and compare positive and negative ion spectra with those of the methyl esters.

Essentially no success resulted from attempts to trap and characterize either the methyl esters or the methanol-pesticide reaction products. It was first throught that this difficulty was due only to the high volatility of the methyl esters. However, when the Plasma Chromatograph was coupled to the gas chromatograph through a 10-in. long piece of heated 1/16-in. stainless steel tubing, it was obvious that essentially no material was getting through to the PC other than solvent, even when 100-ng amounts of methyl ester standard were injected into the gas chromatograph. The PC was easily able to detect 1 ng of pesticide or methyl ester standard using the direct probe approach. Either the organophosphate pesticides and methyl esters were irreversibly absorbed onto the metal surfaces or were decomposed to products that the PC could not detect.

Thin-layer chromatography of both the pesticides and methyl esters after trapping from the gas chromatographic column showed a large number of spots containing phosphorus. This occurred even when the transfer line running from the end of the column to the liquid N_2 trap was an 8 in. × 1/8 in. piece of heated glass tubing. However, the Porapak columns themselves appeared to produce no detectable breakdown when the detector was in place, other than a diminution of peak heights when the column temperature ranged over 230°.

The somewhat unexpected high precision with which retention times of both the ester standards and products from pesticide injections could be measured enhanced the credibility of structure assignments. Retention times were timed with a stopwatch from point of injection. Peaks with retention times as long as 8.5 min could be reproduced upon five serial injections within 2 sec. Retention times on the order of 5 min or less could be reproduced

within 1 sec. The retention times for Azodrin, Ronnel, and compound 4072 and their corresponding esters were all coincident within ±1 sec of variation. Parathion and diazinon, however, consistently gave retention times 2 sec later than diethyl monomethyl thiophosphate when the Porapak Q column was operated at 60 ml/min and 250°. By operating the column at 20 ml/min and 260°, the DEMMTP peak split into two components, the second coinciding exactly with the 8-min 24-sec peak of parathion and diazinon. The first peak was probably a decomposition product of the thiolate, since it increased in size with increasing column temperature while the thiolate peak diminished.

The coupling of the rubidium sulfate AFID, which is highly sensitive to organic nitrogen and phosphorus, to an on-column transesterification step and subsequent separation of the products would seem to be of value as a one-step screening technique for the presence of both organophosphate and carbamate pesticides.

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Human Exposure to Organophosphorus Pesticides. A Modified Procedure for the Gas–Liquid Chromatographic Analysis of Alkyl Phosphate Metabolites in Urine

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A previously described method for analysis of alkyl phosphates (Shafik and Enos, 1969) has undergone a major modification. The new method is more suitable for use in a monitoring program designed to determine human exposure to organophosphate pesticides. Improvements in the methodology include preparation of a less volatile ester derivative of the alkyl phosphate and selection of gas chromatographic columns and conditions which are more compatible with systems used in a routine chlorinated hydrocarbon pesticide residue program. The average recovery of the six dialkyl phosphates from human urine fortified at the 0.1-ppm level was 98.3%. The amyl derivatives of the dialkyl phosphates showed a remarkable increase in sensitivity compared to the methyl and ethyl derivatives on two gas chromatographic columns normally used in pesticide residue analysis. Data illustrating the application of the new method to a human monitoring program are presented.

The gas chromatographic determination of various dialkyl phosphates has been based on preparation of their respective methyl and ethyl ester derivatives and analysis by gc employing a phosphorus sensitive detector (Askew et al., 1960; Shafik et al., 1971; St. John and Lisk, 1968). The available methods are lengthy, especially the gas

chromatographic step, the stability of the derivatives is questionable, and urinary inorganic phosphate interferes with the quantitation of low levels of the derivatized dialkyl phosphates. The application of these methods to large numbers of urine samples indicated the urgent need for certain modifications in order that the method could be used in a monitoring program.

