Biosynthesis of the Pyrimidinyl Amino Acid Lathyrine by *Lathyrus tingitanus* L.

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The biosynthesis of the pyrimidinyl amino acid lathyrine by seedlings of *Lathyrus tingitanus* L. was shown to be stimulated by uracil. [6-¹⁴C]Orotate, [2-¹⁴C]uracil and [3-¹⁴C]serine were incorporated into lathyrine; the incorporation of [6-¹⁴C]orotate was substantially decreased in the presence of uracil. Chemical degradation to locate the ¹⁴C incorporated from labelled precursors showed that 90% of the radioactivity incorporated into lathyrine from [3-¹⁴C]serine could be recovered in the alanine side chain. Over 80% of the radioactivity incorporated from [2-¹⁴C]uracil was shown to be located in C-2 of lathyrine. It is concluded that under the conditions studied, lathyrine arises from a preformed pyrimidine arising via the orotate pathway. Paradoxically, it was also possible to confirm previous reports that radioactivity from L-[guanidino-¹⁴C]homoarginine is incorporated into lathyrine and γ -hydroxyhomoarginine. However, as homoarginine and γ -hydroxyhomoarginine are also both labelled by [2-¹⁴C]uracil, it is suggested that they are products of the ring-opening of lathyrine and that reversibility of this process accounts, at least in part, for their observed experimental incorporation into lathyrine.

Occurrence of an unusual pyrimidinyl amino acid in seeds of *Lathyrus tingitanus* (Tangier peas) was reported by Bell (1961), who named the compound lathyrine. Concurrently and independently, Nowacki & Przybylska (1961) isolated a compound with similar properties from *L. tingitanus* and from seeds of other species of *Lathyrus*. This compound, which they named tingitanine, was subsequently found to be identical with lathyrine and to have the structure β -(2-aminopyrimidin-4-yl)alanine (Bell & Foster, 1962). Bell (1962) found that lathyrine is a major constituent of the seeds of 12 species of *Lathyrus* and is present to a lesser extent in the seeds of five other species. Przybylska (1962) reported that lathyrine occurs also in stems, leaves, pods and unripe seeds of *L. tingitanus*.

Information on the biosynthesis and metabolism of lathyrine is scant, although, on the basis of the incorporation of radioactivity from [¹⁴C]homoarginine into lathyrine by *L. tingitanus*, it has been postulated by Bell (1963), Nowacki & Nowacka (1963) and Bell & Przybylska (1965) that the pyrimidinyl amino acid is formed by cyclization of γ -hydroxyhomoarginine as shown in Scheme 1. If confirmed, this would represent an unusual departure from use of the orotate pathway by which most pyrimidine constituents of plants arise, as in animals and micro-organisms. However, a recent observation (Brown, 1975) that radioactivity from [¹⁴C]orotate is significantly incorporated into lathyrine, suggests that either the hypothetical cyclization of γ -hydroxyhomoarginine does not occur and there is another

explanation for the incorporation of radioactivity from [¹⁴C]homoarginine into lathyrine, or the cyclization route functions in addition to the orotate pathway in producing lathyrine. The present work was undertaken in the context of a wider study of pyrimidine metabolism in plants and aimed to elucidate the biosynthetic route by which the pyrimidine ring of lathyrine arises.

Experimental

Materials

Seeds of *Lathyrus tingitanus* L., kindly supplied by Mr. R. Isherwood of University College of Swansea Botanic Garden, were germinated in moist vermiculite and grown at 25°C in an alternate cycle of 16 h light (6 klx)/8 h dark.

Scheme 1. Cyclization of γ -hydroxyhomoarginine in the formation of lathyrine as suggested by Bell (1963)

Analytical-grade chemicals were purchased from Fisons Scientific Ltd., Loughborough, Leicestershire, U.K., and BDH Ltd., Poole, Dorset, U.K. Radiochemicals were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Uracil, orotic acid, 2-amino-4-hydroxypyrimidine and 2-aminopyrimidine were purchased from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. Norit OL was supplied by Hopkin and Williams Ltd., Chadwell Heath, Essex, U.K., and prepared for use as described by Brown (1962). A sample of lathyrine was generously provided by Professor E. A. Bell, Department of Plant Sciences, King's College, London, U.K.

