Evidence That 13-Hydroxycapsidiol Is Not an Intermediate in Capsidiol Degradation in Peppers

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ABSTRACT

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13-Hydroxycapsidiol [^{14}C] was isolated from pepper fruits (*Capsicum frutescens* L.) following incubation with capsidiol[^{14}C]. No further metabolism of 13-hydroxycapsidiol[^{14}C] could be demonstrated when this compound was incubated with pepper fruits under the same conditions. These results suggest that 13-hydroxycapsidiol is unlikely to be an intermediate in a process of capsidiol degradation or metabolism to other products in healthy pepper fruits.

Capsidiol is a sesquiterpenoid phytoalexin produced by peppers (6), tobacco (2), and jimsonweed (10). When solutions of capsidiol[^{14}C] were incubated in pepper fruits, only part of the radioactivity could be recovered subsequently as capsidiol[^{14}C] by solvent extraction (5). A major portion of the radioactivity remained in the water phase. This suggested that capsidiol was metabolized to water-soluble products and was possibly a normal, although transitory, metabolite in healthy pepper tissues. Subsequently, 13-hydroxycapsidiol was identified as an ether-soluble metabolic product of capsidiol (9) that might serve as an intermediate in a pathway leading from capsidiol to more water-soluble products. In addition, preliminary observations indicated that production of 13-hydroxycapsidiol was inhibited in fruits inoculated with fungi, suggesting that capsidiol accumulation in infected tissue could be mediated by blockage of the hydroxylation step (7). In the present study, 13-hydroxycapsidiol[^{14}C] was prepared and supplied to pepper fruits in an attempt to obtain information about subsequent steps in such a pathway. The results indicate, however, that 13-hydroxycapsidiol is not metabolized by pepper fruits; hence the further metabolism of capsidiol by this route and its accumulation during infection due to blockage of the hydroxylation step are improbable.

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MATERIALS AND METHODS

Preparation of capsidiol[^{14}C]. The procedure was, in general, that of Baker and Brooks (1). One hundred pepper fruits (*Capsicum frutescens* L., 'Keystone Resistant Giant') were washed in tap water, cut into halves lengthwise, and inoculated by dispensing 10 ml of a spore suspension of *Monilinia fructicola* (Wint.) Honey (2×10^5 spores per milliliter) into each half. After incubation at room temperature for 18 hr, 1 ml of a solution of sodium acetate [2-^{14}C] (New England Nuclear, 41 mg in 200 ml; approximately 0.5 mCi) was added and incubation continued for a

further 30 hr. The diffusates and a water rinse of the inside of the fruits were collected and extracted three times with half volumes of ether. The combined ether fractions were dried with sodium sulfate, evaporated under reduced pressure to dryness (206.4 mg), and redissolved in 10 ml of methanol. Liquid scintillation analysis, gas chromatography (GLC), and thin-layer chromatography (TLC) radioscans of this extract indicated that it contained 24.3 μCi of radioactivity and about 70 mg of capsidiol. More than half of the radioactivity (about 60%) was associated with the capsidiol.

Preparation of 13-hydroxycapsidiol[^{14}C]. The methanol solution containing the crude capsidiol[^{14}C] was diluted with 3 L of sterile distilled water to give a capsidiol concentration of about 1 $\times 10^{-4}$ M. Ten milliliters of this solution was dispensed into each of 300 pepper fruit halves and incubated at room temperature for 48 hr. Diffusates and rinsings were extracted five times with half volumes of ether. The combined extracts were dried as above (residue, 233 mg), redissolved in methanol, and examined by TLC radioscan for distribution of radioactivity. The remainder of the extract was separated by preparative TLC. The 13-hydroxycapsidiol and the capsidiol bands were scraped from the plates, transferred to small columns, and eluted with chloroform:methanol (80:20). The entire process was repeated twice more using the residual crude capsidiol[^{14}C] as the feeding solution. The crude 13-hydroxycapsidiol[^{14}C] eluates from the three runs were combined (18 mg). A TLC radioscan of this material indicated that the bulk of the activity was associated with the 13-hydroxycapsidiol spot and minor amounts with two contaminants. The entire sample was run on preparative TLC plates, first in chloroform:methanol (90:10), and then, in the same direction, in ethyl acetate:tertiary butanol (95:5). The 13-hydroxycapsidiol[^{14}C] band was scraped off, eluted from a small column with chloroform:methanol (80:20), dried as above, and dissolved in 10 ml of methanol. A TLC radioscan of an aliquot of this solution demonstrated only a trace of radioactive contaminant (<5%), and GLC indicated that it contained 3.3 mg of 13-hydroxycapsidiol. The sample was then combined with about 3.0 mg of unlabeled 13-hydroxycapsidiol available from previous work, and the combined samples were subjected to preparative TLC and elution as above. Only one radioactive peak was detected by TLC radioscan of the eluted material. A contaminating peak 1/200 the height of the 13-hydroxycapsidiol[^{14}C] peak would have been measurable. No other contaminating material could be detected chromogenically on TLC plates or by GLC. The sample contained 6.04 mg of 13-hydroxycapsidiol by GLC and 520 nCi of radioactivity.

