Biosynthesis of Willardiine and Isowillardiine in Germinating Pea Seeds and Seedlings

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The synthesis of the pyrimidinyl amino acids willardiine and isowillardiine was studied *in vivo* and *in vitro*. Uracil derivatives stimulate the biosynthesis of both compounds; the free base is the most effective. Significant incorporation of [2-¹⁴C]uracil and [6-¹⁴C]orotate into willardiine and isowillardiine was found. Incorporation of [6-¹⁴C]orotate was substantially decreased in the presence of uracil, and to a lesser extent by uridine and UMP. [3-¹⁴C]serine was incorporated into the alanine side chain of the two uracilylalanines but not into the ring. The effect of a number of uracil analogues and inhibitors of pyrimidine metabolism was examined. Some were shown to stimulate the biosynthesis; the most noticeable effects were obtained with 6-azauracil and 2-thiouracil. Attempts to obtain extracts capable of synthesizing the uracilylalanines from uracil and serine were unsuccessful, but weak activity was observed when serine was replaced by *O*-acetylserine.

Two isomeric uracilylalanines have been found in plants. The first of these was isolated from seeds of *Acacia willardiana* by Gmelin (1959) and given the name willardiine. It was later reported to occur in other species of *Acacia* and in *Mimosa asperata* (Gmelin, 1961). Structurally, willardiine (I) is β -(2,4-dihydroxypyrimidin-1-yl)alanine (Gmelin, 1959); identification was confirmed by synthesis by Kjaer *et al.* (1961), Shaw & Dewar (1961) and Dewar & Shaw (1962). The occurrence of a second uracilylalanine was recognized by Brown & Silver (1966), who isolated it from pea seedlings and tentatively identified it as β -(2,4-dihydroxypyrimidin-5-yl)alanine. Subsequent n.m.r. and mass-spectrometric studies (Brown & Mangat, 1969) necessitated revision of this structure, reassigning the alanine side chain to N-3 of the pyrimidine ring to give β -(2,4-dihydroxypyrimidin-3-yl)alanine (II). Concurrently but independently, Lambein & Van Parijs (1968) deduced formula (II) for a compound they had also isolated from pea seedlings. Although the substance was itself unknown at the time, structure (II) had been earlier described as isowillardiine (Shvachkin & Azarova, 1964).

Two initial problems are posed by the biosynthesis of willardiine and isowillardiine: (a) whether these compounds are produced from a preformed heterocyclic nucleus or by cyclization of an aliphatic precursor, and (b) whether one uracilylalanine arises from the other. Whereas analogy with the formation of other heterocyclic β -substituted alanines, such as tryptophan, indicated utilization of a preformed ring system, the work of Bell (1962, 1963) and Rao *et al.* (1963) suggested that lathyrine, another pyrimidinylalanine, arises from the cyclization of γ -hydroxy-homoarginine. The occurrence of γ -hydroxyhomoarginine together with lathyrine in *Lathyrus tingitanus* (Bell, 1964) supports this view.

Experimental

Materials

Seeds of *Pisum sativum* L. var. Meteor from a single batch supplied by Carter's Seeds Ltd., London S.W.20, U.K., were used throughout the work. After brief treatment with 0.1% (v/v) Stergene detergent to ensure wetting, seeds were surface-sterilized by immersion for 5 min in 0.1% (w/v) HgCl₂. After being well washed in several changes of sterile water, they were soaked aseptically with aeration for 16 h in either sterile water or a test solution that had been sterilized by filtration. Seeds were germinated at 25°C in washed, sterilized vermiculite and allowed to grow under a lighting regime of alternating periods of light (4000 lx; 18 h) and dark (6 h).

[2-¹⁴C]Uracil, [6-¹⁴C]orotic acid, [U-¹⁴C]aspartic acid and [3-¹⁴C]serine were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

(I) Willardiine

(II) Isowillardiine

Terbacil (5-chloro-6-methyl-3-t-butyluracil) was a gift from the Du Pont Co. (U.K.) Ltd., London E.C.4, U.K. It was recrystallized twice from acetone-water before use. Alloxan and barbituric acid were obtained from British Drug Houses Ltd., Poole, Dorset, U.K., 6-azauracil and 6-azauridine from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K., 2-thiouracil from Sigma Chemical Co., St. Louis, Mo., U.S.A., and maleimide from Fluka A.G. Buchs, Switzerland.

