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Metabolism of [14C]Naphthaleneacetic Acid in Kinnow Mandarin

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Kinnow mandarin fruits were dipped in aqueous solutions of [14C]α-naphthaleneacetic acid and extracts of the tissue were examined for radioactive constituents. The total amount of radioactivity per fruit remained relatively constant for the 240 days of the experiment, but concentrations decreased during fruit growth and development. A significant amount of the NAA was converted into 1-naphthaleneacetylaspartic acid and 1-β-D-glucose-α-naphthaleneacetate. Smaller amounts of two additional metabolites were found, but they were not identified. Naphthaleneacetic acid and the two major metabolites were identified by gas–liquid chromatography–mass spectrometry.

Among the various known fruit thinning agents, α-naphthaleneacetic acid (NAA) shows the most promise for use on citrus (Hield and Hilgeman, 1969). Before it can be approved for use, however, it is necessary to develop residue and metabolite information. Disappearance and residue studies on citrus have been completed (Coggins *et al.*, 1972). This investigation was undertaken to determine the metabolites of NAA in Kinnow mandarin fruits. Similar work has been reported for various tissues in more than 25 species of plants (Klämbt, 1961; Shindy *et al.*, 1971; Veen, 1966; Zenk, 1962). The objective of this study was to identify the major metabolites and quantitatively determine the relative changes that occur among the metabolites during fruit growth and development.

MATERIALS AND METHODS

A solution containing [14C]NAA was applied during May of 1970 to Kinnow mandarin fruiting branches on mature trees grown in Coachella Valley. The small young fruits were approximately 10 mm in diameter at the time of application.

[14C]NAA Solution and Its Application. A supply of carboxyl-labeled [14C]α-NAA with a specific activity of 16.0 mCi/m*M* was obtained from Tracer Lab, Waltham, Mass., and a purity of greater than 98% was confirmed. The treatment solution was prepared by adding 6.4 mg of the radioactive material (containing 85.9 μCi/mg) and 0.1 ml of Tween-20 to 200 ml of 400 ppm cold NAA. Fruit Fix Super Concentrate 800 (a commercial formulation containing the ammonium salt of NAA) was used as the source of cold NAA. This gave a ratio of cold to labeled material of 12.5:1 or a specific activity of 1.18 mCi/m*M*. The terminal 3–4 in. of fruit and leaf-bearing branches were dipped into the resulting solution. An average of 0.7 ml of the treatment solution was retained per treated unit.

Sample Preparation. Samples were collected first at 6 hr and then at 4, 8, 15, 30, 60, and 240 days after treatment. The number of fruits per sample varied from 36 to 9, depending upon fruit size. Fruits were washed three times with 80% ethanol for 2–3 min and the combined wash solution was evaluated for radioactivity. Fruits were divided into three subsamples, weighed, and analyzed as whole fruit, except for the last sample where fruit were separated into peel and pulp tissue. The fruit tissue was homogenized with 150 ml of 80% ethanol and transferred to a 250-ml round-bottomed flask with an additional 100 ml of fresh 80% ethanol. The macerate was refluxed on a steam bath for 2 hr and cooled, and the supernatant was decanted. The solids were boiled again for 1 hr in 100 ml of fresh 80% ethanol and the supernatant was decanted again. This procedure was repeated a third time. The supernatant was then filtered through Whatman no. 42 filter paper. The filter paper and residue were homogenized with 50 ml of 80% ethanol and filtered again. The total extracts were combined and concentrated to 25 ml under vacuum. A fraction of the solid material was examined for radioactivity to assess extraction efficiency.

The concentrated extract was centrifuged at 16,300 × *g* for 10 min and the supernatant was decanted and saved. A 5-ml aliquot of 80% ethanol was added to the precipitate, stirred, and recentrifuged. This procedure was repeated. The amount of radioactivity in an aliquot of the combined supernatant was used to calculate total radioactivity/gram of fruit tissue and the precipitate was examined for radioactivity to determine whether significant losses were associated with this step in the procedure.

