Table III. Comparative Examination of Diphenyl Absorption by Washed and Waxed vs. Unwashed Honey Tangerines Using the *t* Statistic for Two Means

harvest	storage time, weeks	no. of diphenyl pads	level of significance, %, for storage at 4 °C	21 °C
A	2	1	0.5	0.5
A	2	2	1.0	NS[a]
A	4	1	0.5	NS
A	4	2	0.5	NS
B	2	1	0.5	0.5
B	2	2	0.5	NS
B	4	1	0.5	0.5
B	4	2	0.5	NS

[a] NS = nonsignificant.

with two pads, between washed and unwashed, showed no significant differences in diphenyl absorption levels at 2 and 4 weeks. Apparently the diphenyl vapor pressure from two pads at 21 °C was high enough to overcome the barrier protection of the natural wax coating. This premise appears valid in light of past results by Hayward and Edwards (1964), who showed that waxed and unwaxed oranges packed with two pads absorbed similar amounts of diphenyl when stored at 21 °C.

This study investigated the extent of diphenyl absorption on two tangerine harvests because previous investigations by Rajzman (1965) and Wardowski et al. (1979) indicated that less mature fruit absorbed more diphenyl than more mature fruit. Lemons and oranges with green peel color, for example, absorb more diphenyl than fully colored fruit (Norman et al., 1969, 1971). The results in Tables I and II show with some minor exceptions that fruit harvested in January absorbed higher average amounts of diphenyl than fruit of the February harvest. However, statistical evaluation of the data was inclusive. Further studies on other tangerine cultivars are required to verify the influence of maturity on diphenyl absorption.

Honey tangerines should be stored and shipped at cool temperatures (~4–10 °C) because of the decay rate and the extent of diphenyl absorption at the higher temperatures. Storage of fruit with two diphenyl pads affords the greatest protection against decay but transit temperature and storage time must be carefully monitored because the fruit is exposed to a greater vapor concentration of diphenyl.

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Metabolism of 2,5-Dichloro-4-hydroxyphenoxyacetic Acid in Plants

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The 2,4-dichlorophenoxyacetic acid (2,4-D) metabolite, 2,5-dichloro-4-hydroxyphenoxy[1-^{14}C]acetic acid ([*acetic*-1-^{14}C]-4-OH-2,5-D) was prepared. The labeled compound was taken up by red currant and carrot shoots and by the roots of intact bean, wheat (durum), and corn plants. After 48 h the tissue was freeze-dried and extracted. From 19 to 32% of the label remained in the residual plant material. A small portion of the radioactivity, 1–10%, was recovered as [^{14}C]carbon dioxide. From 9 to 31% of the [^{14}C]-4-OH-2,5-D was found unchanged and 15–50% was recovered after treatment of the extracts with emulsin. A large portion of the radioactivity, 29–40%, was highly polar material and not identified. The chromatograms of the plant extracts suggested that the metabolic products were similar.

The metabolism of 2,4-dichlorophenoxyacetic acid (2,4-D) in plants has been studied by many laboratories over the past three decades. This, in part, reflects the complexity of the herbicide's metabolism and the variation in activity of the enzymes which affect the compound in different plant species. As recently as 1977 a previously undiscovered metabolite of the herbicide was identified in plant tissue (Chkanikov et al., 1977), and there are yet plant metabolites of 2,4-D which have not been chemically

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Table I. Recovery of ^{14}C in Fractions Obtained from Plants Fed [*acetic*-1-^{14}C]-4-OH-2,5-D as the Percent of Radioactivity Absorbed

plant	$Ba^{14}CO_3$	bound to plant residue	fraction I	fraction II	fraction III	fraction IV	^{14}C recovered
corn	3	27	27	12	<1	29	98
bean	10	32	21	3	<1	24	90
carrot shoots	1	20	11	13	<1	52	97
red currant shoots	6	19	18	19	<1	37	99
wheat (durum)	6	19	35	15	2	17	94

characterized (Feung et al., 1978).

