

## Metabolism of Capsidiol by Sweet Pepper Tissue: Some Possible Implications for Phytoalexin Studies

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### ABSTRACT

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The sweet pepper (*Capsicum frutescens*) phytoalexin capsidiol is metabolized to an appreciable extent by healthy pepper tissue. Gas-chromatographic analyses, and tracer experiments employing  $^{14}\text{C}$ -capsidiol, showed the disappearance of one-third to two-thirds of capsidiol that

had been added aseptically to pepper fruit. Almost all of the radioactivity not recovered in the form of capsidiol was recovered in a hydrophilic form. Some implications of these findings for current work on phytoalexins are discussed.

*Additional key words:* *Capsicum frutescens*, secondary metabolites, metabolic turnover.

Capsidiol, a sesquiterpene with the structure shown in Fig. 1 (4, 6) is produced by peppers in response to infection with a range of fungi (8) and has many of the properties postulated for phytoalexins (7, 8, 10). Like many other phytoalexins, the compound has been known for some time to be metabolized by certain fungi (9). The present paper presents evidence that capsidiol is metabolized also, to a significant extent, by healthy tissue of the plant that produces it. Although the nature of the product still is unknown, the available data have implications which are relevant to studies of phytoalexins in general.

### MATERIALS AND METHODS

Peppers (*Capsicum frutescens* L. 'Keystone Resistant Giant') were grown in the greenhouse. The fruits were harvested when semi-ripe and surface-sterilized with 70% alcohol before use.

Chemically pure, nonradioactive capsidiol was available from earlier studies (8). Radioactive capsidiol was prepared essentially as described by Brooks and associates (1, 2). A solution of sodium acetate- $1\text{-}^{14}\text{C}$  (New England Nuclear; 1.0 mCi in 37.3 mg) in water (40 ml) was added in equal portions to peppers (75 fruit halves) 18 hours after they have been inoculated as usual (8) with *Monilinia fructicola* spore suspensions. After incubating the fruit for a further 24 hours, crystalline, radioactive capsidiol (6.4 mg) was isolated from the diffusates by extraction with ether and chromatography of the extract on alumina (20 g; Camag DSF5) in methanol-chloroform (2:98, v/v). After dilution with cold capsidiol (51.5 mg),

the product was recrystallized from ethyl acetate to constant activity (39.5 mg;  $1.93 \times 10^5$  dpm/mg).

Radioactivities were assayed in a Nuclear Chicago scintillation counter (MK1) using the following solvent-fluor systems: (i) Oxifluor- $\text{CO}_2$  (New England Nuclear NEF 945); (ii) Aquasol (New England Nuclear NEF 934); (iii) Omnifluor (New England Nuclear NEF 906; 4 g/liter in toluene). Counting efficiencies were based on internal standardization with standard *n*-hexadecane- $1\text{-}^{14}\text{C}$ .

All solvents used were redistilled; alcohol was 96% ethanol. Capsidiol solutions in 4% alcohol were prepared by injecting 1 ml of a solution of the requisite amount of capsidiol in 96% ethanol into 24 ml of sterile water. Gas chromatography (GLC) was done as described previously (9). Thin-layer chromatography (TLC) employed methanol/chloroform (1:9, v/v) as irrigant and silica gel (Camag DF5) as adsorbent (0.30 mm on  $20 \times 20$  cm plates); phosphomolybdate (5%, w/v in alcohol) was used as spray reagent, with development at 110 C; a Panax RTSL-7A radiochromatogram scanner was employed to locate radioactive compounds. Evaporations were done below 30 C under reduced pressure provided by a water aspirator.

### RESULTS

Both an analytical method employing GLC and a radioactive tracer method were used to demonstrate changes in the concentration of capsidiol added to fruits:

**Estimation by gas chromatography (GLC).**—Eight fruits were halved lengthwise and the calyx and seeds were removed. Under sterile conditions, 3.0 ml of capsidiol solution ( $1.00 \times 10^{-3}\text{M}$ , in 4% alcohol) was added to one half of each fruit; and 3.0 ml of 4% alcohol was added to the other half as a control. The fruit halves then were

