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# BIOSYNTHESIS OF ORCYLALANINE*

L. A. HADWIGER,† H-G. FLOSS, J. R. STOKER and E. E. CONN

Department of Biochemistry and Biophysics, University of California, Davis, California

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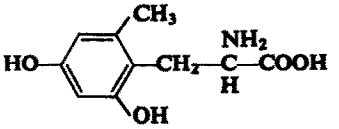
**Abstract**—Serine and actate have been established as precursors of orcylalanine in *Agrostemma githago* by feeding experiments and subsequent degradation studies. Serine provides the amino acid side chain while the aromatic ring and C-methyl group are derived from acetate. A biosynthetic mechanism is proposed involving condensation of serine with orsellinic acid with simultaneous decarboxylation of the latter.

## INTRODUCTION

THE amino acid orcylalanine (I) was first detected in the maturing seeds of *Agrostemma githago L.* (corn cockle) by Schneider.[1] Its structure has been confirmed by chemical synthesis[2] but its physiological role is still uncertain.[3]




Several biogenetic routes for the formation of this unusual amino acid may be envisaged. One possibility would be the modification of the aromatic ring of a shikimic acid derived precursor. On the other hand, the substitution pattern of the aromatic ring strongly resembles that of a polyacetate derived compound such as orsellinic acid, suggesting the involvement of the acetate pathway.[4] In this case the acetate pathway could either account for the whole of the carbon skeleton or alternatively the side chain could be completely or partly derived from some other source such as serine or glycine. This paper describes experiments which have been undertaken to elucidate the biosynthesis of orcylalanine. A preliminary report of these studies has been presented.[5]

## RESULTS

In order to establish the most suitable time to carry out biosynthetic experiments, plant material at various stages of growth was assayed for orcylalanine. Figure 1 shows that

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† Present address: Department of Plant Pathology, Washington State University, Pullman, Washington.

[1] G. SCHNEIDER, *Naturwissenschaften* **15**, 1 (1957).
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[3] G. SCHNEIDER, *Physiol. Plantarum* **14**, 638 (1961).
[4] A. J. BIRCH, *Fortschr. Chem. Org. Naturstoffe* **14**, 186 (1957).
[5] L. A. HADWIGER and E. E. CONN, *Plant Physiol.* **39**, xix (1964).

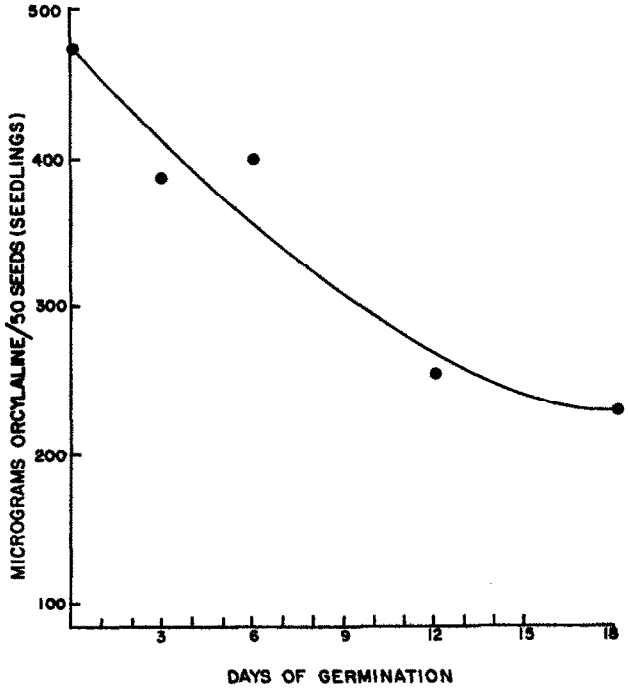


FIG. 1. DEPLETION OF ORCYLALANINE WITH AGE OF SEEDLING.

although the orcylalanine content was highest in the mature seed it appears that the accumulated product becomes depleted as the seedling develops. In 2–3-month-old plants the amino acid could not be detected with the α-nitroso-β-naphthol reagent. A very active period of biosynthesis is initiated in the seeds approximately 30 days after flowering (Table 1). This short period of orcylalanine production could be correlated with the coloration of the maturing seeds and this served as a reliable guide in this study.