The purposes of this investigation were to speed up gas chromatographic analysis, find a more suitable column packing for the separation of dialkyl phosphate derivatives, prepare more stable derivatives, increase the num-

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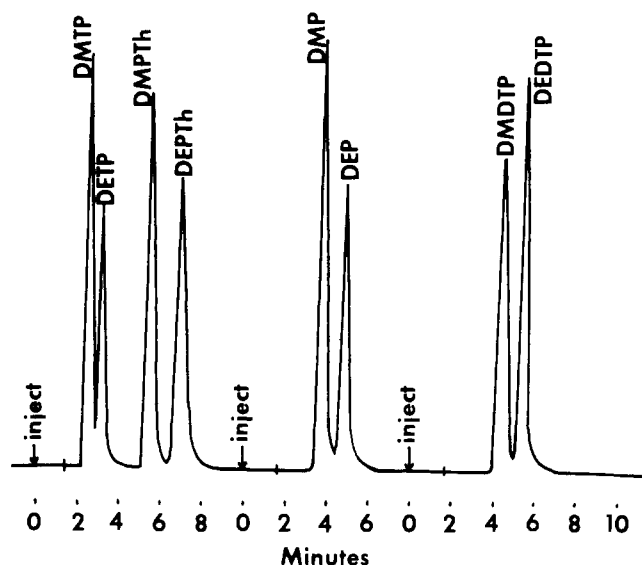
Figure 1. Chromatograms of the amyl derivatives of the three working standard mixtures of the dialkyl phosphates using the 5% OV-210 column and the flame photometric phosphorus detector.

ber of samples analyzed per week (up to 20–30 samples a week), and eliminate the interference caused by the derivatization of any inorganic phosphate present in the urine extract.

EXPERIMENTAL SECTION

Apparatus and Glassware. A Micro-Tek MT-220 gas chromatograph equipped with a dual flame photometric detector (FPD) operating in the phosphorus and sulfur modes is used in the analysis. A Valco =CV 4 HT switching valve is interfaced between the gas chromatographic column and the detector and heated by the transfer block. Glass columns, 6 ft × ¼ in. packed with 5% OV-210 on Chromosorb W, H.P., 80/100 mesh or 4% SE-30/6% OV-210 on Gas Chrom Q, 80/100 mesh, were prepared according to Thompson *et al.* (1969) and used in this investigation. Both columns were conditioned and treated with Carbowax 20M according to Ives and Guiffrida (1970) to reduce tailing of the derivatized alkyl phosphates and increase their detector response. The operating conditions of the gas chromatograph were: column, 165–175°; inlet, 200°; detector, 200°; transfer line and switching valve, 200°; nitrogen (carrier) and nitrogen (purge) flow rates, 30–40 ml/min; hydrogen, 180 ml/min; air, 80 ml/min; and oxygen, 10 ml/min.

The samples were concentrated with a nitrogen evaporator equipped with a water bath maintained at 40° (N-EVAP, Organomation Associates, Worcester, Mass.). Size 23 Chromaflex columns (Kontes, =K-420100) were used for the silica gel column chromatography. Mixing of solutions was accomplished with a Vortex-Genie mixer (Scientific Industries, Inc., Springfield, Mass.) and a Roto-Rack, Model 96 (Fischer, =14-057).

Reagents. The formic acid reagent was a 1% solution of formic acid in benzene. The extracting solvent was a 1:1 (v/v) mixture of acetonitrile and diethyl ether. Anhydrous sodium sulfate, AR, was washed with benzene and stored in a 170° oven. Silica gel, activity grade I (Woelm, Waters Associates, Inc., Framingham, Mass.) was activated at 170° for at least 24 hr before use.

The diazopentane reagent was prepared in the following manner. Dissolve 2.3 g of KOH in 2.3 ml of distilled water in a 125-ml Erlenmeyer flask. Cool in a freezer, add 25 ml of cold diethyl ether (AR with 2% ethanol), cover flask with foil, and return to freezer for 15 min. In a high draft hood, add 2.1 g of N-amyl-N'-nitro-N-nitrosoguanidine

(Aldrich Chemical Co., Milwaukee, Wis.) to the solution in the flask in four or five portions, swirling the flask vigorously after each addition. Decant the ethyl ether layer into a 1-oz-capacity reagent bottle fitted with a Teflon-lined screw cap and store in a freezer. The precautions supplied by Aldrich Chemical Company for preparation and handling of these reagents must be followed closely.