Methods

Extraction and determination of willardiine and isowillardiine. By using a chilled mortar and pestle, weighed batches of freshly harvested seedlings were extracted with ice-cold HClO_4 (0.3M). Approx. 1 ml of HClO_4 was used/g fresh wt. of tissue. Dry seeds were milled in a C580 microhammer mill (Glen Creston Ltd., Stanmore, Middx., U.K.) and then homogenized in ice-cold 0.3M- HClO_4 (10ml/g of powdered seed). Homogenates obtained from either seeds or seedlings were centrifuged at 12000g for 20min at 4°C and the supernatants decanted. Residues were re-extracted twice more in a similar way and the combined supernatants were largely freed from ClO_4^- by precipitation at 4°C with KOH followed by centrifuging at 4000g for 15min. After the pH of the supernatant had been adjusted to pH 3.5 with acetic acid, the supernatant was subjected to a preliminary purification by adsorption on to charcoal and subsequent elution (Brown & Silver, 1966).

The eluate from the charcoal column was fractionated by cation-exchange chromatography on a column (25cm × 2cm diam.) of Dowex 50W (X8; H^+ form; 200–400 mesh). Elution was at a rate of 40ml/h, with a linear gradient of HCl (0–2M) and a 1-litre mixing chamber; 10ml fractions were collected. Three contiguous peaks were recorded (fractions 65–71, 71–78 and 78–86). By using the chromatographic, electrophoretic and spectrophotometric methods described previously (Brown & Silver, 1966; Brown & Mangat, 1969), comparisons with authentic samples showed that peak (1) consisted largely of willardiine, peak (2) of isowillardiine and peak (3) of β -(isoxazolin-5-oxo-2-yl)alanine (Lambein *et al.*, 1969). For routine extraction of willardiine and isowillardiine, peaks (1) and (2) were separately evaporated *in vacuo* and sequentially chromatographed on Whatman no. 1 paper in (1) butan-1-ol–acetic acid–water (12:3:5, by vol.), (2) propan-2-ol–ammonia (sp.gr. 0.88)–water (7:1:2, by vol.), and (3) propan-2-ol–conc. HCl–water (130:33:37, by vol.). Willardiine and isowillardiine, separated in this way, were shown to be chromatographically homogeneous in three further solvent systems, i.e. (4) 2-methylpropionic acid–ammonia (sp.gr. 0.88)–water (33:1:66, by vol.), (5) water and

(6) ethanol–1M-ammonium acetate solution, pH 7.6 (7:3, v/v). Homogeneity of chromatographic bands was examined by using both u.v. light and 0.2% (w/v) ninhydrin in acetone. Recovery experiments showed that, from extraction to determination, there was a 12% loss of willardiine and a 11% loss of isowillardiine; both losses were reproducible. These losses are mainly attributable to the charcoal-adsorption and elution steps.

For preparative-scale separations, the method was essentially similar but a larger cation-exchange column (90cm × 4cm diam.) was used.

After purification, the uracilyl amino acids were determined spectrophotometrically; the ϵ_{max} values used were those given by Shvachkin & Azarova (1964) and Lambein & Van Parijs (1968) for willardiine and isowillardiine respectively.

Extraction of β -(isoxazolin-5-oxo-2-yl)alanine. By using the same cation-exchange separation procedure as that described above for willardiine and isowillardiine, peak (3) material was collected from seedling extracts and evaporated to dryness. The residue was redissolved in water and chromatographed on Whatman no. 1 paper in solvent (1). The main band was eluted and rechromatographed on paper in solvent (3). The single band of β -(isoxazolin-5-oxo-2-yl)alanine so obtained was eluted in water. High-voltage paper electrophoresis for 30min in formic acid–acetic acid buffer (1.5M; pH 2), with a voltage gradient of 120V/cm, gave a single band as judged by viewing in u.v. light and after treatment with 0.2% (w/v) ninhydrin in acetone.

