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-dihydroxyxpyrimidin-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-dihydroxyxpyrimidin-5-yl)alanine. Subsequent n.m.r. and mass-spectrometric studies (Brown & Mangat, 1969) necessitated revision of this view, reassigning the alanine side chain to N-3 of the pyrimidine ring to give β-(2,4-dihydroxyxpyrimidin-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-hydroxyhomoarginine. 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 (4000lx; 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.
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
20 min at 4°C and the supernatants decanted. Resi-
dues 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 fol-
lowed by centrifuging at 4000g for 15 min. After the
pH of the supernatant had been adjusted to pH3.5
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 frac-
tioned 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 spectro-
photometric methods described previously (Brown
& Silver, 1966; Brown & Mangat, 1969), compar-
isons with authentic samples showed that peak (1)
consisted largely of willardiine, peak (2) of isowillar-
diine and peak (3) of β-(isoxazolin-5-oxo-2-yl)alalnine
(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
(150:33:37, by vol.). Willardiine and isowillardiine,
separated in this way, were shown to be chromato-
graphically 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, pH7.6
(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 isowillar-
diine; both losses were reproducible. These losses are
mainly attributable to the charcoal-adsorption and
ebulion steps.

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

After purification, the uracil amino acids were
determined spectrophotometrically; the e$_{\text{max}}$
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)alalnine. By
using the same cation-exchange separation pro-
cedure 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 chromatog-
graphed 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)alalnine so obtained was eluted in water.

Examination for albizziin. Seedlings (10g fresh wt.)
were immersed for 10 min in boiling 80% (v/v)
ethanol (50ml) and then homogenized in the same
solution. After filtration through a Millipore mem-
brane filter (0.45μm 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 aspara-
gine, which otherwise runs with, and masks, albizziin
(1,2-amino-3-ureidopropionic acid). Two-dimen-
sional paper chromatography, in solvent (1) as the
first system andaq. 80% (v/v) phenol as the second,
showed no detectable trace of albizziine 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) nin-
hydrin in acetone.

Enzymic formation of isowillardiine from O-acetyl-
seline and [2-14C]uracil. A concentrated extract of
5-day pea seedlings was prepared by grinding them
together with a mixture of solid K$_2$HPO$_4$ and
KH$_2$PO$_4$ in a chilled mortar and pestle. The amounts
of K$_2$HPO$_4$ and KH$_2$PO$_4$ were calculated to give a
final concentration of 0.1 M and a pH of 7 when dis-
solved 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-acetylseryline (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-acetylseryline 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**

**Uracilalanine 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 uracilalanine, 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 atrophic cotyledons). Attention was turned to the pyrimidines as possible direct precursors of the uracilalanines. 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 uracilalanines 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.

<table>
<thead>
<tr>
<th>Pyrimidine</th>
<th>Willardiine (nmol/seedling)</th>
<th>Isowillardiine (nmol/seedling)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Treated</td>
</tr>
<tr>
<td>Uracil</td>
<td>5</td>
<td>203</td>
</tr>
<tr>
<td>Uridine</td>
<td>5</td>
<td>37</td>
</tr>
<tr>
<td>UMP</td>
<td>5</td>
<td>53</td>
</tr>
<tr>
<td>Orotate</td>
<td>4</td>
<td>7</td>
</tr>
</tbody>
</table>

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; o, 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; o, 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 & Bevers, 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 isowellardiine 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.

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

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).

<table>
<thead>
<tr>
<th>Precursor</th>
<th>Specific radioactivity (mCi/mmol)</th>
<th>Willardiine</th>
<th>Isowellardiine</th>
</tr>
</thead>
<tbody>
<tr>
<td>[U-14C]Aspartate</td>
<td>208.0</td>
<td>252</td>
<td>2021</td>
</tr>
<tr>
<td>[6-14C]Orotate</td>
<td>60.8</td>
<td>25649</td>
<td>562869</td>
</tr>
<tr>
<td>[2-14C]Uracil</td>
<td>52.5</td>
<td>35520</td>
<td>196709</td>
</tr>
<tr>
<td>[3-14C]Serine</td>
<td>48.0</td>
<td>38749</td>
<td>218807</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25040</td>
<td>243726</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1800</td>
<td>13325</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1721</td>
<td>17386</td>
</tr>
</tbody>
</table>