incubated at room temperature, in semi-darkness, in previously sterilized covered glass trays lined with moistened filter paper. After 48 hours, four of the eight halves in each of the two sets were homogenized with alcohol (100 ml) in a Waring Blendor for 75 seconds, to yield the "treated" and "control" homogenate, respectively. The other four halves from each set were homogenized likewise for 60 seconds, then 3.0 ml of  $10^{-3}$ M capsidiol solution was added to each set, and homogenization was resumed for an additional 15 seconds. These preparations were termed the "spiked and treated" and "spiked control" homogenates. Each of the homogenates was filtered and the filter was washed with alcohol (200 ml). The filtrate and washings were evaporated and the concentrate was distributed between methylene chloride ( $4 \times 100$  ml) and water ( $4 \times 100$  ml). The residue obtained from the methylene chloride layers was dissolved in chloroform (25 ml) and a portion (0.40 ml) was fractionated by preparative thin-layer chromatography (TLC) (10 plates). The zones containing capsidiol were scraped off and exhaustively eluted with methanol-chloroform (1:4, v/v). The filtered eluate was evaporated, the residue was taken up in methanol (0.50 ml), and its capsidiol content was determined by GLC. The results (Table 1) show that about 65% of the capsidiol fed to the peppers at the beginning of the experiment had disappeared, whereas the capsidiol used to spike the homogenates was recovered nearly quantitatively. Physical adsorption on particulate matter could not, therefore, account for the observed losses.

**Comparative radioactivity measurements.**—A dilute radioactive stock solution of capsidiol was prepared by dissolving capsidiol- $^{14}$ C (3.65 mg) and cold capsidiol (26.0 mg) in alcohol and diluting to 5.00 ml. A portion (4.00 ml) of this solution was used to prepare 100 ml of a  $1.00 \times 10^{-3}$ M solution of capsidiol in 4% alcohol. Aliquots (3.75 ml) of the latter solution were applied to each of 23 fruit halves (total of  $4.86 \times 10^5$  dpm applied) which then were incubated for two days at room temperature in a sterilized vacuum desiccator equipped with inlet and outlet tubes. The tubes were connected on the inlet side to a wash-bottle containing sterile water and on the outlet side to a tower containing calcium chloride, a wash-bottle containing toluene, and finally, a wash-bottle charged with Oxifluor- $\text{CO}_2$  solution (100 ml). Air was drawn slowly through the system during the incubation period.

At the end of the experiment, the radioactivity of the Oxifluor- $\text{CO}_2$  solution was indistinguishable from that of a blank, indicating that radioactive carbon dioxide had not been evolved.

The incubated fruits were homogenized with alcohol (1,000 ml), filtered, and the filtrate and alcohol washings were concentrated until mainly aqueous (225 ml). The product was extracted with methylene chloride ( $4 \times 250$  ml) and the extracts were washed in turn with three successive 50-ml portions of water. The methylene chloride extracts were combined, dried over sodium sulfate, filtered, and the filter was washed with more of the solvent. An aliquot (10 ml) of the combined filtrate and washings (1,100 ml) was evaporated under nitrogen and the apparent radioactivity (334 cpm) of the nonvolatile residue was measured in Omnifluor (10 ml). In order to correct for the strong quenching effects of pigments in the solution, a second, equal aliquot was spiked with 0.050 ml ( $7.04 \times 10^3$  dpm) of the dilute radioactive stock solution and counted likewise (1,150 cpm). Thus, the counting efficiency was 11.6%, indicating the presence of  $2.90 \times 10^3$  dpm of  $^{14}$ C in 10 ml, or of  $3.17 \times 10^5$  dpm in the total of the methylene chloride extract. This corresponds to a loss of 35% of the  $4.86 \times 10^5$  dpm contained in the capsidiol applied initially to the peppers. A narrow zone corresponding to capsidiol contained all the radioactivity that was detected by radio-TLC of the methylene chloride extract; peaks corresponding to one-twentieth of this activity would have been readily detectable elsewhere on the chromatogram.

The aqueous residue and washings from the methylene chloride extraction were combined and diluted to 450 ml. An aliquot (1 ml) was mixed with Aquasol (18 ml) and counted (250 cpm); a second aliquot was spiked with 0.050 ml of the dilute radioactive stock solution and also counted (6,010 cpm). Hence, by a calculation similar to the above (counting efficiency, 82%), 28% of the radioactivity originally supplied was found to be present in the aqueous fraction.

