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# FORMATION OF VICINE AND CONVICINE BY *VICIA FABA*

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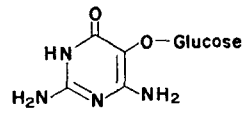
**Key Word Index**—*Vicia faba*; Leguminosae; vicine; convicine; pyrimidine glucosides; biosynthesis from orotate.

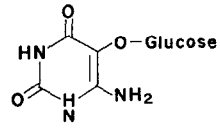
**Abstract**—Methods are described for the quantitative extraction and separation of the pyrimidine glucosides, vicine and convicine. The contents of these two substances in germinating seeds and young seedlings of *Vicia faba* remain constant for the first 2 weeks. Net synthesis and accumulation of vicine and convicine occurs in developing seeds. That the synthesis occurs within the pod and the pyrimidine glucosides are not translocated into them, was shown by injection of $^{14}$C-labelled precursors into the pods. [1-$^{14}$C]- and [2-$^{14}$C]-acetate were weakly incorporated but much greater incorporation was observed with [U-$^{14}$C]-aspartic acid and [6-$^{14}$C]-orotic acid. The results indicate that the orotic acid pathway is involved in the formation of the pyrimidine ring of both vicine and convicine.

## INTRODUCTION

VICINE (I) was first isolated from seeds of *Vicia sativa*[1-3] where it occurs with a related pyrimidine glucoside, convicine (II).[4] The structure of (I) was elucidated by Bendich and Clements,[5] and that of (II) by Bien *et al.*[6] In addition to its presence in *V. sativa*, vicine has been found in *V. faba*,[7] in beet juice,[8] and in peas.[9] It has been implicated as a causative agent of 'favism'.[10,11] Convicine occurs in *V. sativa*,[4] and *V. faba*.[10]




Vicine (I)




Convicine (II)

Despite vicine being the first naturally occurring pyrimidine isolated, nothing is known of its metabolic origins or site of synthesis. The main problem concerning the formation of vicine and convicine is the origin of the pyrimidine ring system. Lathyrine, β-(2-amino-pyrimidin-4-yl)alanine,[12] another unusual pyrimidine occurring in plants, appears to derive

[1] H. RITTHAUSEN and U. KREUSLER, *J. Prakt. Chem.* **2,** 333 (1870).
[2] H. RITTHAUSEN, *J. Prakt. Chem.* **7,** 374 (1873).
[3] H. RITTHAUSEN, *Ber. dt. chem. Ges.* **9,** 301 (1876).
[4] H. RITTHAUSEN, *J. Prakt. Chem.* **24,** 202 (1881).
[5] A. BENDICH and G. C. CLEMENTS, *Biochim. Biophys. Acta* **12,** 462 (1953).
[6] S. BIEN, G. SALEMNIK, L. ZAMIR and M. ROSENBLUM, *J. Chem. Soc.* **C,** 446 (1968).
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[8] E. O. VON LIPPMAN, *Ber. dt. chem. Ges.* **29,** 2653 (1896).
[9] E. SCHULTZE, *Hoppe-Seyler's Z. Physiol. Chem.* **17,** 215 (1893).
[10] J. MAGER, G. GLAZER, A. RAZIN, G. IZAK, S. BIEN and M. NOAM, *Biochem. Biophys. Res. Commun.* **20,** 235 (1965).
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[12] E. A. BELL and R. G. FOSTER, *Nature, Lond.* **194,** 91 (1962).

from the cyclization of an aliphatic precursor, namely γ-hydroxyhomoarginine,[13,14] whereas willardiine and isowillardiine [β-(2,4-dihydroxypyrimidin-1-yl)alanine and β-(2,4-dihydroxypyrimidin-3-yl)alanine, respectively] arise from preformed uracil produced by the orotic acid pathway.[15]

Before the formation of vicine and convicine could be studied, it was necessary to develop suitable extraction and analytical techniques. Earlier extraction methods involved prolonged acid treatment, e.g. 2% $H_2SO_4$ for 12 hr,[5] but the present work indicated that this produces significant hydrolysis of the pyrimidine glucosides. Gmelin and Hasenmaier[16] and later Bien *et al.*[6] used alcoholic extractants. The present extraction technique is based on that of Gmelin and Hasenmaier.[16]

## RESULTS AND DISCUSSION

### *Vicine and Convicine Metabolism during Germination and Early Growth*

Seeds were allowed to germinate and over the first 2 weeks of growth, batches of 10 seedlings were harvested at intervals. The vicine and convicine contents were extracted and estimated spectrophotometrically. The results indicate that the amounts of vicine and convicine remain substantially constant (at about 0·02 and 0·005 μmol/seedling respectively) throughout the first 2 weeks of growth. As there was no net synthesis, the suitability of germinating seeds and young seedlings of *V. faba* for a study of vicine and convicine synthesis was not considered further.