Two major pathways of the herbicide in plants are the conjugation of the carboxyl group of 2,4-D to amino acids (Andreae and Good, 1957; Klämbt, 1961; Feung et al., 1971, 1972, 1973, 1975) and the hydroxylation and conjugation of the aromatic moiety at the 4 position (Thomas et al., 1964; Fleeker and Steen, 1971; Feung et al., 1975). The 2,4-D–amino acid conjugates have been found not to be end products but to be metabolically active substances which can undergo further chemical alteration in plant tissue (Feung et al., 1973; Davidonis et al., 1980). This has led us to examine the metabolic activity of a major product of 2,4-D from the hydroxylation pathway, 2,5-dichloro-4-hydroxyphenoxyacetic acid (4-OH-2,5-D).

MATERIALS AND METHODS

Ethyl bromo[*carboxyl*-^{14}C]acetate and 4-methoxy-2,5-dichlorophenol were used in the preparation of [*acetic*-1-^{14}C]-4-OH-2,5-D. The synthesis has been described by Drinkwine (1979) in detail. The specific activity of the product was 1.60 μCi/mg, and the substance found to be radiochemically pure as determined by thin-layer chromatography (TLC) and high-pressure liquid chromatography.

Corn (*Zea mays*), wheat (*Triticum durum*), and bean (*Phaseolus vulgaris*) were grown in Vermiculite under incandescent and fluorescent light at $\sim$160 lx and with a 16-h photoperiod. The plants were watered with half-strength Hoagland's solution (Hoagland and Arnon, 1938). At the 3–4 leaf stage the roots were rinsed free of Vermiculite and the plants used in the metabolism studies. Carrot (*Daucus carota*) shoots were cut from garden grown plants which were 5–6 weeks old. The red currant (*Ribes sativum*) shoots were the most recent growth from garden shrubs.

The shoots or roots of the whole plants, 18–20 g fresh weight, were allowed to take up a solution of 1.0 mg/mL [*acetic*-1-^{14}C]-4-OH-2,5-D in one-quarter strength Hoagland's solution, pH 6.5, for a period of 6 h. The excess precursor solution was rinsed off and the plants were transferred to fresh one-quarter strength nutrient solution. The plants were then placed in a 2-L glass chamber. Air was drawn through the chamber at $\sim$100 mL/min, and from the chamber it was drawn through two gas spurging bottles containing 1 M NaOH. Incubation in the chamber continued 48 h, with the traps being changed at 12-h intervals. $BaCl_2$ (1 M) was added to the trapping solution to precipitate $Ba^{14}CO_3$. The $Ba^{14}CO_3$ was collected by centrifugation, washed with water twice and then once with 95% ethanol, dried, and weighed. The plants were immediately frozen and later freeze-dried.

The plant tissue was ground in a mortar and extracted in a Soxhlet thimble for 18 h each with methylene chloride, benzene–95% ethanol (2:1), and 95% ethanol. Extraction with 95% ethanol did not further dissolve significant amounts of radioactivity. The combined extracts were concentrated to a small aqueous volume and diluted with water to 30–40 mL. The fractionation of this extract essentially followed the protocol described by Feung et al. (1976). The pH of the extract was adjusted to 2 with 1 M H_3PO_4, and the mixture extracted 3 times with diethyl ether. The ether extract was designated fraction I. The aqueous phase was further extracted 3 times with 1-butanol. The remaining aqueous mixture was designated fraction II. The butanol extracts were evaporated to dryness on the rotary evaporator at 30 °C, and the residue was taken up in 15–20 mL of water. After adjustment of the pH to 5 with 1% $NaHCO_3$ (w/v), the mixture was treated with emulsin (Nutritional Biochemical Corp.) for 24 h at 25 °C. The mixture was acidified to pH 2 and was extracted twice with diethyl ether. The remaining aqueous phase was designated fraction III and the ether extract, fraction IV.

The residual plant material remaining after extraction was dried at 50 °C. A 200–300-mg portion was placed in a culture tube with 20 mL of 2 M HCl, and the tube sealed and heated in a steam bath 2 h. The hydrolysate was cooled and centrifuged, and the supernatant chromatographed on paper and silica gel G plates.