Examination for albizziin. Seedlings (10g fresh wt.) were immersed for 10min in boiling 80% (v/v) ethanol (50ml) and then homogenized in the same solution. After filtration through a Millipore membrane filter (0.45 μm pore size), the residue was re-extracted twice with 50ml portions of boiling 80% ethanol. The combined extracts were evaporated to dryness *in vacuo*, redissolved in 2.5M-HCl and kept at 100°C for 3h. This ensured hydrolysis of asparagine, which otherwise runs with, and masks, albizziin (L-2-amino-3-ureidopropionic acid). Two-dimensional paper chromatography, in solvent (1) as the first system and aq. 80% (v/v) phenol as the second, showed no detectable trace of albizziin although a reference spot of this substance clearly separated from a similarly treated extract to which it had been added. Spots were detected with 0.2% (w/v) ninhydrin in acetone.

Enzymic formation of isowillardiine from O-acetylserine and [2- ^{14}C]uracil. A concentrated extract of 5-day pea seedlings was prepared by grinding them together with a mixture of solid K_2HPO_4 and KH_2PO_4 in a chilled mortar and pestle. The amounts of K_2HPO_4 and KH_2PO_4 were calculated to give a final concentration of 0.1M and a pH of 7 when dissolved in the expressed juice. The homogenate was

centrifuged at 4000g for 20 min at 4°C and the supernatant was used directly as a source of enzyme.

The incubation mixture (final volume 1.5 ml) consisted of *O*-acetylserine (1 mM), uracil (1 mM) containing [2-¹⁴C]uracil (1 μCi), ATP (1 mM), CoA (0.1 mM), pyridoxal 5-phosphate (0.1 mM), phosphate buffer (0.02 M; pH 7.6), and 1 ml of the enzyme extract. Where *O*-acetylserine was replaced by serine, the same final concentration was used.

Measurement of radioactivity. All samples were counted for radioactivity as 0.5 ml aqueous solutions in 5 ml portions of a commercial dioxan-based scintillant containing 2,5-diphenyloxazole, 1,4-bis-(5-phenyloxazol-2-yl)benzene and naphthalene (NE250; Nuclear Enterprises Ltd., Edinburgh, U.K.). A Beckman LS200B automatic liquid-scintillation counter was used; under the specified conditions, the efficiency was 65%. Each sample was counted to a pre-set error of 1%.

Results

Uracilylalanine synthesis in relation to germination and growth

Pea seedlings were examined at various stages of growth for their contents of willardiine and isowillardiine. The results, shown in Fig. 1, indicate a continuing synthesis throughout the early development of the plant. No detectable amount of willardiine or isowillardiine was, however, found in mature dry pea seeds before germination, nor in the shoots and immature pods of 5-week-old plants; the shoots, pods and fully developed seeds of 10-week-old plants appeared similarly devoid of these substances.

The effect of light on production of willardiine and isowillardiine was examined by germinating and growing separate batches of pea seedlings in moist vermiculite under different light regimes. These were complete darkness, continuous light (4000 lx), and an alternating light/dark cycle of 18 h of light (4000 lx) followed by 6 h of darkness. No significant differences were observed in the willardiine and isowillardiine content of these batches.

Effect of uracil and related compounds on formation of willardiine and isowillardiine

Previous work with pea seedlings (Brown & Silver, 1966; Silver & Gilmore, 1969) had indicated that GMP stimulated the production of a uracilylalanine, now known to be isowillardiine (Brown & Mangat, 1969), but this could not be reproduced in the present studies and is believed to have been attributable to a generalized response by seeds suffering from a nutritional deficiency. A contemporary batch of pea seeds from the previous source were found to exhibit 'marsh spot' (a Mn²⁺-deficiency resulting in atro-

Fig. 1. Willardiine (●) and isowillardiine (○) content of pea seedlings during growth

Imbibition by seeds began at zero-time. Batches of 20 seedlings were used for each assay. For further details see the text.

