, I. (1953) *Nature* **172**, 1100.
38. Cerutti, P., Kondo, Y., Landis, W. R. and Witkop, B. (1968) *J. Am. Chem. Soc.* **90**, 771.
39. Fink, R. M., McGaughey, C., Cline, R. E. and Fink, K. (1956) *J. Biol. Chem.* **218**, 1.
40. Bradford, M. M. (1976) *Analyt. Biochem.* **72**, 248.