Feeding of 13-hydroxycapsidiol[^{14}C]. The chromatographically pure 13-hydroxycapsidiol[^{14}C] was supplied to pepper fruit halves as described above for the capsidiol feeding at a concentration of 10^{-4} M in sterile distilled water, 10 ml per fruit half, and the fruits were incubated at room temperature for 24 hr.

Extraction and analysis of diffusates and pepper tissue. The diffusates and washings of the fruit cavities were partitioned five times with half volumes of ether or ethyl acetate (depending upon the experiment). The tissue (referred to as whole tissue) was steeped in 50% methanol overnight. The methanol was combined with rinsings of the tissue and taken to dryness; the residue was redissolved in water. The tissue was then homogenized in 50% methanol (referred to as homogenized tissue), and the homogenized tissue was separated by centrifugation and dried at room temperature. The methanol extract of the homogenized tissue was reduced to dryness and the residue redissolved in water. The water solutions from the dried methanol extracts of the whole and homogenized tissue were each partitioned five times with half volumes of ether or ethyl acetate. Ether or ethyl acetate extracts were dried, and the residues were taken up in appropriate volumes of methanol.

GLC was performed as described previously (8). Column and detector temperatures for capsidiol were 160 and 250 C, respectively (retention time, 265 sec), and for 13-hydroxycapsidiol, 200 C and 250 C (retention time, 170 sec).

Radioactivity in methanol solutions and lightly colored water phases was determined by liquid scintillation directly in Omnifluor (New England Nuclear MEF 906; 4 g/L toluene) with *n*-hexadecane[1-^{14}C] as an internal standard. Strongly colored water phases and dried tissues after extraction were first converted to carbon dioxide in a liquid scintillation sample oxidizer (Intertechnique, Oxymat LN 4101) with methyl methacrylate[^{14}C] as standard. Radioactivity on TLC plates was located and estimated with a Panax RTSL-7A radiochromatogram scanner and recorder.

TLC was done on silica gel (200-μm thick, Polygram, precoated plastic plates, Macherey-Magel Co.) with chloroform:methanol (90:10) and ethyl acetate:tertiary butanol (95:5) as solvent systems. Compounds on the plates were detected by spraying with 0.5% vanillin in 80% sulfuric acid in ethanol and heating at 110 C for a few minutes until colors developed and by reference to standards available from previous work (6,9). Preparative TLC was done on silica gel (1-mm thick, Analtech, Uniplate precoated glass plates) using the same solvent systems.

RESULTS AND DISCUSSION

In the first experiment 3.0 mg of 13-hydroxycapsidiol (245 nCi) in 120 ml of water were supplied to 12 fruit halves. The diffusate was extracted with ether and the water solubles from the methanol extract of the whole tissue were partitioned with ether also. The two combined ether fractions contained 1.73 mg of 13-hydroxycapsidiol and about 130 nCi of radioactivity (Table 1), indicating that no significant dilution with cold 13-hydroxycapsidiol from the fruits had occured. No radioactive compounds other than 13-hydroxycapsidiol were detected in radioscans of TLCs of these two fractions. About half of the remainder of the radioactivity supplied was in the two water phases and the rest was unaccounted for. These results were consistent with previous assumptions that metabolism of capsidiol to water-soluble products might occur via 13-hydroxycapsidiol (7,9). However, when the water phase of the diffusate was partitioned subsequently with ethyl acetate, virtually all the radioactivity was recovered in the ethyl acetate phase and 13-hydroxycapsidiol was the only radioactive compound detected.