Determination of Radioactivity Levels. An aliquot of the extract was dissolved in approximately 14 ml of a solution (consisting of 5 g of PPO and 100 g of naphthalene/liter of dioxane) for direct counting with an Ansitron liquid scintillation spectrometer. All counts were corrected for background and quenching by using 14C-labeled toluene as an internal standard.

Thin-Layer Chromatography (tlc). Glass plates (20 × 20 cm) coated with silica gel G (0.50 mm) and the following developing solvents were used: A, isopropyl alcohol–ammonium hydroxide–water (10:1:1 v/v); B, isopropyl alcohol–benzene–water (8:1:1 v/v); C, isopropyl alcohol–ammonium hydroxide–water (3:1:1 v/v).

Distribution of Radioactivity in Various Metabolites. To separate various metabolites, an aliquot of 100 μl from the extract was spotted on tlc and developed for 2–3 hr in solvent A. The air-dried plates were scanned for radioactivity by using a Varian Aerograph Scanner (Model LB-2723) and four radioactive zones were located. The silica gel from each zone was carefully scraped off and placed into 15-ml centrifuge tubes and shaken for 1 min after adding 3 ml of 80% ethanol. Each tube was then centrifuged and the supernatant was saved. Another 3 ml

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Table I. Radioactivity Extracted from Kinnow Mandarin Fruits after Treatment with [^{14}C]NAA

Time after treatment	Surface radioactivity,[a] cpm/fruit	Total extracted radioactivity,[b] cpm/g	Percent of radioactivity contained in zones[c]			
			I	II	III	IV
6 hr	13,920	37,884	16.3	11.1	35.1	21.6
4 days	8,372	45,326	27.9	8.9	29.5	31.1
8 days	8,009	43,841	26.1	9.3	34.9	30.5
15 days	5,355	22,911	28.8	9.4	34.1	34.5
30 days	3,649	6,403	24.9	10.3	30.1	21.7
60 days	4,022	6,207	28.5	10.4	38.7	11.5
240 days	58	3,262	23.2	18.1	41.2	0.5

[a] Amount of radioactivity removed from fruit surfaces with three washes of 80% ethanol. [b] Total radioactivity extracted from fruits. Extraction efficiency was approximately 99% in the early samples and gradually decreased to 95% by the end of the study. [c] Expressed as percentage of total extracted radioactivity shown in column 3. These zones accounted for 83% or more of the extracted amount.

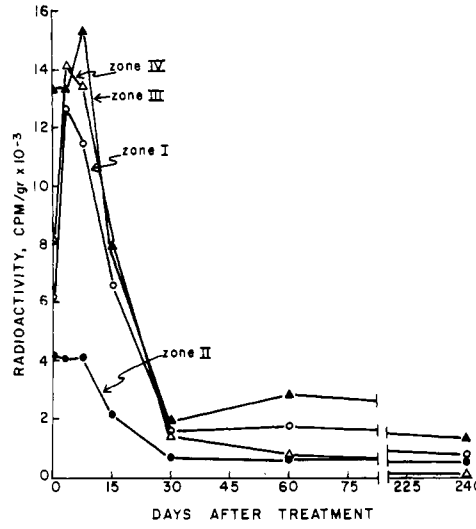

Figure 1. Concentration of NAA and its metabolites extracted from Kinnow mandarin fruits at specified times after treatment with [^{14}C]NAA.

of 80% ethanol was added and the previous step was repeated. Finally the ethanol extracts were combined and the final volume was made up to 10 ml. A 3-ml aliquot was taken from the ethanol extract for each zone and counted for radioactivity. Data were reported as averages of three separate tlc runs.

Identification of Radioactive Metabolites. In order to obtain sufficient quantities of different metabolites, the above separation techniques were used except that a number of tlc plates were streaked with larger quantities of fruit extract (0.5–1.0 ml). After developing and scanning, silica gel from equivalent zones was collected, combined, extracted with 80% ethanol, shaken, and filtered through filter paper. After concentrating the ethanol extract, new tlc plates were streaked and the process was repeated. Finally, each extract representing a zone was spotted as one spot per plate for development in two-dimensional tlc using solvent A in the first direction and solvent C in the second one. The tlc plates were scanned and the silica gel representing one spot was removed and extracted three times with 80% ethanol. An aliquot of clean metabolite was transferred to a reacti-vial and dried under a nitrogen stream on a warm (50°) hot plate.