**Bioisynthetic interrelationship of willardiine and isowellardiine**

The possibility that willardiine is the precursor of isowellardiine 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 isowellardiine 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 isowellardiine 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 isowellardiine (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 3. Effect of uracil, uridine and UMP on the incorporation of [6-14C]orotate into willardiine and isowillardiine

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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Willardiine</th>
<th>Isowillardiine</th>
</tr>
</thead>
<tbody>
<tr>
<td>[6-14C]Orotate</td>
<td>486584</td>
<td>416631</td>
</tr>
<tr>
<td>[6-14C]Orotate + uracil</td>
<td>400769</td>
<td>323529</td>
</tr>
<tr>
<td>[6-14C]Orotate + uridine</td>
<td>74980</td>
<td>84635</td>
</tr>
<tr>
<td>[6-14C]Orotate + UMP</td>
<td>60114</td>
<td>48206</td>
</tr>
<tr>
<td></td>
<td>171169</td>
<td>133989</td>
</tr>
<tr>
<td></td>
<td>160569</td>
<td>106340</td>
</tr>
<tr>
<td></td>
<td>219005</td>
<td>150435</td>
</tr>
<tr>
<td></td>
<td>169652</td>
<td>111292</td>
</tr>
</tbody>
</table>

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

o, Isowillardiine; ⋄, willardiine. Shoots were fed with [2-14C]uracil for 30min (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 simultaneous 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
Biosynthesis of isoxazoline could be 14C-labelled with seedlings; control and concerned on effects of pyrimidine metabolism above, described Effects of mining and compound. Terbacil contents allowed produced (44% seedlings. tested had None in inhibition. with 6-azauracil (59% Stimulation of for each Table 4. Alloxan gave (5-chloro-6-methyl-3-t-butyluracil) 2-Thiouracil Terbacil grown for Barbituric acid 6-Azauridine 5. Experimental Each incubation mixture Vol. 129

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 [2-14C]uracil and serine. However, replacing serine with O-acetylsyrine 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-ylalalnine) arises from the cyclization of γ-hydroxyhomarginine, 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 imbied in water.

<table>
<thead>
<tr>
<th>Analogue or inhibitor</th>
<th>Fresh wt. per batch (20 seedlings)</th>
<th>Isowillardiine (nmol/g fresh wt.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Treated</td>
</tr>
<tr>
<td>6-Azauridine</td>
<td>17.4</td>
<td>15.8</td>
</tr>
<tr>
<td>6-azauracil</td>
<td>13.7</td>
<td>14.2</td>
</tr>
<tr>
<td>Alloxan</td>
<td>17.1</td>
<td>17.2</td>
</tr>
<tr>
<td>Barbituric acid</td>
<td>18.3</td>
<td>17.7</td>
</tr>
<tr>
<td>Maleimide</td>
<td>17.5</td>
<td>19.3</td>
</tr>
<tr>
<td>Terbacil</td>
<td>17.8</td>
<td>17.1</td>
</tr>
<tr>
<td>2-Thiouracil</td>
<td>15.2</td>
<td>15.8</td>
</tr>
</tbody>
</table>

Table 5. Effect of O-acetylsyrine on the incorporation of [2-14C]uracil into isowillardiine by an extract from pea seedlings

Each incubation mixture (total volume 1.5 ml) contained 1 μCi of [2-14C]uracil. For further details see the Experimental section.

<table>
<thead>
<tr>
<th>Isowillardiine</th>
<th>Total radioactivity (d.p.m.)</th>
<th>Specific radioactivity (d.p.m./μmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extract + [2-14C]uracil</td>
<td>nil</td>
<td>nil</td>
</tr>
<tr>
<td>Extract + [2-14C]uracil + serine</td>
<td>nil</td>
<td>nil</td>
</tr>
<tr>
<td>Extract + [2-14C]uracil + O-acetylsyrine</td>
<td>309</td>
<td>3092</td>
</tr>
</tbody>
</table>

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have suggested that willardiine arises from albizzin (1-2-amino-3-ureidopropionic acid) after ring closure with a malonate-derived unit. Present studies, however, failed to detect albizzin 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'-monophosphatase 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-azuridine 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


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