Distribution of Radiocarbon in Valencia Oranges after Treatment with ^{14}C-Cycloheximide

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A procedure is presented for measuring the amount of radioactivity in various fractions of the orange after treatment with ^{14}C-cycloheximide. The distribution of radiocarbon throughout the orange was found in general to stabilize after approximately 72 hr. The largest amount of radioactivity was found on the peel and the least in the edible portion. Cycloheximide partially degraded on the surface of the orange. One of the degradation products was identified as anhydrocycloheximide.

Cycloheximide has been reported (Cooper *et al.*, 1969) to be an effective abscission agent for oranges. The mechanism by which cycloheximide (CYH) enhances abscission is not well understood. Rasmussen and Cooper (1969) reported CYH stimulated the production of ethylene (a known abscission agent) in some citrus. This investigation was undertaken to elucidate further the behavior of CYH when applied to the surface of an orange. Knowledge concerning both the amount and depth of penetration into the orange of CYH and its breakdown products is desirable in view of the possible use of this protein synthesis inhibitor as an agricultural chemical on oranges.

MATERIALS AND METHODS

A solution of ^{14}C-CYH was applied during September and October of 1970 to Valencia oranges on 4-year-old trees maintained in a greenhouse. This fruit developed from a late bloom and therefore matured at a later time than usual. However, all oranges were at the same stage of maturity.

^{14}C-CYH Solution and Its Application. ^{14}C-CYH (1.15 mCi per mmol; supplied by the Upjohn Co., Kalamazoo, Mich.) was prepared microbiologically. The distribution of radiocarbon was undetermined. This ^{14}C-CYH, radiochemically pure by autoradiography, was held as a stock solution (5.1×10^{-4} mCi per ml, 1.132×10^{6} dpm) in distilled water (pH 5.5) at 10° C.

An aliquot (500 μl, 5.66×10^{5} dpm) of this stock solution was pipetted into a liquid scintillation counting vial and dried in a vacuum desiccator. The residue was quantitatively dissolved in 1.00 ml of the Upjohn Co.'s CYH abscission formulation. The resulting solution was painted onto the surface of an intact orange, except for the area immediately adjacent to the stem, in order to avoid possible preferential penetration through the abscission zone.

The ^{14}C-CYH applied was equivalent to approximately 4.85×10^{5} dpm per orange. This value was obtained as the difference between the activity in disintegrations per minute pipetted into a counting vial and the activity in disintegrations per minute remaining in the vial and on the brush bristles after painting. All counts were corrected for background and quenching. This degree of radioactivity represents about 0.053 mg of CYH per orange.

Sample Preparation. 1. Oranges were picked after periods of 1, 2, 3, 5, and 7 days, respectively.

2. The intact fruit was rinsed with 250 ml of water.

3. The flavedo and albedo were successively removed with a potato peeler.

4. The flavedo was placed in a Waring Blendor with 100 ml of commercial Clorox (5.25% sodium hypochlorite, pH about 12, adjusted to a pH of 7.0 to 7.5 with carbon dioxide). This was to minimize pH deterioration of CYH.

5. The flavedo was then reduced to a fine suspension and decolorized by the Clorox. This minimizes color quenching when measuring radioactivity levels.

6. The pH of the suspension from Step 5 varied from 7.5 to 9 (perhaps due to loss of carbon dioxide). This suspension was adjusted to pH 6 to 7 with 30% hydrogen peroxide, which also destroyed any residual sodium hypochlorite or hypochlorous acid.

7. The resulting mixture was filtered through a supercel mat and the filter cake washed with (3×100 ml) water.

8. The filtrate from Step 7 was extracted three times with an equal volume of chloroform, which removed the radioactivity from the aqueous layer.

9. This chloroform extract was reduced to about 5 ml *in vacuo* at a bath temperature of 26° C. Final evaporation to dryness was in a counting vial equipped with an adapter which allowed evaporation to be carried out *in vacuo* at 26° C.

10. The water rinse of the peel from Step 2 was evaporated under reduced pressure and temperature not exceeding 40° C to approximately one-half the original volume. This final volume was carried through the above procedure starting with Step 8.