Uptake of suspected precursors

Aqueous solutions (pH 5.5) of compounds for examination were dispensed (0.1 ml portions) into Durham tubes. Excised shoots were allowed to take up these solutions (one shoot per tube); transpiration was stimulated by passing a slow current of air over the shoots at 25°C and illuminating at 6 klx. When the solution had been completely absorbed, 0.2 ml of water was added to each tube and this was also taken up. Finally, the cut ends of the shoots were placed in beakers of water and left under the same conditions of light and temperature for 24 h before extraction.

Extraction of lathyrine, homoarginine and γ -hydroxy-homoarginine

When lathyrine alone was required, the most satisfactory procedure for its extraction was found to be homogenization in ice-cold 0.3 M-HClO₄ (1 ml/g fresh wt. of seedlings) by using a chilled mortar and pestle. After removal of ClO₄⁻ by precipitation with KOH, the pH of the extract was adjusted to 3.5 with acetic acid. For each 20 g (fresh wt.) of material extracted, 1 g of Norit OL charcoal was added to the clear extract and stirred continuously for 2 h. The suspension was left to settle for 20 h at 4°C before being centrifuged at 5000g for 30 min. The charcoal was washed twice by resuspension in water and re-centrifuging. Lathyrine was eluted by suspending the charcoal for 2 h in ethanol (25%, v/v) containing 5% (v/v) NH₃ solution (sp.gr. 0.88). The suspension was left for 20 h at 4°C then the charcoal removed by centrifuging at 5000g for 30 min. The elution procedure was repeated three times and the eluates were combined.

For simultaneous extraction of lathyrine and other amino acids, tissues were homogenized in ice-cold 50% (v/v) ethanol (20 ml/g fresh wt.) in a chilled mortar and pestle. The homogenate was stirred continuously for 2 h, left for a further 20 h at 4°C, then centrifuged at 10000g for 20 min. The supernatant was retained and the residue extracted, as above, a

further three times with 50% (v/v) ethanol. The combined supernatants were evaporated to dryness in a rotary-film evaporator and the residue was taken for chromatographic and electrophoretic examination. In contrast with HClO₄, ethanol extraction caused some problem with interfering pigments.

Before dry seeds could be used in either of the above extraction procedures they were first milled to a fine powder in a C580 Microhammer Mill (Glen Creston Ltd., Stanmore, Middx., U.K.).

Chromatography and electrophoresis

Compounds of interest in the present investigation were separated as a routine from the extracts described above by a combination of paper chromatography and high-voltage paper electrophoresis. Chromatography was by the descending method on Whatman 3MM paper in the solvents: (i) butan-1-ol/acetic acid/water (12:3:5, by vol.); (ii) propan-2-ol/NH₃ solution (sp.gr. 0.88)/water (7:1:2, by vol.); (iii) propan-2-ol/conc. HCl/water (130:33:37, by vol.); (iv) ethanol/NH₃ solution (sp.gr. 0.88)/water (8:1:1, by vol.); (v) water. High-voltage paper electrophoresis was effected on Whatman 3MM paper by using an electrode potential of 3 kV (60 V/cm). For most purposes, a formic acid/acetic acid buffer (pH 2) was used. This was 0.8 M with respect to formic acid and 0.7 M with respect to acetic acid. In order of increasing migration under these conditions, homoarginine, lathyrine and γ -hydroxyhomoarginine moved in a close sequence. It was therefore found expedient first to remove lathyrine from extracts by adsorption on charcoal, as described above, before separating homoarginine and γ -hydroxyhomoarginine by electrophoresis. This gave good resolution of the latter two amino acids, and lathyrine could be removed from the charcoal by the elution procedure described above.