Preparation of Standard Solutions. In 15-ml graduated glass-stoppered centrifuge tubes accurately weigh amounts of standard sodium or potassium dialkyl phosphate salts (available from American Cyanamid Co.) equivalent to 10 mg of the potassium salt of each dialkyl phosphate. To each tube, add 2 drops of 6 N HCl and sufficient diazopentane reagent to produce a persistent yellow color (2–5 ml). Allow to stand in a high-draft hood for 20 min, and then remove the excess reagent by adding formic acid reagent dropwise until the yellow color *just* disappears. Dilute the solution(s) exactly to 10 ml with benzene, stopper, mix thoroughly, and store in a freezer.

Pipet aliquots of the individual standards into three volumetric flasks to prepare the following binary mixtures (see Table I for abbreviations used throughout this paper).

I	II	III
DMTP 10 ng/μl	DMDTP 5 ng/μl	DMP 10 ng/μl
DETP 10 ng/μl	DEDTP 5 ng/μl	DEP 10 ng/μl

Prepare working standards by diluting 1 ml of each mixture to 50 ml with hexane. The final concentration of the working standards is dictated by detector response. The sensitivities stated or implied throughout the text can be achieved by optimizing chromatographic and detector parameters.

The quantitation of the derivatized alkyl phosphates was based on peak height using the phosphorus mode of the flame photometric detector. DMTP and DETP isomerize under the conditions of the derivatization procedure, producing a mixture of their respective thionate and thiolate esters. Quantitation is based on DMAP, DEAP, DMATP, DEATP, DMADTP, and DEADTP. Inorganic phosphate is converted to TAP in the analytical procedure. Figure 1 shows chromatograms of the amyl derivatives of dialkylphosphates using the 5% OV-210 column and the phosphorus mode. The presence of sulfur-containing alkyl phosphates may be confirmed by operating the FPD in the sulfur mode.

The Determination of Alkyl Phosphates in Urine. Pipet a 2-ml aliquot of the urine sample into a 15-ml centrifuge tube. Add 2 g of NaCl, 4.0 ml of extracting solvent, and 1 ml of 6 N HCl, in that order. Immediately stopper the tube, mix on a Vortex mixer for 1 min, and centrifuge at 2000 rpm for 1 min. Transfer 2.0 ml of the organic solvent layer to a graduated 13-ml centrifuge tube with a disposable pipet. Working in a high draft hood, add 2 ml of diazopentane reagent to the extract or sufficient reagent to produce a persistent orange color. Allow to stand for 20 min. No interruptions should occur until the completion of derivatization.

In the hood, concentrate the amylated extract to about 0.2 ml using the nitrogen evaporator. Add 5 ml of water, about 5 g of NaCl, and 5 ml of hexane. Stopper and mix vigorously on the Vortex mixer for 1 min. Allow the layers to separate by standing or centrifugation, and transfer the hexane layer to a 13-ml graduated centrifuge tube. Reextract the aqueous layer with 2 ml of hexane, transferring the hexane layer to the centrifuge tube.

Partially deactivate silica gel by adding 2.0 ml of water to 10 g of silica gel in a glass vial which has a Teflon-lined screw cap. Place on the Roto-Rack for 1–2 hr at about 50 rpm. Deactivated silica gel may be stored in a tightly closed container for 1 week without affecting the elution