The radioactivity of $Ba^{14}CO_3$ was determined by the method of Jeffay and Alvarez (1961). Liquid samples were analyzed for ^{14}C in a liquid scintillation counter. The solvent was toluene–Triton X-100 (2:1) containing 1% PPO and 0.1% dimethyl-POPOP. For these samples, [^{14}C]toluene (New England Nuclear, NES-006) was used as the internal standard. The residual plant material was combusted in a Harvey biological oxidizer and the $^{14}CO_2$ collected in a scintillation solvent-trapping solution purchased from Harvey Instruments Inc., Hillsdale, NJ. The eficiency of the biological oxidizer and scintillation counter was determined with [^{14}C]benzoic acid (New England Nuclear, NES-001). Chromatograms were cut into 1-cm strips, placed in scintillation vials, and assayed for radioactivity after addition of scintillation solvent. Reverse-isotope dilution analysis of [^{14}C]-4-OH-2,5-D in extracts was performed as described by Fleeker and Steen (1971).

Paper chromatography was performed on Whatman No. 1 paper, and the chromatograms were developed with 1-butanol–95% ethanol–3 M NH_3 (12:4:15). Silica gel TLC plates (Baker, 4463) were developed with diethyl ether–hexane–formic acid (70:30:2) and with chloroform–methanol–water (60:40:5).

RESULTS AND DISCUSSION

One of the minor metabolic products of [*acetic*-1-^{14}C]-4-OH-2,5-D in the plants was radioactive carbon dioxide (Table I). This may reflect a metabolic process similar to that observed with 2,4-D, in which the carboxymethyl group is removed as a two-carbon fragment that may subsequently be oxidized to carbon dioxide (Fleeker, 1973). Most plants oxidize 1–10% of the carboxymethyl moiety of 2,4-D to carbon dioxide, although a few species, such as red currant, carry out the process at much higher rates (Luckwill and Lloyd-Jones, 1960a,b). The data in Table I indicate that 4-OH-2,5-D as well as 2,4-D can undergo loss of the carboxymethyl group in plants. Dichlorophenol is found in plant tissue treated with 2,4-D (Chkanikov et al., 1965; Steen, 1972); therefore, the de-

Table II. Recovery of [^{14}C]-4-OH-2,5-D Found Bound, Free, and Conjugated in Plants as the Percent of the Absorbed [^{14}C]-4-OH-2,5-D

plant	fraction			released from plant residue by hydrolysis[b]	total [^{14}C]-4-OH-2,5-D recovered
	I[a]	II[b,c]	IV[c]		
corn	24	<1	28	14	66
bean	16	<1	22	14	52
carrot shoots	9	<1	50	9	68
red currant shoots	16	<1	35	8	59
wheat (durum)	31	<1	15	9	55

[a] Found as the free compound. [b] Released by treatment with 2 N HCl at 100 °C for 2 h. [c] Released by emulsin.

carboxymethylation process observed with 2,4-D as a precursor does not require formation of the hydroxylated metabolite prior to removal of the side chain.

Between 19 and 32% of the ^{14}C from [^{14}C]-4-OH-2,5-D was incorporated into plant material which could not be solubilized by extraction with methylene chloride, 95% ethanol, or benzene–95% ethanol (2:1) (Table I). Hydrolysis of the residual material from each plant with 2 N HCl for 2 h released approximately half of the bound radioactivity (Table II). With each species, essentially all of the ^{14}C released was identified as [^{14}C]-4-OH-2,5-D.

The herbicide 2,4-D is incorporated into trichloroacetic acid (Cl_3AcOH) insoluble substances or polymeric plant material to an extent that varies from species to species (Luckwill and Lloyd-Jones, 1960a,b; Rakitin et al., 1966; Chkanikov and Pavlova, 1966; Chkanikov et al., 1968; Dexter et al., 1971). In only a few instances has the insoluble material been characterized as to whether it is the herbicide that is actually incorporated or a metabolite such as 4-OH-2,5-D. Rakitin et al. (1966) estimated that 80% of the label from [^{14}C]-2,4-D was incorporated into the Cl_3AcOH-insoluble material in oat tissue in 3 days. The radioactivity was released by mild acid hydrolysis and found to be 2,4-D. Other plant species are reported to bind ^{14}C to Cl_3AcOH-insoluble material when treated with side-chain-labeled 2,4-D (Chkanikov and Pavlova, 1966; Chkanikov et al., 1968; Dexter et al., 1971). In these cases the chemical nature of the bound ^{14}C was not reported.