Measurement of radioactivity

Duplicate samples were examined for radioactivity as 0.5 ml aqueous solutions in 5 ml of a dioxan-based scintillant containing 2,5-diphenyl-oxazole, 1,4-bis(5-phenyloxazol-2-yl)benzene and naphthalene (NE 250; Nuclear Enterprises Ltd., Edinburgh, Scotland, U.K.). Each sample was counted twice for radioactivity, for 50 min, in a Beckman LS-355 liquid-scintillation spectrometer; quench correction was by the external-standard channels-ratio method. For measurement of specific radioactivity, total lathyrine was determined spectrophotometrically.

Degradation of lathyrine to locate ¹⁴C labelling

To distinguish between labelling in the pyrimidine ring and that in the alanine side chain, lathyrine was

hydrolysed with 6M-HCl for 24h at 150°C. The products were separated by paper chromatography and high-voltage electrophoresis and, by comparison with authentic samples under various conditions, identified as 2-amino-4-hydroxypyrimidine and alanine. Isolation of the guanidine carbon atom (C-2) of lathyrine involved ring opening and catalytic hydrogenation to yield homoarginine, followed by hydrolysis to lysine and urea. Acidification of the hydrolysate released CO₂ from the guanidine-derived urea (Nowacki & Nowacka, 1963). Ring opening and catalytic hydrogenation was effected by hydrogenating a 100mg sample of lathyrine dissolved in 200ml of water at 5.0MPa (50 atm) for 8h at 150°C; the catalyst was 40mg of RuO₂. The resulting homoarginine was refluxed with 33% (w/v) NaOH for 24h, and after cooling, the solution was acidified with 10% (v/v) H₂SO₄. The CO₂ evolved was passed into 5ml of phenethylamine and counted for radioactivity.

Results

Effect of uracil on lathyrine production

The amounts of lathyrine present in seeds and seedlings of *Lathyrus tingitanus* were determined at various stages during germination and growth. The results (control curve, Fig. 1) show a continuing synthesis during the 15-day period examined and indicate the suitability of young *Lathyrus* seedlings as experimental material for the biosynthetic study. Following an

Fig. 1. Effect of uracil on the lathyrine content of *Lathyrus* seedlings during growth

Imbibition, by seeds, of water (controls; ○) or 50mm-uracil solution (□) began at zero time. Batches of 25 seedlings were used for each assay. Further details are given in the Experimental section.

observation (Ashworth *et al.*, 1972) that uracil stimulates production of willardiine and isowillardiine, the pyrimidinyl amino acids of pea seedlings, it was examined for possible effect on the formation of lathyrine. Seeds of *L. tingitanus* were left to imbibe for 24h in an aerated 50mm solution of uracil at pH5.5; a similar, control, batch was simultaneously soaked in aerated water. After the seeds had germinated (see the Experimental section), batches of 25 treated seedlings and 25 control seedlings were harvested at intervals over a period of 15 days and analysed for their lathyrine contents. The results (Fig. 1) show that uracil has a stimulatory effect on lathyrine production.

Incorporation of ¹⁴C-labelled precursors

The observed effect of uracil (Fig. 1) implies that like the pyrimidinyl amino acids willardiine and isowillardiine (Ashworth *et al.*, 1972), lathyrine arises from a preformed pyrimidine ring. This implication was tested by supplying excised shoots of *Lathyrus* seedlings with ¹⁴C-labelled compounds considered to be possible precursors. By using the techniques described in the Experimental section, the lathyrine content of the treated seedlings was extracted and examined for radioactivity. Significant incorporation of the labelled pyrimidines [6-¹⁴C]orotate and [2-¹⁴C]uracil was found (Table 1). By analogy with observed origin of the side chain of willardiine and isowillardiine (Ashworth *et al.*, 1972), [3-¹⁴C]serine was also examined and found to give rise to significant labelling of lathyrine (Table 1).