The experiment was repeated. After the diffusates were removed and the fruit cavities rinsed, the whole tissue was steeped in methanol for 72 hr, homogenized, and then extracted again. All water phases were extracted thoroughly with ethyl acetate, and emulsions were broken by centrifugation. Almost all of the radioactivity supplied (160 nCi in 1.85 mg 13-hydroxycapsidiol) was recovered, most of it directly from the diffusate (Table 1). Most of the residual activity in the water phases was also extractable with ethyl acetate, as a second series of partitions demonstrated (Table 1). No radioactivity was detected in the homogenized tissue after extraction. In the ethyl acetate-soluble material from the diffusate and the whole tissue, 1.78 mg of 13-hydroxycapsidiol was determined by GLC. None was detected in

TABLE 1. Recovery of radioactivity (nCi) from pepper fruits after incubation with 13-hydroxycapsidiol

	Solvent[a]	
	Ether	Ethyl acetate
Diffusate		
Extract	126.7	133.5 (3.31)[b]
H_2O phase	57.7	3.7 (0)
Whole tissue[c]		
Extract	3.9	16.1 (0.19)
H_2O phase	3.3	2.4 (1.27)
Homogenized tissue		
Extract	...	4.0
H_2O phase	...	0
Total recovered	196.6	159.7
Initial		
13-hydroxycapsidiol [^{14}C]	244.6	160.5

[a] Two separate experiments.

[b] Values in parentheses were obtained after a second series of extractions of the water phases.

[c] Whole tissue was extracted by steeping in 50% methanol. In the second experiment, tissue was homogenized in 50% methanol also.

the ethyl acetate-soluble material from the homogenized tissue or in any of the water phases. This, however, was not unexpected, because 13-hydroxycapsidiol would have been below the limits of detection by GLC if it was present in amounts corresponding to the radioactivity in these fractions. 13-Hydroxycapsidiol was the only radioactive compound detected in TLC-radioscans of aliquots of the ethyl acetate-soluble material from the diffusate and whole tissue. A smaller scale repetition of this experiment and an additional experiment in which slices of fruit tissue were used gave essentially the same results. We found no visible indications that 13-hydroxycapsidiol was toxic to pepper tissues at the concentrations used.

These findings indicate that, under the same conditions as those in which capsidiol is converted to 13-hydroxycapsidiol and with the assumption that access to endogenous metabolic pools is similar, the latter is not metabolized further to any significant extent. 13-Hydroxycapsidiol was not diluted appreciably by endogenous material, and hence, capsidiol production also must be very limited in healthy tissues. Capsidiol accumulation in infected tissues as a result of a metabolic block is therefore improbable. Furthermore, no grounds now exist for the earlier suggestion (7,9) that 13-hydroxycapsidiol might be on a pathway of capsidiol degradation or metabolism in healthy pepper fruits. Evidently, the apparent conversion to water-soluble products (5,7,9) was, at least in part, an artifact of the solvents chosen for extraction. Hence, no firm evidence of further capsidiol metabolism by healthy pepper fruits currently exists. Somewhat similar procedures were used in studies of rishitin metabolism in potato tubers (3), and the partitioning of compounds such as 13-hydroxyrishitin (3,9) between ether and water could possibly lead to incorrect conclusions about their further metabolism.

Production of less 13-hydroxycapsidiol in inoculated than in healthy fruits was previously observed (7). However, this may have been an artifact also and is of doubtful significance in view of the present findings that capsidiol accumulation is not due to blockage of a metabolic pathway that involves 13-hydroxycapsidiol. Suggestions that glyceollin accumulation in soybeans treated with abiotic elicitors is due to blockage of a degradative pathway (11,12) also seem unlikely in the light of recent evidence (4). Phytoalexins accumulate following infection and then usually decline to low levels over a period of several days. Consequently, the assumption has been made that they are not end products of plant metabolism. The mechanisms by which they decline and the significance of these, if any, in the biochemistry of the moribund tissues of necrotic lesions, remain uncertain.

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