In the initial experiments, a number of potential precursors were fed by injection into 30-day-old seed pods. As can be seen from Table 2 the shikimic acid pathway does not seem to be involved since shikimic acid-G-$^{14}$C, L-phenylalanine-1-$^{14}$C and L-tyrosine-1-$^{14}$C were

TABLE 1. ORCYLALANINE CONTENT OF SEEDS OF *Agrostemma githago*

| Days after flowering | Orcylalanine μg/25 seeds |
|---|---|
| 0 | 0·0 |
| 5 | 0·0 |
| 10 | 0·0 |
| 15 | 0·0 |
| 20 | 0·0 |
| 25 | 0·0 |
| 30 | 83·5 |
| 33 | 135·0 |
| 35 | 362·9 |

not incorporated. On the other hand, acetate-1-$^{14}$C was appreciably incorporated thereby pointing to the possibility of the compound being formed from a polyacetate precursor.

TABLE 2. COMPOUNDS TESTED AS PRECURSORS OF ORCYLALANINE

| Compound* | mc/mM | Incorporation (%) |
|---|---|---|
| Acetic acid-1-$^{14}$C | 20 | 5·40 |
| L-tyrosine-1-$^{14}$C | 2·64 | <0·2 |
| L-phenylalanine-1-$^{14}$C | 24·3 | <0·2 |
| Shikimic acid-G-$^{14}$C | 8·26 | <0·2 |

* 1·0 ml fed.

To establish that acetate was incorporated specifically and to check whether the whole molecule or only part was derived from acetate a degradation was performed on the orcylalanine derived from acetate-1-$^{14}$C. Originally it was attempted to degrade the amino acid to orsellinic acid, methods being available for the further degradation of this compound.[6] Low yields of orsellinic acid by the alkaline fusion of orcylalanine rendered this impracticable. Therefore the partial degradation outlined in Fig. 2 was performed. Ninhydrin

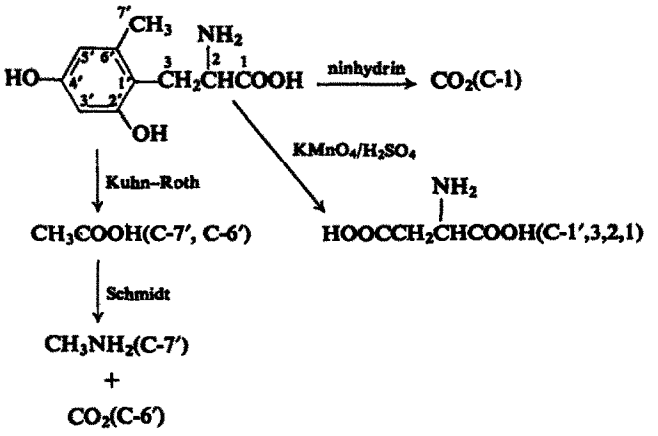


FIG. 2. DEGRADATION OF ORCYLALANINE.

oxidation liberated C-1 of orcylalanine as $CO_2$. The alanine side chain and C-1′ were isolated as aspartic acid after permanganate oxidation according to Schneider.[2] Kuhn–Roth[7] oxidation gave acetic acid which was further degraded by the Schmidt[8] reaction giving $CO_2$ from C-6′ and methylamine from C-7′. The results of this degradation are given in Table 3. The acetic acid from the Kuhn–Roth oxidation has about one-third of the total activity of the orcylalanine derived from acetate-1-$^{14}$C. Since the methylamine from the Schmidt degradation of this acetic acid is unlabeled, all this activity must be in the carboxyl group.

[6] K. MOSBACH, *Acta Chem. Scand.* **14**, 457 (1960).
[7] R. KUHN and H. ROTH, *Ber. Deut. Chem. Ges.* **66**, 1274 (1933).
[8] E. F. PHARES, *Arch. Biochem. Biophys.* **33**, 173 (1951).