phied cotyledons). Attention was turned to the pyrimidines as possible direct precursors of the uracilylalanines. The compounds examined were orotate, uracil, uridine and UMP; each was separately tested for effect on production of willardiine and isowillardiine. This was done by allowing dry pea seeds to imbibe a neutral solution of the pyrimidine concerned (see the Experimental section) and then allowing the seeds to germinate. After 136 h, seedlings were harvested and their contents of the uracilylalanines determined (Table 1). Uracil consistently stimulated production of both willardiine and isowillardiine; uridine and UMP had similar but lesser effects. Orotate gave a slight but reproducible stimulation although, owing to limited solubility, it was necessarily examined at a concentration much lower than that of the other pyrimidines (Table 1). Further examination of the marked effect of uracil showed it to persist for at least 14 days after treatment (Fig. 2) and to increase with the concentration of uracil used (Fig. 3).

Incorporation of ¹⁴C-labelled precursors

By using the same imbibition technique as before, [2-¹⁴C]uracil was supplied to germinating seeds and the willardiine and isowillardiine were isolated on day 6. Both compounds were found to contain significant radioactivity (Table 2). Similarly, [6-¹⁴C]-orotate and, to a much smaller extent, [U-¹⁴C]-aspartate were incorporated (Table 2).

Table 1. *Effect of uracil and some related pyrimidines on the synthesis of willardiine and isowillardiine by pea seedlings*

At zero-time seeds were immersed in water (controls) or in test solution (30 mM; pH 5.8) and after 16 h were transferred to moist vermiculite. Because of limited solubility, orotate was used at 8 mM. At 136 h, seedlings were harvested and their contents of willardiine and isowillardiine assayed. Results are the means of triplicate analyses. Batches of 20 seeds were used for each replicate treatment and each replicate control.

Pyrimidine	Willardiine (nmol/seedling)		Isowillardiine (nmol/seedling)	
	Control	Treated	Control	Treated
Uracil	5	203	36	177
Uridine	5	37	39	89
UMP	5	53	36	95
Orotate	4	7	44	56

Fig. 2. *Stimulatory effect of uracil on synthesis of (a) willardiine and (b) isowillardiine by pea seedlings*

(a) Willardiine synthesis: ●, willardiine content of control seedlings derived from seeds imbibed 0–16 h in water; ○, willardiine content of treated seedlings derived from seeds similarly imbibed in 30 mM-uracil. Each analysis was on a sample of 20 seedlings. (b) Isowillardiine synthesis: ●, isowillardiine content of control seedlings, derived from seeds imbibed 0–16 h in water; ○, isowillardiine content of seedlings derived from seeds similarly imbibed in 30 mM-uracil. For further details see the text.

Incorporation of radioactivity from [6-¹⁴C]orotate into willardiine and isowillardiine was substantially diluted in the presence of unlabelled uracil, uridine, or UMP (Table 3). The biggest dilution was that observed with uracil, followed in order of decreasing magnitude by that with uridine and UMP.

By analogy with the synthesis of other β-substituted alanines in plants (see, e.g., Fowden, 1970), serine was considered to be a likely precursor of the alanine moiety of willardiine and isowillardiine. Experiments showed a significant incorporation of

radioactivity from [3-¹⁴C]serine into the two pyrimidine amino acids (Table 2), although the extent of incorporation was much less than that obtained with the ¹⁴C-labelled pyrimidines. However, this may be primarily attributable to the relatively large pool of unlabelled serine in pea seedlings (Larson & Beevers, 1965). To confirm that the label from [3-¹⁴C]serine had been incorporated into the side chain rather than into the pyrimidine ring of willardiine and isowillardiine, radioactive samples of the two latter compounds were isolated and separately hydrolysed

in 6M-HCl at 150°C for 24h in sealed tubes. The liberated uracil was separated from the hydrolysate by paper chromatography in solvent (1) and further chromatographed sequentially in several different solvent systems until chromatographically homogeneous. Examination showed it to be devoid of radioactivity.

Fig. 3. Effect of exogenous uracil concentration on willardiine and isowillardiine synthesis by pea seedlings

○, Isowillardiine content of seedlings derived from seeds imbibed in uracil solutions of various concentrations; seedlings were extracted at 136h; ●, willardiine content of similarly derived pea seedlings. Batches of 20 seedlings were used for each analysis. For further details see the text.