Degradative procedures were developed to locate the positions in the lathyrine molecule of incorporated ¹⁴C. After acid hydrolysis of lathyrine (see the Experimental section), the products were separated by paper chromatography and high-voltage electrophoresis. Identification was made from chromatographic, electrophoretic, and where appropriate, spectrophotometric comparison with authentic samples. Apart from a small residual amount of unchanged lathyrine, the only u.v.-absorbing component of the hydrolysate was isocytosine (2-amino-4-hydroxypyrimidine). After hydrolysis, in this way, of labelled lathyrine extracted from shoots previously supplied with [3-¹⁴C]serine, 90% of the total radioactivity incorporated was recovered (Table 2) in the alanine side chain and none was detected in the pyrimidine ring of isocytosine.

To facilitate location of ¹⁴C incorporated from [2-¹⁴C]uracil into the pyrimidine ring of lathyrine, the latter was converted into homoarginine by catalytic hydrogenation and ring opening (see the Experimental section). The resulting [¹⁴C]homoarginine was then degraded to a mixture of lysine and urea as described in the Experimental section and the

Table 1. *Incorporation of ^{14}C -labelled precursors into lathyrine by Lathyrus seedlings*

Batches of 25 excised shoots were supplied with $2\mu\text{Ci}$ of the appropriate precursor and lathyrine extracted after 24 h. For further details see the Experimental section.

Precursor	Specific radioactivity (mCi/mmol)	Lathyrine	
		$10^{-3} \times \text{Total radioactivity (d.p.s.)}$	$10^{-1} \times \text{Sp. radioactivity (d.p.s./}\mu\text{mol)}$
[6- ^{14}C]Orotic acid	61.0	2282	4134
[2- ^{14}C]Uracil	59.0	3978	4974
[3- ^{14}C]Serine	55.7	168	1266

Table 2. *Location of ^{14}C incorporated into lathyrine from [3- ^{14}C]serine and [2- ^{14}C]uracil*
Details of degradation procedures are given in the Experimental section.

Precursor	Lathyrine isolated		Degradation product	
	$10^{-3} \times \text{Total radioactivity (d.p.s.)}$	$10^{-1} \times \text{Sp. radioactivity (d.p.s./}\mu\text{mol)}$	Name and origin	$10^{-3} \times \text{Total radioactivity (d.p.s.)}$
[3- ^{14}C]Serine	2678	1398	Alanine (C-1-C-2-C-3 of side chain)	2406
[2- ^{14}C]Uracil	40002	5034	CO_2 (C-2 of pyrimidine ring)	32070

solution cooled and acidified with 10% (v/v) H_2SO_4 . The CO_2 evolved, derived from the guanidine carbon atom (Nowacki & Nowacka, 1963), was passed into 5ml of phenethylamine and counted for radioactivity. It was found to contain about 80% of the total radioactivity incorporated into lathyrine (Table 2).

Effect of uracil on the incorporation of [6- ^{14}C]orotic acid into lathyrine

Table 1 shows that [2- ^{14}C]uracil is more efficiently incorporated into lathyrine than is [6- ^{14}C]orotate. To assess which of these two pyrimidines is the more direct precursor of lathyrine, an isotope-dilution experiment was carried out as follows. Two batches (25 each) of 8-day-old *Lathyrus* seedlings were selected and the excised shoots of one batch allowed to take up a solution containing $2\mu\text{Ci}$ of [6- ^{14}C]orotic acid (specific radioactivity 61 mCi/mmol). The other batch was supplied with $2\mu\text{Ci}$ of [6- ^{14}C]orotic acid of the same specific radioactivity dissolved in 2ml of 30mm-uracil. By using dil. HCl or dil. KOH as appropriate, the pH of each solution was adjusted to 5.5 before use. After 24h, the lathyrine content of each batch was extracted for examination. The results showed that under the conditions described [6- ^{14}C]orotate gave rise to lathyrine with a specific radioactivity of 13143 d.p.s./ μmol . In the presence of

30mm-uracil, this decreased to 6181 d.p.s./ μmol . That the decrease is attributable to dilution of the labelled precursor by passage through the relatively large pool of unlabelled uracil and not to inhibition of orotate utilization, was indicated by an analogous experiment in which the specific radioactivity of UMP was estimated. Incorporation of [6- ^{14}C]orotate ($2\mu\text{Ci}$) into UMP was not significantly decreased in the presence of 30mm unlabelled uracil (sp. radioactivity, 18262 d.p.s./ μmol as against 18045 d.p.s./ μmol in the presence of uracil).