TABLE 3. DEGRADATIONS ON ORCYLALANINE

| Degradation products* | Per cent of total radioactivity of orcylalanine (OA) from | | | |
|---|---|---|---|---|
| | acetate-1-$^{14}$C (186 dpm/$\mu$mole OA) | serine-U-$^{14}$C (82·4 (a) or 154 (b) dpm/$\mu$mole OA) | | serine-1-$^{14}$C (16·1 dpm/$\mu$mole OA) |
| | | a | b | |
| $CO_2$ from ninhydrin oxidation (C-1) | 0·4 | 23·3 | 30·3 | 95·4 |
| Aspartic acid from permanganate oxidation (C-1′,1,2,3) | 3·3 | — | — | — |
| Acetic acid from Kuhn–Roth oxidation (C-6′,7′) | 30·6 | 8·2 | 8·1 | — |
| $CO_2$ from Schmidt degradation of acetic acid (C-6′) | 23·3 | — | — | — |
| Methylamine from degradation of acetic acid (C-7′) | 0·0 | — | — | — |

* For numbering see Fig. 2.

The observed value for C-6′ is considerably lower than expected, probably due to dilution of the carbon dioxide obtained from the Schmidt degradation. The alanine side chain of the amino acid and C-1′ are almost devoid of activity as shown by the ninhydrin reaction and the oxidation to aspartic acid. These results demonstrate that at least the three carbon side chain is not directly derived from acetate. Although it has not been established by complete degradation of the molecule it seems highly likely from the data obtained that C-2′, C-4′, and C-6′ each carry one-third of the label after feeding acetate-1-$^{14}$C.

One reasonable precursor of the alanine side chain would seem to be serine. This compound when fed to seeds of corn cockle is incorporated into orcylalanine; in Experiment (a) in Table 3 where serine-U-$^{14}$C was fed, 0·67 per cent incorporation occurred. This value can not be compared with the values listed for acetate incorporation in Table 2 however, since similar plant material was not used in both experiments. Degradation of material from two experiments with L-serine-U-$^{14}$C showed that C-1 had about one-quarter to one-third of the total activity. On the other hand, the acetic acid isolated by Kuhn–Roth oxidation also carried some label. To check whether this was due to cross contamination during the Kuhn–Roth oxidation orcylalanine-2-$^{14}$C was synthesized and subjected to this reaction. The acetic acid in this case had only 1·1 per cent of the total activity so cross contamination does not account for all of the activity in the acetic acid. It must be assumed therefore that serine undergoes rapid metabolism in the plant—this giving rise to some label in the acetate. The degradation values are in accord with the labeling pattern where each acetate derived carbon has about 4 per cent of the total activity and each side chain carbon carries about one-third of the remaining label.

The results with serine-U-$^{14}$C were confirmed by degrading orcylalanine obtained from an experiment with serine-1-$^{14}$C (Table 2). Here the carboxyl carbon accounted for almost all of the activity of the compound thereby showing that serine was incorporated specifically.

## DISCUSSION

The results obtained here demonstrate that the biosynthesis of orcylalanine involves both acetate and serine. Serine provides the alanine side chain whereas the aromatic ring and C-methyl group appear to be formed from acetate. The labeling pattern in orcylalanine after feeding acetate-1-$^{14}$C is best explained by assuming alternate labeling in the ring which is characteristic of polyacetate derived compounds. C-1′ must come from the methyl group of acetate unless one makes the highly unlikely assumption that it is provided by an extra one carbon unit.

A reasonable biosynthetic mechanism which fits all the experimental results would be the condensation of orsellinic acid with serine as outlined in Fig. 3. This reaction could proceed

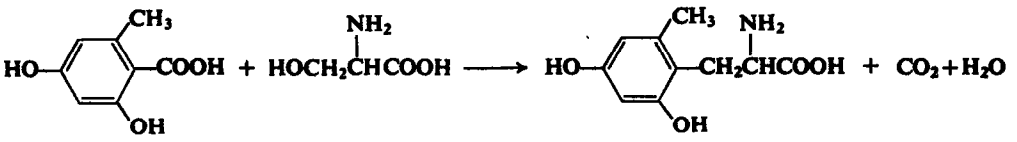


FIG. 3. CONDENSATION OF ORSELLINIC ACID WITH SERINE.

with simultaneous decarboxylation of the orsellinic acid or else the orsellinic acid could be first decarboxylated, the resulting orcinol then reacting with serine. From an energetic point of view the first possibility would seem to be more likely. This would constitute a novel mixed type of polyacetate biosynthesis. Nucleophilic substitution reactions on the $\beta$-carbon of serine are well known, for example the formation of tryptophan from serine and indole glycerol phosphate[9] and also the formation of $\beta$-cyanoalanine from serine and cyanide.[10] An analogy for the condensation with simultaneous decarboxylation may be seen in the formation of indole glycerol phosphate by cyclization of 1-(*o*-carboxyphenylamino)-1-desoxyribulose-5-phosphate.[11] Further experiments are necessary to firmly establish this mechanism.