Biosynthetic interrelationship of willardiine and isowillardiine

The possibility that willardiine is the precursor of isowillardiine or vice versa was examined by a 'pulse-chase' experiment. For this, the cut ends of shoots excised from 8-day pea seedlings were placed in a solution of uracil (5mM) and serine (5mM) and allowed to take up this solution for 2h in light (6000lx). The shoots were then rapidly transferred to a solution of the same concentration but which also contained [2-¹⁴C]uracil (5μCi/ml). After 30min, they were removed, lightly blotted with tissue paper and replaced in the original non-radioactive solution. At intervals over the next 20h, batches of ten shoots were taken from the latter solution and their contents of willardiine and isowillardiine were extracted for assay and radioactivity counting. The results (Fig. 4) show that [2-¹⁴C]uracil is incorporated simultaneously into the two compounds and that the incorporated radioactivity is not lost from either when the radioactive precursor is replaced by non-radioactive uracil. The indications that neither willardiine nor isowillardiine is the precursor of the other and that both are relatively inert metabolically were confirmed in a second experiment. This involved supplying ¹⁴C-labelled willardiine and isowillardiine (prepared biosynthetically from [2-¹⁴C]uracil) to separate batches of excised pea shoots and, after 8h, preparing extracts of these shoots for chromatography. Paper chromatography in solvent (1), followed by radioautography, revealed a single radioactive band in each extract. These were eluted and separately subjected to high-voltage paper electrophoresis (1.5M-formic acid-acetic acid buffer, pH2; 120V/cm); radioautograms were again prepared and showed that the sole band obtained from the seedlings that had been supplied with willardiine was unchanged willardiine.

Table 2. Incorporation of ¹⁴C-labelled precursors into willardiine and isowillardiine

For each substance examined, 20 pea seeds were allowed to imbibe in 5ml of a solution containing 10μCi of the precursor. Approx. 4.7ml of this was taken up by the seeds. Seedlings grown from these seeds were extracted at 136h (imbibition began at zero-time).

Precursor	Specific radioactivity (mCi/mmol)	Willardiine		Isowillardiine	
		Total radioactivity (d.p.m.)	Specific radioactivity (d.p.m./μmol)	Total radioactivity (d.p.m.)	Specific radioactivity (d.p.m./μmol)
[U- ¹⁴ C]Aspartate	208.0	252	3605	2021	2926
[6- ¹⁴ C]Orotate	60.8	25 649	400 769	562 869	416 630
		35 520	486 584	196 709	323 529
[2- ¹⁴ C]Uracil	52.5	38 749	232 030	218 807	207 597
		25 040	281 365	243 726	296 866
[3- ¹⁴ C]Serine	48.0	1800	27 137	13 325	23 458
		1721	23 909	17 386	20 745

Table 3. *Effect of uracil, uridine and UMP on the incorporation of [6-¹⁴C]orotate into willardiine and isowillardiine*

[6-¹⁴C]Orotate (10 μ Ci) in 5 ml of water or 30 mM-pyrimidine solution was used in each treatment; batches of 20 seeds were separately imbibed and grown for 136 h before extraction.

Treatment	Specific radioactivity (d.p.m./ μ mol)	
	Willardiine	Isowillardiine
[6- ¹⁴ C]Orotate	486 584	416 631
	400 769	323 529
[6- ¹⁴ C]Orotate+uracil	74 980	84 635
	60 114	48 206
[6- ¹⁴ C]Orotate+uridine	171 169	133 989
	160 569	106 340
[6- ¹⁴ C]Orotate+UMP	219 005	150 435
	169 652	111 292

Fig. 4. *Incorporation of a 'pulse' of radioactivity from [2-¹⁴C]uracil into willardiine and isowillardiine by excised pea shoots*

○, Isowillardiine; ●, willardiine. Shoots were fed with [2-¹⁴C]uracil for 30 min (see the text for further details) and then transferred to non-radioactive uracil solution (zero-time). Each analysis is of a batch of 10 shoots.

Similarly, the only band obtained from the seedlings that had been supplied with isowillardiine was unmetabolized isowillardiine.