Examination of possible intermediates in lathyrine biosynthesis

It was considered that either isocytosine (2-amino-4-hydroxypyrimidine) or 2-aminopyrimidine may be an intermediate in lathyrine biosynthesis. To test this, three batches (25 each) of shoots were excised from 8-day-old *Lathyrus* seedlings and one batch was allowed to take up a solution containing $2\mu\text{Ci}$ of [2- ^{14}C]uracil (sp. radioactivity 59 mCi/nmol). The second and third batches were supplied with solutions containing $2\mu\text{Ci}$ of [2- ^{14}C]uracil of the same specific radioactivity but dissolved in 30mm-isocytosine and 30mm-2-aminopyrimidine respectively. All the solutions were adjusted to pH 5.5 before use. After 24h, the lathyrine content of each batch was extracted and

Table 3. Incorporation of ^{14}C -labelled precursors into lathyrine, homoarginine and γ -hydroxyhomoarginine. Batches (25 each) of excised *Lathyrus* shoots were supplied with $2\mu\text{Ci}$ of the respective precursors. The three amino acids were extracted after 24h as described in the Experimental section.

Precursor	$10^{-3} \times \text{Total radioactivity (d.p.s.)}$		
	Lathyrine	Homoarginine	γ -Hydroxyhomoarginine
[2- ^{14}C]Uracil	4128	181	42
L-[guanidino- ^{14}C]Homoarginine	166	27330	30

its specific radioactivity determined. The incorporation of radioactivity from [2- ^{14}C]uracil into lathyrine was not, however, significantly diluted by either isocytosine or 2-aminopyrimidine.

Incorporation of [2- ^{14}C]uracil and [6- ^{14}C]orotate into lathyrine (Table 1) and dilution by uracil of incorporation of [6- ^{14}C]orotate into lathyrine contrast with the hypothesis of Bell & Przybylska (1965). The latter authors describe the incorporation of [^{14}C]homoarginine into lathyrine through the intermediate formation of γ -hydroxyhomoarginine. In the present study, their findings were re-examined. L-[guanidino- ^{14}C]Homoarginine was prepared from L-[2- ^{14}C]lathyrine by catalytic hydrogenation and ring-opening (see the Experimental section), then purified by paper chromatography and high-voltage electrophoresis. It was supplied to batches of excised shoots, or through the roots, to intact seedlings of *L. tingitanus*. After 24h, lathyrine, homoarginine and γ -hydroxyhomoarginine were extracted and shown to be radioactively labelled (Table 3). The same three compounds were also found to be labelled in similar experiments in which [2- ^{14}C]uracil replaced L-[guanidino- ^{14}C]homoarginine (Table 3).

Metabolism of [2- ^{14}C]lathyrine by *L. tingitanus*

When [2- ^{14}C]lathyrine, prepared biosynthetically from [2- ^{14}C]uracil, was supplied either to excised shoots taken from 8-day-old seedlings or, through the roots, to intact seedlings, no radioactive products could be detected after paper chromatography of ethanolic (50%, v/v) extracts. The only radioactive spot on each chromatogram was that of unmetabolized [2- ^{14}C]lathyrine. Since, however, it had already been shown that [2- ^{14}C]uracil gives rise both to [^{14}C]homoarginine and [^{14}C]lathyrine in these seedlings, it was considered likely that the failure of exogenously supplied [2- ^{14}C]lathyrine to be metabolized in this experiment was due to its inability to penetrate to the relevant metabolic site.