Luckwill and Lloyd-Jones (1960a) found that 10–30% of the radioactivity of [*acetic*-1-^{14}C]-2,4-D taken up by red and black currant leaves was bound to the tissues in a form which could not be removed by solvent extraction or mild hydrolysis. A pesticide analogue of 2,4-D, 2-methyl-4-chlorophenoxyacetic acid (MCPA), was found to undergo extensive cleavage at the ether linkage in *Galium aparine* (Leafe, 1962). When [*acetic*-1-^{14}C]MCPA was applied to the plants, the radioactivity was found predominantly in the protein, nucleic acids, and tissue residues. When the precursor was [^{36}Cl]MCPA, most of the label remained in the water-soluble fraction.

These reports again reflect the complexity of the metabolism of 2,4-D and other phenoxy herbicides in plant tissues. They suggest that more than one route exists for the incorporation of the carbon atoms of 2,4-D into the polymeric substances or Cl_3AcOH-insoluble fraction of plant tissues. Our data suggest yet another route exists, i.e., via the metabolite of the herbicide, 4-OH-2,5-D. This route may become significant in the plant over a period of time as the metabolite accumulates in the tissues.

Analysis of fraction I by paper chromatography and reverse-isotope dilution indicated that most of the radioactivity in this fraction was [^{14}C]-4-OH-2,5-D in all five plant species (Tables I and II). The radioactivity not moving with the precursor on the chromatograms remained at or near the origin. Feung et al. (1978), in their studies on 2,4-D metabolism, observed substances with an R_f of 0.42–0.51, slightly ahead of 4-OH-2,5-D, which were tentatively identified as the amino acid conjugates of 4-OH-2,5-D and other hydroxylated metabolites of 2,4-D. We employed similar chromatographic conditions and did not observe substances with comparable chromatographic mobility. This may be a result of the experimental conditions. We used a 2-day incubation period and their study employed a 14-day period.

Amino acid conjugates of 4-OH-2,5-D were first discovered in plants as the glycosides (Chkanikov et al., 1977) and may account for some of the [^{14}C]-4-OH-2,5-D present in fraction IV (Tables I and II). Most of the radioactivity in fraction IV was [^{14}C]-4-OH-2,5-D, which was released by emulsin treatment (Table II). Emulsin cleaves the amide bonds of the derivatives as well as the β-glycosidic bond (Feung et al., 1978); thus, these substances could not be identified by the methods used here. On the other hand, the absence of these compounds in fraction I may be explained if the 2,4-D–amino acid conjugates are necessary precursors of the corresponding 4-OH-2,5-D–amino acid conjugates. In this case, the latter could not arise in plant tissues where 2,4-D was not present.

Much of the radioactivity in all five plant species was present in chemical forms that did not release [^{14}C]-4-OH-2,5-D on brief hydrolysis. This uncharacterized ^{14}C, represented by the difference between the total radioactivity recovered (Table I) and the total [^{14}C]-4-OH-2,5-D recovered (Table II), ranged from 29% in carrot tissue to 40% in red currant shoots. This material is of a diverse chemical nature as it was present in all the soluble fractions, especially fraction II, and the residual material after extraction and hydrolysis. Most of the unidentified soluble material in fraction II had a much lower mobility on TLC and paper chromatography than [^{14}C]-4-OH-2,5-D and thus appears to be of greater polarity than the precursor.

This study has demonstrated that 4-OH-2,5-D can undergo extensive metabolic change in plants. The compartmentation of cellular enzymes and the transport mechanisms of the plant cell may result in considerable differences in metabolism when a substance is formed within a cell as opposed to an origin from without. It therefore remains unclear whether the transformations observed here actually reflect the metabolism of 4-OH-2,5-D in plants when it arises in vivo from 2,4-D hydroxylation.