A 100-μl aliquot of *N,O*-bis(trimethylsilyl)acetamide reagent (BSA), Pierce Chemical Company No. 38837, was added to the reacti-vial containing the dried residue. The vial was immediately capped and heated gently (50°) for 30 min before its contents were analyzed by gas–liquid chromatography (glc) using a Varian Aerograph (Model 1200) equipped with a flame ionization detector and linear temperature programmer. This system employed a stainless steel column (⅛ in. × 5 ft) packed with 3% QF-1 on 60/80 mesh Chrom Q operating under the following conditions: nitrogen carrier flow, 66 ml/min; hydrogen flow, 30 ml/min; air flow, 600 ml/min; injector temperature, 200°; and detector temperature, 275°. The temperature programming started at 100° and increased linearly to 250° at the rate of 8°/min. Aliquots of 1.0–5.0 μl were injected into the glc. Authentic standards were handled in the same manner as unknowns.

The gas–liquid chromatography–mass spectrometry (glc–ms) identifications were accomplished with a Finnigan mass spectrometer (Model 1015C) interfaced to a Varian Aerograph glc (Model 1400) with a Goelke all-glass separator. Two-second mass spectra were taken at 70 eV and 300 μA ionizer current. The same column and conditions used before in glc alone were used again for glc–ms identification, except that helium was used as carrier gas instead of nitrogen. The unknowns were identified by comparison of retention times and mass spectra with authentic standards and/or by the interpretation of mass spectral data.

RESULTS AND DISCUSSION

Total and Relative Distribution of Radioactivity. The use of a larger quantity of cold NAA relative to the radioactive NAA served two purposes: first, it was necessary to have the rate of treatment the same as that of a normal field application so that a similar physiological response could be obtained; and second, it was desirable to obtain as much material as possible for identification purposes.

Complete washing of fruit before extraction, with 80% ethanol, was performed in order to remove all surface [^{14}C]NAA and photodecomposition products (Table I). Breakdown products resulting from weathering phenomena and exposure of NAA to uv light would have created products not necessarily associated with metabolism (Crosby and Tang, 1969). It is unlikely that the ethanol wash caused artifacts. This was demonstrated by washing the fruit surface with 80% ethanol right after application of [^{14}C]NAA, spotting, developing, and scanning the tlc plates. All radioactivity was present as NAA.

The amount of radioactivity extracted per fruit was essentially constant throughout the experiment (data not shown). This agrees with the conclusion of other investigators (Crosby *et al.*, 1970; Luckwill and Lloyd-Jones, 1962; Veen, 1966; Zenk, 1962) that the loss of NAA and/or its metabolites is slow. When expressed as the amount/g of fresh weight, however, total extracted radioactivity reached a maximum at 4 days after treatment and then decreased to a low level by the time the 240-day samples were harvested (Table I). The increase probably resulted from translocation of NAA from leaves into fruits (Crosby *et al.*, 1970; Luckwill and Lloyd-Jones, 1962). The decrease probably was due to dilution during growth (Coggins *et al.*, 1972).

The amount of radioactivity associated with NAA and its metabolites is shown in Table I and Figure 1. Appreciable conversion of NAA (zone III) to metabolites was apparent at the earliest sampling date. The relative amounts (Table I) of zone I and II metabolites remained high throughout the experiment. The metabolite that is represented by zone IV, however, decreased substantially by 60 days and was almost absent at the end of the experiment. On a concentration basis (Figure 1), NAA and its metabolites decreased rapidly as the fruits increased in size.