### *Formation of Vicine and Convicine in Developing Seeds*

Following flowering, developing seeds of *V. faba* were harvested periodically and the seeds and pods separately examined for vicine and convicine. With very young pods (up to 10 cm in length), vicine and convicine could not be detected in either the seeds or pods themselves. However, with the larger pods (13–15 cm in length) they were readily detected in the seeds but, again, not in the pods. Because of wide variations and the relatively small sample of pods that could be examined, it was found impossible to relate directly the rate of their accumulation to any parameter such as growth, length of pods, or number and weight of seeds. However, it was clear that vicine and convicine began to accumulate in pods approx. 12–14 cm in length.

### *Incorporation of $^{14}$C-labelled Precursors*

Using the method described in the Experimental, $^{14}$C-labelled materials were introduced into pods actively synthesizing vicine and convicine. After 24 hr, the treated pods were harvested and the seeds removed for vicine and convicine extraction. To locate the site of synthesis, [1-$^{14}$C]- and [2-$^{14}$C]-acetate were used initially. The results given in Table 1, show that the site of synthesis of vicine and convicine is within the pod, i.e. these pyrimidine glucosides are not translocated into the developing seeds from another tissue.

Two separate experiments with the known pyrimidine precursors [U-$^{14}$C]-aspartate and [6-$^{14}$C]-orotate showed that these substances were incorporated into vicine and convicine much more efficiently than was either [1-$^{14}$C]- or [2-$^{14}$C]-acetate. [6-$^{14}$C]-Orotate was

[13] E. A. BELL, *Nature, Lond.* **199,** 70 (1963).
[14] E. A. BELL, *Nature, Lond.* **203,** 378 (1964).
[15] T. S. ASHWORTH, E. G. BROWN and F. M. ROBERTS, *Biochem. J.* in press.
[16] R. GMELIN and G. HASENMAIER, *Arzneimittel-Forsch.* **12,** 755 (1959).

incorporated to a greater extent than [U-$^{14}$C]-aspartate. This is as would be expected if the orotic acid pathway of pyrimidine biosynthesis is the source of the pyrimidine ring of both vicine and convicine.

TABLE 1. INCORPORATION OF $^{14}$C-LABELLED ACETATE, ASPARTATE AND OROTATE INTO VICINE AND CONVICINE BY DEVELOPING SEEDS OF *Vicia faba*

| Labelled precursor (10 $\mu$Ci supplied) | | Vicine Sample extracted ($\mu$mol) | Vicine Specific activity (dpm/$\mu$mol) | Convicine Sample extracted ($\mu$mol) | Convicine Specific activity (dpm/$\mu$mol) |
|---|---|---|---|---|---|
| [U-$^{14}$C]-Aspartate | (A)* | 3·4 | 7923 | 0·66 | 8806 |
| [U-$^{14}$C]-Aspartate | (B) | 7·8 | 3375 | 1·41 | 3092 |
| [1-$^{14}$C]-Acetate | | 5·1 | 835 | 0·83 | 938 |
| [2-$^{14}$C]-Acetate | | 5·3 | 997 | 0·76 | 1181 |
| [6-$^{14}$C]-Orotate | (A) | 4·8 | 21 908 | 0·70 | 21 246 |
| [6-$^{14}$C]-Orotate | (B) | 8·7 | 9611 | 1·72 | 7831 |

* Experiment *B* was carried out 1 month later in the growing season than experiment *A* (see Results and Discussion).

The specific activities of vicine and convicine formed from [U-$^{14}$C]-aspartate and [6-$^{14}$C]-orotate are noticeably lower in the second series of experiments (*B*) than in the first series (*A*). The decrease in specific activity is accompanied by corresponding increases in the content of vicine and convicine in the seeds (Table 1). The first series of experiments were carried out one month earlier in the growing season than the second and although the pods were essentially similar in length, the seeds contained within them were much larger than in the later experiment. The lower specific activities in the later experiments are, thus, largely attributable to the higher content of vicine and convicine already in the seeds before injection of the $^{14}$C-labelled precursors.