Biogenesis of β -(isoxazolin-5-oxo-2-yl)alanine

During cation-exchange chromatography of an extract from pea seedlings, Lambein *et al.* (1969) identified a peak, contiguous with that of isowillardiine, as β -(isoxazolin-5-oxo-2-yl)alanine. The simul-

taneous appearance of this compound with the uracilylalanines during germination led these authors to suggest a possible biogenetic relationship. Since uracil derivatives readily give rise to isoxazoles on treatment with hydroxylamine (see, e.g., Kochetkov & Budowsky, 1969), the possibility was considered in the present work that β -(isoxazolin-5-oxo-2-yl)alanine, like willardiine and isowillardiine, could be derived biochemically from uracil. However, no significant incorporation of radioactivity from [6-¹⁴C]orotate or from [2-¹⁴C]uracil into the

isoxazoline could be detected. Feeding cut shoots with ^{14}C -labelled willardiine or isowillardiine, as described above, also failed to label the isoxazoline.

Effects of pyrimidine analogues and inhibitors

Several pyrimidine analogues and known inhibitors of pyrimidine metabolism were examined for their effects on the production of isowillardiine. Seeds were allowed to imbibe aqueous solutions of the substances concerned and set to germinate. After 6 days, the isowillardiine contents of the seedlings were determined and compared with the values obtained from control seedlings; the results are shown in Table 4. Stimulation of isowillardiine synthesis was observed with 6-azauracil (59% above controls) and 2-thiouracil (44% above controls). Smaller stimulations were produced by maleimide and alloxan. Terbacil (5-chloro-6-methyl-3-t-butyluracil) gave a slight inhibition. None of the analogues and inhibitors tested had significant effect on the growth of the seedlings.

Enzyme extracts

Extracts prepared from pea seedlings or cucumber seedlings, either by the method of Dunnill & Fowden (1963) or that described by Nair & Vaidyanathan (1964), proved incapable of detectable synthesis of willardiine or isowillardiine from $[2-^{14}\text{C}]$ uracil and serine. However, replacing serine with *O*-acetylserine led to a small but reproducibly significant amount of isowillardiine synthesis (Table 5); insufficient willardiine was present for examination.

Discussion

The work of Bell (1963) and Rao *et al.* (1963) indicated that lathyrine (2-aminopyrimidin-4-ylalanine) arises from the cyclization of γ -hydroxyhomo-arginine, and not through the orotate pathway of pyrimidine biosynthesis. Similarly, following synthetic studies and considering the concomitant occurrence of albizziin and willardiine in several species of *Acacia* and *Mimosa*, Shvachkin & Azarova (1964)

Table 4. *Effects of inhibitors of pyrimidine metabolism and pyrimidine analogues on isowillardiine synthesis in pea seedlings*

For each substance examined, except terbacil, 20 pea seeds were allowed to imbibe for 16 h in a 1 mM solution of the compound. Terbacil was examined at a concentration of 0.2 mM. After imbibition, seeds were germinated and grown for 6 days before analysis. Each control represents a separate batch of 20 seeds imbibed in water.

Analogue or inhibitor	Fresh wt. per batch (20 seedlings)		Isowillardiine (nmol/g fresh wt.)	
	Control	Treated	Control	Treated
6-Azauridine	17.4	15.8	43	49
6-Azauracil	13.7	14.2	42	67
Alloxan	17.1	17.2	34	44
Barbituric acid	18.3	17.7	30	27
Maleimide	17.5	19.3	40	57
Terbacil	17.8	17.1	50	40
2-Thiouracil	15.2	15.8	36	52

Table 5. *Effect of O-acetylserine on the incorporation of $[2-^{14}\text{C}]$ uracil into isowillardiine by an extract from pea seedlings*

Each incubation mixture (total volume 1.5 ml) contained $1\mu\text{Ci}$ of $[2-^{14}\text{C}]$ uracil. For further details see the Experimental section.