11. The albedo was treated the same as the flavedo from Step 4.

12. The endocarp remaining from Step 3 was squeezed with a household rotary extractor.

13. The expressed juice was centrifuged and the resulting clear supernate decolorized with Clorox as in Step 4.

14. The pH of the decolorized juice was then adjusted as in Step 6.

15. This juice was then carried through Steps 8 and 9.

16. The rag resulting from Step 12 and the pellet from Step 13 were combined and processed from Step 4.

17. All filter cakes from Step 7 were air-dried and extracted in a Soxhlet with chloroform for 24 hr. These chloroform extracts were added to their respective chloroform fractions from Step 8.

Each of the five experiments, comprising samples taken after 1, 2, 3, 5, and 7 days, was performed in triplicate (Table I).

Efficiency of Radiocarbon Recovery. Known amounts of

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Table I. Percentage Distribution of Radiocarbon in Valencia Oranges

Days after application	Water wash of peel surface		Peel				Endocarp				% of Activity accounted for	
			Flavedo		Albedo		Rag		Juice			
	Mean % recovered	Std dev	Mean % recovered	Std dev	Mean % recovered	Std dev	Mean % recovered	Std dev	Mean % recovered	Std dev	Mean % recovered	Std dev
1	83.3	3.8	4.5	0.3	2.2	0.9	1.4	0.9	0.2	<0.1	91.7	1.7
2	72.0	1.1	12.7	0.8	5.0	0.2	1.4	0.3	0.7	0.1	92.0	0.4
3	59.3	0.8	17.7	1.7	9.9	0.8	0.9	<0.1	0.7	0.1	88.6	2.3
5	58.0	0.7	19.1	0.9	10.6	0.1	0.7	<0.1	0.8	0.1	89.4	0.2
7	58.2	0.9	19.7	0.4	10.8	1.6	0.9	0.2	0.7	<0.1	90.5	1.5

^{14}C-CYH were added to ^{14}C-free control samples. These individual samples were carried through their steps of the procedure. Recoveries of radiocarbon from triplicate determinations of the individual fortified controls averaged 90% or better.

Determination of Radioactivity Levels. The residue from Step 9 above was dissolved in approximately 19 ml of a solution consisting of 5.0 g of PPO per l. of toluene for direct counting with a Beckman liquid scintillation spectrometer (Model LS-100). All counts were corrected for background and quenching. The quench curve was prepared from a solution of ^{14}C-labeled toluene standardized in disintegrations per minute. The samples were stored in the dark for at least 24 hr prior to counting in order to eliminate errors due to phosphorescence.

Thin-Layer Chromatography (tlc). Both 20- × 20-cm plates coated with silica gel G (0.25 mm) and polyamide plates prepared according to Nordby *et al.* (1966) were used.

Developing solvents employed with the silica gel G were: A, ethyl acetate–isopropyl alcohol (98:2); B, ethyl acetate–methyl alcohol (98:2); C, dimethylformamide–methylene chloride (5:95); D, nitromethane–benzene (1:1); E, methyl alcohol–methylene chloride (10:90); F, methyl alcohol–methylene chloride (2:98); G, methyl alcohol–chloroform (5:95); H, pyridine–hexane (1:2) with the polyamide; I, ethyl acetate–methyl alcohol (95:5); J, methylene chloride–methyl alcohol (98:2).

Identification of Anhydrocycloheximide (AnCYH). A concentrated chloroform extract of the peel water wash obtained from an orange which had been treated with CYH 3 days previously was streaked on silica gel tlc plates and developed in solvent system A. The area marked AnCYH in Figure 1 was located by autoradiography, scraped from the plate, and eluted with methyl alcohol. Portions of the concentrated eluate were rechromatographed with unlabeled, authentic AnCYH (The Upjohn Co.) in the ten tlc systems described above. The chromatographed material was located by a combination of autoradiography followed by spraying with 10% (w/v) phosphomolybdic acid in 95% ethyl alcohol. Heating the sprayed plates at 100° C for several minutes gave a blue spot on a yellow background.