Discussion

Fig. 1 shows that seedlings of *Lathyrus tingitanus* synthesize lathyrine by a mechanism that is stimulated by uracil. This finding, coupled with the observed incorporation of [6- ^{14}C]orotate and of [2- ^{14}C]uracil into lathyrine (Table 1), indicate that the pyrimidine

moiety of this pyrimidinyl amino acid is formed from uracil or a closely related compound arising via the orotate pathway. The operation of the latter in higher plants has not only been amply demonstrated [see, e.g., Reifer *et al.* (1960); Buchowicz & Reifer (1961, 1962); Ross *et al.* (1971); Ross & Murray (1971)], but is also known to be involved in the formation of other pyrimidinyl amino acids (Ashworth *et al.*, 1972) and in the biosynthesis of the unusual pyrimidine glucosides vicine and convicine (Brown & Roberts, 1972). Indications that the biosynthesis of lathyrine occurs via the orotate pathway would not therefore be unexpected, except for the reports of Bell (1963), Nowacki & Nowacka (1963) and Bell & Przybylska (1965) suggesting that lathyrine is formed by cyclization of γ -hydroxyhomoarginine arising from homoarginine.

That a pyrimidine formed through the orotate pathway is the direct precursor of lathyrine was confirmed by demonstrating (a) that [2- ^{14}C]uracil specifically labels C-2 of lathyrine (Table 2) and (b) that the incorporation of radioactivity from [6- ^{14}C]orotate into lathyrine is significantly decreased in the presence of uracil whereas that into UMP remains unaffected. The greater extent of incorporation into lathyrine of [2- ^{14}C]uracil than was obtained with [6- ^{14}C]orotate (Table 1) suggests that uracil is a more direct precursor than is orotate. This is also supported by the observed decrease by uracil of the specific radioactivity of lathyrine formed from [6- ^{14}C]orotate. The immediate pyrimidine precursor of lathyrine is not known. Certainly, isocytosine and 2-aminopyrimidine cause no significant decrease of the specific radioactivity of lathyrine formed from [2- ^{14}C]uracil, although this may be due to these compounds failing to penetrate to the biosynthetic site. The mechanism by which the alanine side chain is attached to the pyrimidine precursor is at present obscure, but results (Table 2) indicate that it originates from serine or a close derivative of serine. Analogy with the biosynthesis of other heterocyclic β -substituted alanines of plant origin suggests that the side-chain precursor is *O*-acetylserine (Murakoshi *et al.*, 1972; Ashworth *et al.*, 1972).

Although the results presented show that lathyrine is biosynthesized from a preformed pyrimidine ring originating from the orotate pathway, paradoxically it was also possible to confirm an earlier observation

Orotate $\rightarrow$ uracil $\rightarrow$ lathyrine $\rightleftharpoons$ 5,6-dihydrolathyrine $\rightleftharpoons$ γ -hydroxyhomoarginine $\rightleftharpoons$ homoarginine

Scheme 2. *Postulated relationship between orotate, lathyrine and homoarginine*

by Bell (1963), Rao *et al.* (1963) and Bell & Przybylska (1965) that the ^{14}C label from L-[guanidino- ^{14}C]homoarginine is incorporated into lathyrine (Table 3). These observations could be interpreted in terms of two independent biosynthetic routes to lathyrine. However, incorporation of [^{14}C]uracil not only into lathyrine but additionally into homoarginine and γ -hydroxyhomoarginine reveals a relationship between the two routes, the simplest explanation of which is shown in Scheme 2. In this Scheme, the steps from lathyrine to γ -hydroxyhomoarginine are analogous to the known ring-opening of uracil, via 5,6-dihydrouracil to carbamoyl- β -alanine, the reversibility of which has been demonstrated in animal tissues (Fritzson, 1957; Wallach & Grisolia, 1957) and in wheat seedlings (Buchowicz *et al.*, 1963). Scheme 2 does not exclude the postulated intermediary role of γ -oxohomoarginine between γ -hydroxyhomoarginine and lathyrine (Hider & John, 1973).

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