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INCORPORATION OF MOLECULAR OXYGEN INTO CAFFEIC ACID BY GREEN *HELIANTHUS ANNUUS*

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Abstract—The results of experiments in which *Helianthus annuus* were grown in the light in an atmosphere enriched with oxygen-18 indicated that the hydroxyl oxygen atoms in caffeic acid are derived from molecular oxygen

INTRODUCTION

CAFFEIC acid (3,4-dihydroxycinnamic acid) is one of the most widespread of plant phenolics.[1] This compound appears to have more than one role in the metabolism of higher plants. Caffeic acid is an intermediate in the biosynthesis of the coumarins and the phenylpropanoid moieties of the flavonoids and the lignins.[2–4] Caffeic acid has been reported to inhibit the activity of indoleacetic acid oxidase.[5] Esters of caffeic acid are present in relatively large amounts in the stigmatic exudates of certain plants[6] and may have a role in determining whether stigmas stimulate the germination of some pollens or inhibit the germination of others.[7]

Although the enzyme which catalyzes the hydroxylation of the aromatic ring of *p*-coumaric acid (4-hydroxycinnamic acid) to caffeic acid has been extracted from plant tissues and some of its properties studied,[8] no definitive experiments concerning the source of oxygen atoms for the hydroxylation reaction have been reported. This paper is concerned with the demonstration that the hydroxyl oxygen atoms in caffeic acid synthesized by green *H. annuus* are derived from molecular oxygen.

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RESULTS AND DISCUSSION

In preliminary experiments, caffeic acid was isolated from green *H. annuus* grown in unenriched atmospheres. The identity of caffeic acid isolated from these seedlings was confirmed by comparing its MS with that of authentic material. Caffeic acid isolated from the shoot-root axes of green *H. annuus* grown in an atmosphere enriched with 10 at. % oxygen-18 was analyzed mass spectrometrically. The MS analysis of the average of four scans, when corrected for natural abundance isotopes, indicated that the phenolic oxygen atoms in caffeic acid were labeled to the extent of 8·7 ± 1·0 at. %; the carboxyl oxygen atoms were not labeled.

The presence of oxygen-18 in phenolic oxygen atoms only was confirmed by comparing the abundance of ^{18}O in the molecular ion (*m/e* 180, 182) with that in the $(M-CO_2)^+$ ion (*m/e* 136, 138). The fact that *m/e* 136 corresponded to $C_8H_8O_2^+$ was confirmed by high resolution mass measurement. The loss of CO_2 as a unit was established by an appropriate metastable ion at *m/e* 102·8. This unit is virtually certain to correspond to the carboxyl group. These results suggest that both of the phenolic oxygen atoms in caffeic acid are derived directly from molecular oxygen. The small discrepancy in the labeling of the caffeic acid hydroxyls and the labeling of the atmospheric oxygen (8·7 ± 1·0 at. % vs 10·0 at. %) is very close to the limits of experimental error, and may be due either to preformed caffeic acid in the tissue or to an ^{18}O isotope effect.

TABLE 1. INCORPORATION OF OXYGEN-18 INTO TISSUE WATER IN 8-DAY-OLD GREEN *Helianthus annuus* GROWN IN AN ATMOSPHERE ENRICHED WITH 10 AT. % $^{18}O_2$

Fraction	Shoot-root axes*		Cotyledons†	
	Quantity of water recovered (ml)	Atom per cent excess‡	Quantity of water recovered (ml)	Atom per cent excess‡
First	2·3	0·13	1·3	0·10
Second	0·5	0·13	0·3	0·10
Third	0·4	0·14	0·2	0·10
Fourth	0·3	0·13	0·1	0·06

* 7 g fresh wt

† 4·5 g fresh wt

‡ On the basis of determinations of the oxygen-18 content of several different samples of water of known enrichment, the accuracy of measurement was estimated to be ±1%.

To exclude the possibility that the observed enrichment in caffeic acid was an indirect effect caused by the reduction of $^{18}O_2$ to H_2O during respiration followed by subsequent incorporation of $H_2{}^{18}O$ into caffeic acid, it was necessary to analyze the oxygen-18 content of tissue water. The oxygen-18 enrichment in H_2O recovered from shoot-root axes and cotyledons of *H. annuus* seedlings grown in atmospheres enriched with 10 at. % oxygen-18 are shown in Table 1. These enrichment values are very small, in the order of 0·1 at. % excess. Therefore it can be concluded that the hydroxyl oxygen atoms in caffeic acid are derived from molecular oxygen.

EXPERIMENTAL

Sunflower seeds (*Helianthus annuus* L.) were purchased locally and soaked for 6 hr in tap H_2O. Then they were sown in vermiculite in elongated glass flasks each of 330 cc vol., with 10 seeds per flask. The vermiculite was wetted with sufficient H_2O at the start of the growth period, so that further additions of H_2O were not needed. To prevent excessive accumulation of respiratory CO_2, a vial containing 5 ml 40 per cent aq. KOH

together with a filter paper wick, was placed upright inside each flask Each flask was sealed with a rubber stopper into which a short piece of glass tubing was fitted, the opening of the glass tubing was sealed with rubber tubing and a clamp Germination and subsequent growth for 8 days took place in a room lighted during daylight hours

Oxygen gas containing 10 at % oxygen-18 was prepared by electrolysis of ^{18}O-enriched water, purchased from BioRad Laboratories Enriched oxygen gas was mixed with nitrogen (vol 21 79), This gas mixture was introduced into each flask at the beginning of the growth period by evacuating the flask with a H_2O aspirator and then filling to 1 atm pressure Oxygen gas absorbed by the respiring seedlings was replaced every 24 hr with pure oxygen gas containing 10 at % ^{18}O

At the end of the growth period, the cotyledons were separated from the shoot-root axes Caffeic acid was isolated from 25 g (fresh weight) of shoot-root tissue according to the method of Swain and Williams,[9] except that separation from other plant constituents was achieved by TLC, not PC The adsorbent was powdered cellulose without a binder (MN 300, Brinkmann Instruments) The location of caffeic acid on the plates was visualized under short wave UV and the spots were scraped off, eluted with alcohol, and re-chromatographed four times. Cotyledonary tissue was not used for the isolation of caffeic acid from seedlings grown in an oxygen-18 atm, because preliminary investigation showed that these tissues contained relatively large quantities of caffeic acid on the first day of seed germination

Caffeic acid was analyzed with an AEI Scientific Apparatus MS-902 spectrometer, Samples were introduced directly into the mass spectrometer by use of a probe inlet Source temperature was 200°

H_2O which was present in the shoot-root axes and in the cotyledons at the end of the growth period in $^{18}O_2$ was extracted by freezing and drying the tissues *in vacuo* by means of a mechanical vacuum pump The drying process was interrupted at intervals, so that successive fractions of tissue H_2O were obtained Oxygen-18 content of tissue water was determined by the method of Boyer *et al* [10] using a Hitachi–Perkin–Elmer mass spectrometer, model RMU-6E

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