Table I. List of Dialkyl Phosphates and Derivatives

DMP	O,O-Dimethyl phosphate
DEP	O,O-Diethyl phosphate
DMTP	O,O-Dimethyl phosphorothionate
DETP	O,O-Diethyl phosphorothionate
DEDTP	O,O-Diethyl phosphorodithioate
DMDTP	O,O-Dimethyl phosphorodithioate
TMP	O,O,O-Trimethyl phosphate
DEMP	O,O-Diethyl O-methyl phosphate
DEMTP	O,O-Diethyl O-methyl phosphorothionate
DEMPTh	O,O-Diethyl S-methyl phosphorothiolate
DEMDTP	O,O-Diethyl O-methyl phosphorodithioate
TMDTP	O,O,O-Trimethyl phosphorodithioate
DMEP	O,O-Dimethyl O-ethyl phosphate
DMETP	O,O-Dimethyl O-ethyl phosphorothionate
DMEPTh	O,O-Dimethyl S-ethyl phosphorothiolate
TEP	O,O,O-Triethyl phosphate
DMAP	O,O-Dimethyl O-amyl phosphate
DEAP	O,O-Diethyl O-amyl phosphate
DMATP	O,O-Dimethyl O-amyl phosphorothionate
DEATP	O,O-Diethyl O-amyl phosphorothionate
DMAPTh	O,O-Dimethyl S-amyl phosphorothiolate
DEAPTh	O,O-Diethyl S-amyl phosphorothiolate
DMADTP	O,O-Dimethyl S-amyl phosphorodithioate
DEADTP	O,O-Diethyl S-amyl phosphorodithioate
TAP	O,O,O-Triamyl phosphate

Table II. Recoveries of Dialkyl Phosphates from Human Urine Fortified at the 0.1-ppm Level

Alkyl phosphate	Avg recovery, % (three determinations)	Range, %
DMTP	95.8	94.0–97.2
DETP	100.5	98.9–102.0
DMDTP	98.7	98.3–99.2
DEDTP	99.8	99.5–100.0
DMP	97.9	97.0–98.5
DEP	97.9	96.0–99.1

patterns of alkyl phosphates. Weigh 2.4 g of the deactivated silica gel and transfer to a size 23 Chromaflex column plugged with glass wool. Tamp firmly and top with about 1 in. of anhydrous Na₂SO₄. Prewash with 10 ml of hexane and discard the eluate.

Evaporate the hexane extract of derivatized alkyl phosphates to 1 ml. If standards are being chromatographed, evaporate an aliquot of the mixed standards to 0.1 ml and dilute to 3 ml with hexane.

Transfer the hexane solution to the silica gel column just as the last of the prewash sinks into the column. Rinse the tube with 2-ml portions of hexane, adding serially to the column until a total of 10 ml of hexane has been added. Place a 13-ml graduated centrifuge tube under the column and elute with 10 ml of 30% methylene chloride–hexane (fraction I). Continue elution with 20 ml of 1% acetone–methylene chloride, discarding this eluate. Place a 25-ml concentrator tube under the column and elute with 20 ml of 5% acetone–methylene chloride (fraction II). Fraction I contains amyl derivatives of the thionate isomers of DMTP and DETP. Although DMDTP and DEDTP are not commonly detected in urine, if present they will appear in this fraction. Fraction II contains the amyl derivatives of DMP and DEP. The discarded fraction contains interfering material, triamyl phosphate arising from alkylation of inorganic phosphate, and amyl derivatives of the thiolate isomers of DMTP and DETP.

Elution patterns of the three standard mixtures and spiked urine extracts may vary from one laboratory to another, depending on conditions such as temperature and relative humidity. This emphasizes the need for establishing elution patterns under local conditions before at-

Table III. Comparison of Detector Response to Derivatives of Alkyl Phosphates

	Detector sensitivity to ester derivative (4:1 signal-to-noise ratio), ng		
Dialkyl phosphate	Methyl	Ethyl	Amyl
DMTP		1.5	0.11
DETP	1.0		0.14
DMP		1.5	0.07
DEP	0.4		0.09
DMDTP	15.0		0.06
DEDTP	1.0		0.07

Table IV. Relative Retention Times of Amyl and Hexyl Derivatives of Dialkyl Phosphates on Two Columns

Dialkyl phosphate	5% OV-210[a]		4% SE-30/6% OV-210[b]	
	Amyl	Hexyl	Amyl	Hexyl
DMTP	0.61	0.66	0.73	0.75
DETP	0.77	0.77·	1.00	0.98
DMPTh	1.47	1.36	1.61	1.55
DEPTh	1.85	1.66	2.21	2.07
DMP[c]	1.00	1.00	1.00	1.00
DEP	1.28	1.18	1.42	1.30
DMDTP	1.05	1.02	1.46	1.44
DEDTP	1.32	1.23	2.00	1.88

[a] Column was operated at 173° for amyl derivatives and at 190° for hexyl derivatives. Flow rates for both derivatives are 35 ml/min. [b] Column was operated at 173° for amyl derivatives and at 190° for hexyl derivatives. Flow rates for both derivatives are 60 ml/min. [c] The retention time of DMP was approximately 3.9 min.

tempting to analyze samples. Volumes may be changed if necessary to prevent splitting between fractions.