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Tetrachlorvinphos Metabolism in Laying Hens

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Tetrachlorvinphos metabolism was studied, with the aid of a ^{14}C-labeled compound, in laying hens fed 50 ppm of the insecticide. Approximately 71% of the radioactivity was eliminated in 24 h in excreta. After the final dose, an additional 1.3 and 2.6% of total ^{14}C was excreted during the next 3 and 7 days. Eggs laid within 24 h of the treatment contained radioactivity. ^{14}C was also detected in tissues and organs at an insecticide equivalent in the ppm range in kidney, liver, and abdominal fat but in the ppb range elsewhere. After dosing was discontinued, the ^{14}C content of excreta, eggs, tissues, and organs gradually decreased. Compounds identified in excreta were desmethyl tetrachlorvinphos (25%), 2,4,5-trichloromandelic acid (30.5%), and tetrachlorvinphos (1.0%). During the treatment period, all tissues examined contained small amounts of the insecticide, but only traces were detected in tissues and organs from hens killed 7 days after the last dose.

The use of pesticides in poultry and animal production has increased considerably in recent years. Therefore, considerable effort is being made to ensure that edible tissues, organs, milk, and eggs marketed for human consumption are free of pesticide residues.

It is well documented that, on ingestion, a pesticide is absorbed from the gastrointestinal tract into the blood or lymph and thus distributed to various tissues, organs, eggs, milk, etc. and finally eliminated by excretion in feces and urine or excreta (Loomis, 1968). During this cycle, the pesticide may face a variety of biochemical attacks which could result in the formation of products or metabolites which are different in nature and behavior than the original insecticide.

Tetrachlorvinphos (Rabon, Stirofos, etc.) is an organophosphate insecticide which has shown great promise in the control of ectoparasites in poultry. Previous studies on the fate of tetrachlorvinphos fed to laying hens indicated only a very limited transfer of residues to tissues and eggs (Sherman and Herrick, 1971; Wasti and Shaw, 1971; Yadava and Shaw, 1970). A sufficient amount of the insecticide was reported to be eliminated in the excreta of hens to control ectoparasites (Wasti et al., 1970). Residues of the insecticide were detected in body fat and egg yolk of hens after their dust boxes and litter were treated with a tetrachlorvinphos formulation (Ivey et al., 1969).

The following is a report detailing the metabolism and distribution of tetrachlorvinphos and metabolites in various tissues, organs, and eggs of laying hens which had free access for 7 consecutive days to a standard laying ration fortified with the insecticide at the 50-ppm level and given a daily oral dose of [^{14}C]tetrachlorvinphos during this period.

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EXPERIMENTAL SECTION

Chemicals. Pesticide-grade solvents (Caledon Laboratories Ltd., Georgetown, Ontario, Canada) were used as received. Esterification reagents, 14% (w/v) BF_3–MeOH and 10% (w/v) BCl_3–2-chloroethanol, were obtained from Applied Science Laboratories, State College, PA. Bovine liver β-glucuronidase (5000 Sigma Units/mL) was purchased from Sigma Chemical Co., St. Louis, MO. Pure samples of ^{14}C-labeled and unlabeled tetrachlorvinphos (I), desmethyl tetrachlorvinphos (II), 2,4,5-trichlorophenacyl chloride (III), 2,4,5-trichloroacetophenone (IV), 1-(2,4,5-trichlorophenyl)ethanol (V), 2-chloro-1-(2,4,5-trichlorophenyl)ethanol (VI), (2,4,5-trichlorophenyl)ethane-1,2-diol (VII), and 2,4,5-trichloromandelic acid (VIII) and its methyl ester were available from a previous study (Akhtar and Foster, 1977).

Chicken Treatment and Collection of Tissues. Eight Single Comb White Leghorn hens, approximately 1.5 years old and at 70% production, were kept in individual laying cages as described previously (Foster et al., 1972). They were fed a standard laying ration for 7 days while the daily feed intake was recorded. This was followed by a 7-day period during which the hens were fed the standard laying ration containing 50 ppm of tetrachlorvinphos and were given a daily oral dose (1.86×10^5 dpm, 450 μg) of radiolabeled [*vinyl*-^{14}C]tetrachlorvinphos in Risella oil (Shell Canada Ltd.). This was followed by a further 7-day period during which the hens were fed the standard laying ration. In both latter periods, records were kept of daily feed intake.

Excreta and eggs were collected on 24-h basis. Eggs were stored at 4 °C and excreta at −20 °C in closed glass jars

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