	Isowillardiine	
	Total radioactivity (d.p.m.)	Specific radioactivity (d.p.m./ μmol)
Extract + $[2-^{14}\text{C}]$ uracil	nil	nil
Extract + $[2-^{14}\text{C}]$ uracil + serine	nil	nil
Extract + $[2-^{14}\text{C}]$ uracil + <i>O</i> -acetylserine	309	3092

have suggested that willardiine arises from albizziin (L-2-amino-3-ureidopropionic acid) after ring closure with a malonate-derived unit. Present studies, however, failed to detect albizziin in pea seedlings and show that both willardiine and isowillardiine arise from a preformed pyrimidine ring derived from the orotate pathway.

That the stimulatory effects of uracil and its derivatives (Table 1) were due to direct incorporation of the pyrimidine ring into willardiine and isowillardiine is indicated by the results in Table 2. Incorporation of radioactivity from [6-¹⁴C]orotate into willardiine and isowillardiine could be the result of orotate degradation to β -[3-¹⁴C]alanine and subsequent synthesis of pyrimidines from this by the scheme suggested by Buchowicz *et al.* (1963). This is, however, unlikely since [2-¹⁴C]uracil is similarly incorporated yet would yield ¹⁴CO₂ if degraded in the same way, and, although ¹⁴CO₂ is a precursor of pyrimidines, its specific radioactivity would be considerably lowered by dilution before being re-incorporated into pyrimidines.

The results in Table 2 suggest that either orotate is a more immediate precursor than uracil or that it penetrates to the site of synthesis more easily. The first possibility is eliminated, however, by the observation that uracil substantially lessens the incorporation of orotate into willardiine and isowillardiine (Table 3). Further, UMP and uridine, although effective in this respect too, produce much less dilution than uracil, as would be expected from the sequence of the orotate pathway, which is known to operate in higher plants (Reifer *et al.*, 1960; Buchowicz *et al.*, 1961; Buchowicz & Reifer, 1961, 1962). The dilution results do not, however, exclude the direct decarboxylation of orotate to uracil, reported to occur in wheat seedlings (Buchowicz & Reifer, 1961, 1962; Buchowicz & Lesniewska, 1970). On the contrary, the effect of 6-azauracil on isowillardiine synthesis (Table 4) strongly supports the existence of direct decarboxylation of orotate, for 6-azauracil is a potent inhibitor of orotidine 5'-monophosphate decarboxylase (Handschumacher & Pasternak, 1958; Pasternak & Handschumacher, 1959) yet it stimulates production of isowillardiine. This effect is attributable to the shunting, by direct decarboxylation, of orotate from pyrimidine nucleotide production into the formation of uracil, which is the limiting factor in isowillardiine synthesis *in vivo* (Figs. 2 & 3). As 6-azauracil and 6-azauridine exert their effects on orotidine 5'-monophosphate decarboxylase only after conversion into the corresponding 5'-nucleotide (Handschumacher, 1960), the observed lesser effect of 6-azauridine suggests that uracil phosphoribosyltransferase is more active under the conditions described than is uridine kinase. The effect may, however, be due to selective permeability. As 2-thiouracil is known to block uracil degradation (Newmark *et al.*, 1962), the stimulatory effect of

2-thiouracil on isowillardiine biosynthesis (Table 4) is also ascribable to increased availability of uracil. Maleimide and alloxan may also exert their stimulatory effects (Table 4) in a similar way.

Consideration of the biosynthesis of tryptophan (Mudd & Zalik, 1958; Greenberg & Galston, 1959), of β -pyrazol-1-ylalanine (Dunnill & Fowden, 1963), and of other heterocyclic β -substituted alanines (see, e.g., Fowden, 1967), suggested that the side chain of the uracilylalanines arises from serine or a close derivative. This is confirmed by the present findings that [3-¹⁴C]serine contributes to the side chain but not to the ring system of both willardiine and isowillardiine.

Failure to detect synthesis of willardiine and isowillardiine in variously prepared enzyme extracts or in the non-enzymic pyridoxal 5-phosphate systems of Metzler *et al.* (1953), suggests that serine or uracil, or both of them, are not the immediate substrates of the condensing enzyme but must undergo preliminary modification. That serine is required to be *O*-acetylated first, indicated by the work of Murakoshi *et al.* (1972), is supported by the results in Table 5. Present findings do not exclude the possibility that uracil may, too, require structural modification before condensation.