Aliquots of 5–25 μl may be injected into the gas chromatograph from fractions I and II. In the case of low levels of alkyl phosphates in the sample (0.01 ppm), fractions I and II may be concentrated to 1 ml using the nitrogen evaporator and 5-μl injections can be made.

RESULTS AND DISCUSSION

Limits of detectability and recovery of known amounts of dialkyl phosphates from rat and human urine have been established in previous work (Shafik and Enos, 1969; Shafik et al., 1971). Recoveries from human urine spiked at levels of 0.01–0.05 ppm and rat urine spiked at levels of 0.05–0.25 ppm were in the range of 80–100%.

In a recovery study using the modified method, human urine was spiked at 0.1 ppm. The results of this study are shown in Table II. The levels of alkyl phosphates in control human urine were high enough to preclude spiking at lower levels.

Table III compares detector response of methyl and ethyl derivatives of dialkyl phosphates (as determined by the previous method) with amyl derivatives (by the modified method). The modified method permits detection of appreciably smaller amounts of trialkyl phosphates. The remarkable increase in sensitivity of the amyl derivative of DMDTP over the methyl derivative indicates that the amyl derivative is perhaps much more stable and less inclined to undergo decomposition during gas chromatography.

The relative retention times of n-amyl and n-hexyl derivatives of the dialkyl phosphates on two different chromatographic columns are shown in Table IV. The combination of the 5% OV-210 column and amyl derivatives of the dialkyl phosphates was found to give the most satisfactory results. The amyl derivatives elute rapidly from the gc, giving sharp peaks with adequate resolution. This in turn allows many more samples to be injected into the

Table V. Concentration of Alkyl Phosphates in Representative Urine Samples from Individuals with No Record of Exposure, ppm

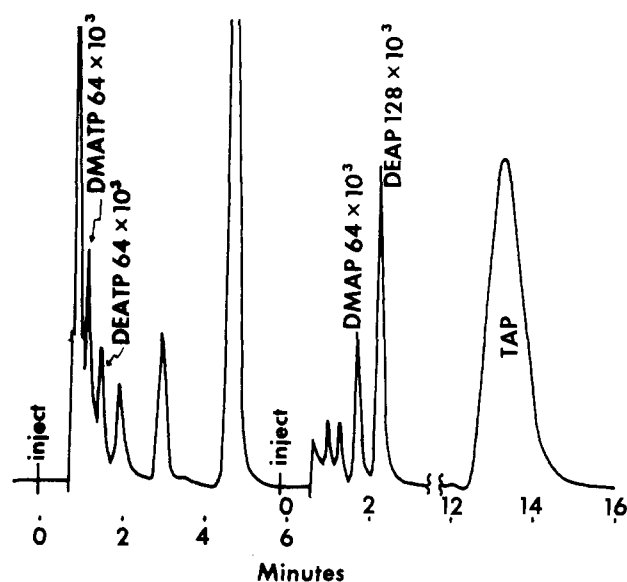| Urine samples	DMP	DEP	DMTP	DETP
1	0.01	0.08	0.09	ND[a]
2	0.04	0.04	0.10	ND
3	<0.005	0.05	0.07	ND
4	0.01	0.08	0.03	ND
5	<0.005	0.04	0.02	ND
6	0.01	0.03	0.06	0.01

[a] ND, not detected.