Ultraviolet spectra were obtained in 95% ethyl alcohol with a Beckman Model DK-2 spectrophotometer. Mass spectra were obtained with a Bendix (TOF) Model 3012 mass spectrometer. The source was operated at 70 eV and 80° C.

RESULTS AND DISCUSSION

The distribution gradient of radioactivity from the peel surface to the juice (Table I) indicates that neither CYH nor its degradation products are readily translocated into the endocarp. This persistence of the radiocarbon on or in the peel supports the view by Cooper *et al.* (1969) that the abscission enhancing property of CYH is associated with the rind.

The percent distribution of radioactivity throughout the orange (Table I) should not be considered as exclusively representing cycloheximide. In fact, autoradiography of a sample prepared from the water wash of the orange peel exposed to CYH for 3 days showed several radioactive areas in addition to CYH (Figure 1). The orange exposed to CYH for 7 days displayed the same pattern with a slight increase in the intensity of the AnCYH area.

Since the water wash of the peel samples was not exposed to the pH, Clorox, and hydrogen peroxide treatment during the sample preparation, it is unlikely that these samples contain artifacts. This was demonstrated by rinsing the peel 15 min after application of the ^{14}C-CYH solution. Processing of the rinse water, followed by autoradiography, showed only CYH.

The predominant spot in Figure 1 labeled CYH corresponds to cycloheximide. The compound recovered from the region designated AnCYH and authentic anhydrocycloheximide (the dehydration product of cycloheximide) displayed identical R_f values in the same tlc system. The R_f values in systems A, B, C, D, E, F, G, H, I, and J were 0.55, 0.72, 0.53, 0.26, 0.83, 0.20, 0.49, 0.40, 0.80, and 0.85, respectively. The two compounds were coincident in all the above systems when cochromatographed.

Both compounds exhibited identical uv and mass spectra. The uv spectra showed maximum absorption at 241.5 nm. The molecular ion was at m/e 263 (78%).

Cycloheximide (mol wt 281) readily loses water under electron impact to give a strong anhydro ion (m/e 263). However,

Figure 1. Tlc autoradiogram showing the degradation of ^{14}C-CYH 3 days after application to the surface of an orange

the unknown material was recovered from the tlc plate in an area less polar than CYH and produced a uv spectrum which CYH does not. This precludes the possibility that the molecular ion *m/e* 263 (78%) was actually obtained as the electron impact dehydration product of CYH.

AnCYH applied at 0.05 mg/orange to Valencia oranges showed no evidence of abscission by pull force measurements. Also, the Upjohn formulation without CYH did not stimulate abscission.

Attempts by tlc and autoradiography to isolate the compound or compounds responsible for the radioactivity in the endocarp and peel were unsuccessful. This failure was primarily due to the presence of interfering unlabeled compounds that were co-isolated and caused streaking. There is the possibility that if ^{14}C compounds had been separated they could be artifacts produced by the sample preparation.

The approximate 90% recovery of radiocarbon from both the individual fractions fortified with ^{14}C-CYH and the ^{14}C-CYH solutions painted on the oranges (Table I) shows that the procedure does not convert the CYH molecule to volatile or chloroform insoluble compounds to a degree that would prohibit use of the method. An aliquot of the aqueous layer after chloroform extraction (Step 8 of the sample preparation) was dried and counted. This aliquot after correction for the batch showed insignificant radioactivity above background.

An abscission agent must of necessity be applied near harvest, so it is imperative that the chemical be either innocuous, leave little residue in edible material, or leave a residue which can be easily removed. The above results with ^{14}C-CYH under the conditions described have some practical significance in that 58 to 83% of the applied radiocarbon was removed by simple washing and 2% or less was found in the endocarp.

ACKNOWLEDGMENT

The author thanks Joseph A. Hawarah and Sue G. Bartlett for technical assistance.

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Received for review March 4, 1971. Accepted May 7, 1971. Florida Agricultural Experiment Stations Journal Series No. 3899.