## EXPERIMENTAL

### *Plant Material*

*Agrostemma githago* plants were grown to maturity both in the field and in the greenhouse.

### *Radioactive Compounds*

Acetic acid-1-$^{14}$C (20 mc/mM), L-serine-U-$^{14}$C (120 mc/mM), L-serine-1-$^{14}$C (4·0 mc/mM), L-tyrosine-1-$^{14}$C (2·64 mc/mM), L-phenylalanine-1-$^{14}$C (24·3 mc/mM), glycine-2-$^{14}$C (3 mc/mM), and shikimic acid-G-$^{14}$C (8·26 mc/mM) were purchased from New England Nuclear Corporation, Boston, Mass. Carrier orcylalanine was synthesized according to the method of Schneider.[2] The synthesis of orcylalanine-2-$^{14}$C followed the same route starting from glycine-2-$^{14}$C.

### *Administration of Radioactive Compounds*

Radioactive compounds were injected into the seed pod and peduncle of flowers of intact plants approximately 30 days after flowering. To obtain larger quantities of biosynthetically

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[10] S. N. NIGAM and C. RESSLER, *Biochim. et Biophys. Acta* **93**, 339 (1964).
[11] C. YANOFSKY, *Biochim. et Biophys. Acta* **20**, 438 (1956).

labeled orcylalanine for degradation, 5 g of light tan to light brown seeds were slightly sliced and placed immediately into a solution of the precursor (5–10 $\mu$c) in a volume of water sufficient to submerge the seeds. After the liquid was taken up it was replenished once and the seeds were allowed to dry and turn dark. Orcylalanine was extracted after 7 days.

*Isolation of Orcylalanine*

The dried seeds of *Agrostemma githago* were ground to a powder with liquid nitrogen in a mortar. The powder was extracted with boiling 80% ethanol (1 part tissue/20 ml 80% ethanol) for 10 min and filtered hot. The residue was washed three times with 20 ml of hot 80% ethanol and the wash solutions combined with the filtrate and reduced to 1 ml *in vacuo*. Aliquots of the extracts from some experiments were separated two dimensionally on Whatman No. 1 filter paper with butanol: acetic acid: water 4:1:1 (solvent 1) and 80% phenol (solvent 2). The developed chromatograms were exposed to X-ray films and the orcylalanine was located by spraying with ninhydrin and $\alpha$-nitroso-$\beta$-naphthol. Orcylalanine was eluted from other chromatograms and further identified and its concentration determined by its u.v. absorption spectrum.[1] To obtain labeled orcylalanine for degradation, the ethanol extracts were chromatographed in solvent 1 under nitrogen. The orcylalanine was eluted immediately under nitrogen with 5% acetic acid and carrier material (100–200 mg) added to the eluate. The eluate was acidified with hydrochloric acid, treated with charcoal and filtered. The orcylalanine was precipitated by adjusting the filtrate to pH 5·0 with ammonia. This crystallization was repeated until the specific activity was constant.

*Degradation of Orcylalanine*

Kuhn–Roth oxidation and Schmidt degradation were performed by standard procedures[7,8] except that the methylamine from the Schmidt degradation was counted as the hydrochloride. C-1 was obtained as $CO_2$ by oxidation with excess ninhydrin in dilute phosphoric acid. Oxidation of orcylalanine to aspartic acid with permanganate in sulphuric acid was carried out as described by Schneider.[2] Aspartic acid was isolated using a Dowex-50 column and was purified by chromatography in solvent 1. It was quantitatively determined by its color with ninhydrin.[12] The yield in this reaction is less than 1%.

*Determination of Radioactivity*

$CO_2$ from decarboxylation reactions was either precipitated as barium carbonate and counted on planchets in a Nuclear Chicago Gas-Flow Counter ($\sim$ 25% efficiency), corrections for self absorption being made. Alternatively it was trapped in ethanolamine and counted in a Packard Tricarb Scintillation Counter ($\sim$ 45% efficiency) using internal standards.

All other compounds were counted in Brays solution[13] using a Packard Tricarb Scintillation Counter. Benzoic acid-$^{14}$C was used as an internal standard in all measurements.

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