Table VI. Concentration of Alkyl Phosphates in Representative Urine Samples from Individuals Exposed to Dasanit, Thimet, and Di-Syston, ppm

Urine samples	DMP	DEP	DMTP	DETP
1	0.01	0.42	0.09	0.18
2	0.06	2.41	0.22	1.62
3	0.02	1.16	0.08	0.80
4	0.01	0.34	0.06	0.16
5	0.02	0.79	0.06	0.32
6	<0.005	0.70	0.06	0.52

Figure 2. Chromatograms of a urine sample containing low levels of DMP (0.02 ppm), DEP (0.17 ppm), DMTP (0.06 ppm), and DETP (0.06 ppm).

gas chromatograph than was possible in the previous method. Confirmation of any particular compound may be accomplished by preparing the hexyl derivative. The method describing the preparation of diazopentane is followed, except that N-hexyl-N'-nitro-N-nitrosoguanidine is used as the diazoalkane precursor. Further confirmation may be achieved by injecting the samples on a 4% SE-30/6% OV-210 column.

Fifty-five urine samples were provided by the State Services Program, Office of Pesticide Programs, Environmental Protection Agency. These represented: 14 samples from persons with no record of exposure to organophosphorus pesticides; 27 samples from persons employed in a formulation plant where they were exposed to Dasanit, Thimet, and Di-Syston; and 14 samples with less clearcut exposure histories. The urine samples were analyzed by the modified method presented above, and representative results from the exposed and unexposed groups are presented in Tables V and VI. The four dialkyl phosphates—DMP, DEP, DMTP, and DETP—are the only ones found in these samples, even though DEDTP may theoretically result from metabolism of Thimet and Di-Syston. All urine samples, regardless of exposure history, were found to contain at least some dialkyl phosphates. A typical chromatogram of a urine sample from an "unexposed" individual with low levels of four dialkyl phosphates is shown in Figure 2.

By the previous method, both methylation and ethylation of each urine sample were necessary to try to circumvent interferences caused by inorganic phosphates. Despite this, low levels of a derivative eluting just after the inorganic phosphate peak (TMP or TEP) were difficult to quantitate accurately. Preparation of amyl derivatives of the dialkyl phosphates results in less volatile and more thermally stable derivatives. The use of the amyl derivative coupled with silica gel cleanup eliminates the interference formerly encountered from inorganic phosphates. Traces of inorganic phosphate which are not removed by silica gel elute from the gas chromatographic column as triamyl phosphate and are well separated from the derivatized dialkyl phosphates. The derivatives may be gas chromatographed on standard columns at temperatures in the range of 170–190°, which are compatible with conditions employed in a pesticide residue laboratory. The in-

creased detector response permits the use of smaller urine samples (1 ml instead of 5 ml) and smaller injections (5-μl equivalents of urine instead of 500 μl), thus prolonging the life of the column.

Silica gel column chromatography has become an essential part of the modified method. The new gas chromatographic system gives incomplete resolution of DMAP from DMADTP and DEAP from DEADTP. It is therefore necessary to separate these compounds before glc analysis. In addition, amylation of urine extracts results in an increase in compounds which interfere with the gas chromatographic analysis of alkyl phosphates, and silica gel chromatography is necessary to remove most of these interfering substances. A convenient result of silica gel chromatography is the separation of triamyl phosphate and the two thiolate isomers from the fractions containing the remaining alkyl phosphates.

As has been noted, under the conditions of the modified method described above, DMTP and DETP each react with diazopentane to produce a mixture of thionate and thiolate isomers. This is in contrast to the previous method where the thiolate isomer of DMTP was not evident (Shafik et al., 1970). Quantitation of DMTP and DETP are based on the thionate isomers DMATP and DEATP, respectively.

The modifications described in this paper improve two features of the method which are critical to a routine monitoring program. First, one chemist can analyze 25–30 samples per week by the method, as compared to 8–10 with the previous version. Second, the gas chromatographic conditions are the same as those used for other multiresidue determinations of pesticidal compounds.

The excretion of alkyl phosphates in the urine can be detected at organophosphate exposure levels much lower than those which result in cholinesterase inhibition. In addition, the general class of the organophosphate compound involved in the exposure may be deduced through the characterization of the metabolite(s) excreted. Confirmation of the identity of the alkyl phosphate metabolite is obtained by determining the relative retention time for both the hexyl and amyl derivatives on each of two gas chromatographic columns.

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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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