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References

- Bell, E. A. (1962) *Biochem. J.* **85**, 91–93
- Bell, E. A. (1963) *Nature (London)* **199**, 70–71
- Bell, E. A. (1964) *Biochem. J.* **91**, 358–361
- Brown, E. G. & Mangat, B. S. (1969) *Biochim. Biophys. Acta* **177**, 427–433
- Brown, E. G. & Silver, A. V. (1966) *Biochem. Biophys. Acta* **199**, 1–10
- Buchowicz, J. & Lesniewska, A. (1970) *Phytochemistry* **9**, 1083–1085
- Buchowicz, J. & Reifer, I. (1961) *Acta Biochim. Pol.* **8**, 25–34
- Buchowicz, J. & Reifer, I. (1962) *Acta Biochim. Pol.* **9**, 63–70
- Buchowicz, J., Reifer, I. & Makowski, J. (1961) *Acta Biochim. Pol.* **8**, 377–385
- Buchowicz, J., Reifer, I. & Geric, I. (1963) *Acta Biochim. Pol.* **10**, 157–162
- Dewar, J. H. & Shaw, G. (1962) *J. Chem. Soc.* 583–585
- Dunnill, P. M. & Fowden, L. (1963) *J. Exp. Bot.* **14**, 237–248
- Fowden, L. (1967) *Annu. Rev. Plant Physiol.* **18**, 85–106
- Fowden, L. (1970) in *Progress in Phytochemistry* (Reinhold, L. & Liwschitz, Y., eds.) vol. 2, pp. 203–266, Interscience, London
- Gmelin, R. (1959) *Hoppe-Seyler's Z. Physiol. Chem.* **316**, 164–169
- Gmelin, R. (1961) *Acta Chem. Scand.* **15**, 1188–1189

- Greenberg, J. B. & Galston, A. W. (1959) *Plant Physiol.* **34**, 489-494
- Handschumacher, R. E. (1960) *J. Biol. Chem.* **235**, 2917-2919
- Handschumacher, R. E. & Pasternak, C. A. (1958) *Biochim. Biophys. Acta* **30**, 451-452
- Kjaer, A., Knudsen, A. & Olesen-Larsen, P. (1961) *Acta Chem. Scand.* **15**, 1193-1195
- Kochetkov, N. K. & Budowsky, E. I. (1969) *Progr. Nucl. Acid Res. Mol. Biol.* **9**, 403-438
- Lambein, F. & Van Parijs, R. (1968) *Biochem. Biophys. Res. Commun.* **32**, 474-479
- Lambein, F., Schamp, N., Vandendriessche, L. & Van Parijs, R. (1969) *Biochem. Biophys. Res. Commun.* **37**, 375-382
- Larson, L. A. & Beevers, H. (1965) *Plant Physiol.* **40**, 424-432
- Metzler, D. E., Ikawa, M. & Snell, E. E. (1953) *J. Amer. Chem. Soc.* **76**, 648-652
- Mudd, J. B. & Zalik, S. (1958) *Can. J. Bot.* **36**, 467-472
- Murakoshi, I., Kuramoto, H., Haginiwa, J. & Fowden, L. (1972) *Phytochemistry*, **11**, 177-182
- Nair, P. M. & Vaidyanathan, C. S. (1964) *Arch. Biochem. Biophys.* **104**, 405-415
- Newmark, P., Stephens, J. D. & Barrett, H. W. (1962) *Biochim. Biophys. Acta* **62**, 414-416
- Pasternak, C. A. & Handschumacher, R. E. (1959) *J. Biol. Chem.* **234**, 2992-2997
- Rao, S. L. N., Ramachandran, L. K. & Adiga, P. R. (1963) *Biochemistry* **2**, 298-300
- Reifer, I., Buchowicz, J. & Toczko, K. (1960) *Acta Biochim. Pol.* **7**, 29-36
- Shaw, G. & Dewar, J. H. (1961) *Proc. Chem. Soc. London* **216**
- Shvachkin, Yu. P. & Azarova, M. T. (1964) *Zh. Obshch. Khim.* **34**, 407-411
- Silver, A. V. & Gilmore, V. (1969) *Phytochemistry* **8**, 2295-2299