## DISCUSSION

The data presented above indicate that exogenous capsidiol is metabolized, to the extent of up to 65%, when it is incubated with healthy tissue of pepper fruit during 2 days. The observed losses were almost certainly not

TABLE 1. Recovery of capsidiol after metabolism by pepper fruit as estimated by gas chromatography (GLC)

	Capsidiol				
	Applied <sup>a</sup> (mg)		Recovered <sup>c</sup> (mg)	Lost	
	At time 0	At 48 hours (spike) <sup>b</sup>		(mg) <sup>d</sup>	(%)
Control	0.00	0.00	0.00	...	...
Spiked <sup>b</sup> control	0.00	0.71	0.66	0.05	7(4) <sup>e</sup>
Treated	2.83	0.00	0.89	1.94	69(62)
Spiked <sup>b</sup> and treated	2.83	0.71	1.78	1.76	62(66)

<sup>a</sup>Amount of capsidiol applied to two fruits.

<sup>b</sup>Spike applied during homogenization of two fruits.

<sup>c</sup>Capsidiol found in extract from two fruits by GLC as detailed in text.

<sup>d</sup>Difference between Applied and Recovered.

<sup>e</sup>Values in parentheses are from a duplicate experiment.

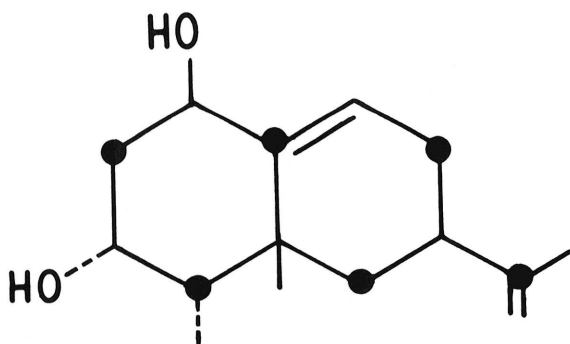


Fig. 1. Stereochemical structure of capsidiol. Heavy dots indicate carbon atoms derived from C-1 of acetate.

artificial because capsidiol is known from many earlier studies (e.g.; 6, 8, 9), to be stable to hydroxylic solvents, acids (including mineral acids) at moderate concentrations, light, and air. The experimental conditions under which the observations were made had been chosen to approximate those used in studies (e.g. 8) on the induction of capsidiol in peppers by fungi. In particular, the solutions applied to healthy pepper fruit in the present experiments were similar, with respect to volume and capsidiol concentration, to those which were collected from infected fruit in the earlier studies. Radioactive capsidiol, biosynthesized from acetate- $1-^{14}\text{C}$  and known to be labeled in the positions marked in Fig. 1, as determined by other workers (1, 2), was used in one experiment, both to confirm that metabolism occurred and also to facilitate the eventual isolation of the products. By these means, a substantial amount of radioactivity (28% of that originally supplied in the form of capsidiol) was located in the aqueous phase when the homogenate of the treated tissue was extracted with methylene chloride. The radioactivity in the organic extracts was associated only with unchanged capsidiol, as far as could be judged by radio-TLC. Together, the aqueous and methylene chloride fractions accounted for most of the radioactivity that had been applied to the peppers, and probably for all of it if unavoidable experimental losses and the errors inherent in the radioactivity measurements are taken into account. A search for radioactive carbon dioxide gave a negative result in this experiment. This, however, does not prove that degradation to carbon dioxide does not take place because this compound could arise from a nonlabeled position in the capsidiol molecule.

It is not yet known whether the metabolism of capsidiol involves extensive chemical changes, such as degradation to smaller fragments, or whether it merely represents a sequestration into a masked form, for instance, as a glycoside. Experiments are in progress to clarify this by the isolation and identification of the radioactive compounds present in the aqueous phase. However, the observations already available have implications of immediate significance. Thus, it can no longer be assumed

that there is a simple correspondence between the amount of capsidiol found and that biosynthesized in any given experiment with pepper tissue. This is likely to be of particular importance when different fungi are compared with respect to their potential to induce capsidiol. Further, it now seems possible that capsidiol might be a normal metabolite of peppers which, in healthy tissue, is metabolized as rapidly as it is formed. Its accumulation in infected pepper tissue might then represent a block of metabolic utilization rather than stimulated biosynthesis. The rapid metabolic turnover of secondary plant substances, a class to which most phytoalexins belong, although often ignored, has been documented before, for instance in mono- and sesquiterpenes (5) and flavonoids (3).

The capsidiol-*Capsicum* situation is unlikely to be unique and the possible metabolism of other phytoalexins by the tissues which produce them clearly will have to be considered in other cases. It is for this reason that we report our results at this preliminary stage.

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