## EXPERIMENTAL

*Materials.* Seeds of *Vicia faba* L. var. Mammoth Windsor, supplied by Carter's Seeds Ltd., London, were surface-sterilized[15] and germinated in moist vermiculite at 25°. Young seedlings were grown at 25° in a light regime of 16 hr light (4000 lx) alternating with 8 hr dark. Mature plants required for fruiting were grown outdoors. [1-$^{14}$C]- and [2-$^{14}$C]-acetate (Na), [U-$^{14}$C]- aspartic acid, and [6-$^{14}$C]-orotic acid were obtained from The Radiochemical Centre, Amersham, Bucks.

*Extraction of vicine and convicine.* Using a chilled mortar and pestle, fresh material was homogenized in MeOH–$H_2O$ (1:1) (10 ml/g fr. wt.). Acid-washed sand was added when necessary to facilitate grinding. Mature dry seeds were first milled in a C580 microhammer mill (Glen Creston Ltd., Stanmore, Middlesex) and then homogenized in a blender using MeOH–$H_2O$ (1:1). Homogenates were boiled for 5 min and filtered through a Millipore membrane filter (pore size 0·45 $\mu$m). Residues were extracted twice in a similar manner. The combined filtrates were concentrated *in vacuo* at 37°.

Methanolic extracts so obtained could not be directly chromatographed because of the presence of relatively large amounts of extraneous material which fluoresced markedly in UV light. Attempts to remove these by selective adsorption and elution from charcoal columns,[17] or by the use of insoluble poly-*N*-vinylpyrrolidone (Polyclar AT) were largely unsuccessful. In the former technique, it was found difficult to elute vicine and convicine from charcoal, and with the latter method, the required low pH caused serious hydrolysis. The procedure eventually adopted was based on that used for preliminary purification of nucleoside extracts.[18] The MeOH extracts were evaporated to dryness *in vacuo* at 37°, redissolved in $H_2O$ (5 ml/g fr. wt. of tissue extracted) and the pH adjusted to 7·6 with dil. ammonia. The extract was percolated through a column (1 cm dia.) of Dowex 1 (formate; X8; 200–400 mesh); 1 ml of resin was used per g fr. wt of tissue

[17] E. G. BROWN, *Biochem. J.* **85,** 633 (1962).
[18] E. G. BROWN, *Biochem. J.* **95,** 509 (1965).

extracted. The column was washed with $H_2O$ (5 × bed vol.) and the washings combined with the non-exchangeable fraction. The solution was evaporated to dryness *in vacuo* at 37° and redissolved in a suitable small volume of $H_2O$ for PC.

*Paper chromatography.* Extracts were chromatographed sequentially as broad bands on Whatman 3 MM paper. The following solvent systems were used, (1) EtOH–M ammonium acetate, pH 7·6,[19] (2) *n*–BuOH–pyridine–$H_2O$,[20] (3) *iso*PrOH–ammonia (sp. gr. 0·88)–$H_2O$ (7:1:2), and (4) *n*–BuOH–$H_2O$ (43:7). The $R_f$ of vicine and convicine respectively were: (1) 0·45 and 0·56, (2) 0·44 and 0·52 (3), 0·18 and 0·24, (4) 0·02 and 0·02. Bands were eluted by the method of Dent.[21]

*Estimation of vicine and convicine.* After extraction and separation as described, vicine and convicine were estimated spectrophotometrically. The $\epsilon_{max}$ values used were those given by Bendich and Clements[5] and Bien *et al.*[6] for vicine and convicine respectively. Recovery of added vicine and convicine was 87–93%.

*Introduction of labelled precursors.* Each labelled material (10 $\mu$Ci) was dissolved in 0·2 ml of sterile water and the pH adjusted to 5·5. Using a very fine hypodermic needle and syringe, the solution was injected directly into the pods whilst these were still on the plant. The needle was introduced between the seeds at several places in any one pod. After 24 hr, treated pods were harvested and the seeds extracted. Radioactivity was measured by liquid scintillation counting as previously described.[15]

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[19] A. C. PALADINI and L. F. LELOIR, *Biochem. J.* **51,** 426 (1952).
[20] R. I. MORRISON, *Biochem. J.* **53,** 474 (1953).
[21] C. E. DENT, *Biochem. J.* **41,** 245 (1947).