Identification of Various Metabolites. The R_f values for zones I, II, III, and IV in solvent A were 0.1, 0.3, 0.5,

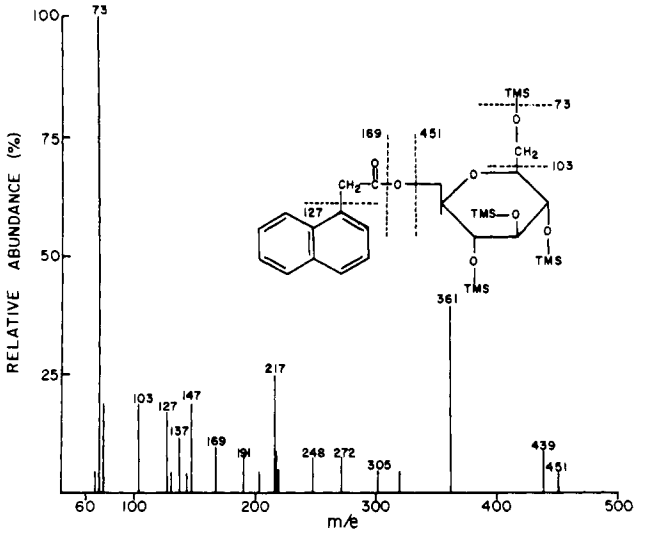

Figure 2. Mass spectrum of the TMS derivative of the metabolite from zone IV. TMS represents $(SiMe_3)^+$.

and 0.8, respectively. Naphthaleneacetylaspartic acid and naphthaleneacetic acid were coincident with metabolites from zones I and III, respectively, when cochromatographed in solvent systems A, B, and C. The sequence and relative intensity of tlc spots were parallel to those isolated by Zenk (1962) from pea epicotyl, Veen (1966) from coleus, and Shindy *et al.* (1971) from olives. Thus, it appears that the same major metabolites occurred in all of these plants.

The trimethylsilyl (TMS) derivatives of NAA and naphthaleneacetylaspartic acid had the same glc retention times as the TMS derivatives of zones III (12.5 min) and I (5.1 min), respectively.

Autoradiographic examinations of tlc plates revealed that zone II contained two metabolites. Although we were unable to obtain enough material for positive identification, circumstantial evidence suggests that these metabolites are 5-hydroxy-NAA (Klämbt, 1961) and glucosyloxy-NAA (Crosby *et al.*, 1970).

Hydrolysis of zones I and IV by HCl caused a shift in R_f value to that similar to zone III, which has been identified as NAA. Furthermore, glc–ms studies of compounds from zones III and I gave mass spectra that were identical to NAA and naphthaleneacetylaspartic acid, respectively. Enzymatic hydrolysis with β-glucosidase had no effect on metabolites from zones I and II, but it released NAA from zone IV. Since we did not have an authentic standard to compare the suspected metabolite of zone IV, the full mass spectrum is shown (Figure 2). From the mass spectrum, in conjunction with the results of the β-glucosidase treatment, we can identify it as 1-β-D-glucose-α-naphthaleneacetate. The molecular ion peak was absent, but the most intense peak (100%) was *m/e* 73 for the $(SiMe_3)^+$ ion. The other most intense peaks are those at *m/e* 217 and 361, corresponding to the ions $(Me_3SiOCH{=}CHCH{=}OSiMe_3)^+$ and $(C_{15}H_{33}O_4Si_3)^+$, respectively. The *m/e* of 361 is the result of the glucosyl ion, 451 less 90, for $[(CH_3)_3SiOH]$ (see Figure 2).

The presence of a peak at *m/e* 127 is an indication of a naphthalene ring. The ion $[Me_3SiOCH{=}C(OSiMe_3)CH{=}OSiMe_3]^+$ having *m/e* 305 is an indication of carbohydrates. Meanwhile, the presence of relatively intense peaks at *m/e* 147 and 103 is also another characteristic of the mass spectra of trimethysilyl ethers of the monosaccharides (Chizhov *et al.*, 1967). The following structures are the most probable for these ions: $(Me_3SiO{=}SiMe_2)^+$ and $(CH_2{=}OSiMe_3)^+$.

All the above evidence indicates that the metabolite present in zone IV is indeed 1-β-D-glucose α-naphthaleneacetate.

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