Uptake, Translocation, and Metabolism of Tirpate in Tobacco *Nicotiana tabacum*

James E. Hill and Robert I. Krieger*

[^{14}C]Tirpate [(2,4-dimethyl-1,3-dithiolane-2-carboxaldehyde *O*-(methylcarbamoyl)oxime] was administered to young tobacco *Nicotiana tabacum* plants in hydroponic culture. The radiolabel was rapidly taken up and translocated throughout the shoot. Young leaves sampled 12 days following treatment were labeled less heavily than older leaves indicating that the Tirpate and its metabolites were not rapidly retranslocated. Tirpate and three major metabolites were extracted with organic solvent and were chromatographed by silica gel TLC. The half-life of Tirpate in the plant was 8–9 hr. The first metabolite was identified as Tirpate sulfoxide. The lyophilized aqueous fraction was hydrolyzed with acid, glucosidase, and sulfatase. Water-soluble conjugates were quantitated but not identified.

Tirpate [(2,4-dimethyl-1,3-dithiolane-2-carboxaldehyde *O*-(methylcarbamoyl)oxime] (Table I, ENT 27696) is an oxime–carbamate with acaricidal, insecticidal, and nematicidal activities. Tirpate is particularly effective against a number of economically important nematode genera. This compound is a potent anticholinesterase and in the male rat it has an LD_{50} of 1.2 mg/kg. The fate and persistence of Tirpate and its potentially toxic metabolites have not been described.

Tirpate is structurally similar to the insecticide Temik [(2-methyl-2-(methylthio)propionaldehyde *O*-(methylcarbamoyl)oxime]. Experiments on the metabolism of Temik in plants have been reported in cotton (Metcalf et al., 1966; Coppedge et al., 1967; Bartley et al., 1970) and potato (Andrawes et al., 1971), and they offered some clues to the fate of Tirpate in plants. This report describes the root uptake, translocation, and metabolism of Tirpate in tobacco plants. The plants were grown in hydroponic culture to facilitate rapid treatment and subsequent study of the kinetics of metabolism.

EXPERIMENTAL SECTION

Chemicals. A preparation of [^{14}C]Tirpate labeled in the tetrasubstituted position (Table I) (specific activity 200 μCi/mmol) was supplied by the Agrichemical Laboratory, 3M Co., Minneapolis, Minn. The radiopurity of the tracer was shown to be greater than 99% by thin-layer chromatography (TLC). Nonlabeled Tirpate standards and some candidate metabolites (Table I) were also supplied by the Agrichemical Laboratory. Their identity was confirmed by infrared and mass spectroscopic analysis.

Hydroponic Culture and Treatment. Tobacco, *Nicotiana tabacum* (var. Coker 319), seeds were planted in a greenhouse (26 °C) in synthetic media consisting of sand–peat (1:1) and the required nutrients in a dry form. Two-week old seedlings were root-washed and transferred to aerated half-strength nutrient solution (Huffaker et al., 1970) in large plastic containers. Five- to six-week old plants were selected for uniformity and treated with [^{14}C]Tirpate as follows. Five milliliters of half-strength nutrient solution containing 1 μCi (about 1 mg) of [^{14}C]Tirpate was placed in a 125-ml filter flask and aerated using capillary tubing through the side arm. Plants were removed from the plastic containers and roots were blotted dry and immersed in the treatment solution. Label was taken up in about 15 min and was followed by two 20-ml chases with nonlabeled nutrient solution. The total treatment time was approximately 2 hr. Following treatment, the plants were transferred back to the original plastic containers and harvested periodically thereafter. Two plants were treated similarly to those described above but with a total of 235 mg of Tirpate (0.59 μCi/mmol) in 350 ml of half-strength nutrient solution. These plants were used to obtain larger quantities of the major metabolites for identification.

Sampling and Harvesting. At the appropriate time after treatment, the shoot and root portions of the plant were separated, immediately frozen in powdered Dry Ice, and stored in a freezer at −20 °C until all plants were harvested. Other plants were removed and prepared for autoradiography. The two plants with high levels of Tirpate were harvested at 36 hr after treatment.

Extraction. The plants were coarsely chopped into small sections with a razor blade and homogenized in 6 vol of ethanol–water (1:1) per g of tissue with a polytron PT3500 homogenizer. The homogenate was centrifuged at 1000*g* and the residue rewashed and recentrifuged two additional times. The supernatants were combined and flash evaporated at 40 °C to about one-half the original volume. This material was extracted with an equal volume of acetonitrile followed by the immediate addition of an equal volume of chloroform (Andrawes et al., 1971). The organic partition was concentrated using a rotary evaporator and the aqueous partition was lyophilized for subsequent separation procedures. Representative samples of the crude homogenates, supernatants, distillates, and concentrated fractions were assayed for ^{14}C radiolabel by scintillation counting. Corrections were made for quenching. The radiolabel in the residue was estimated by difference.

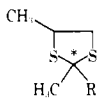
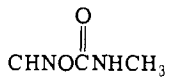
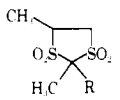
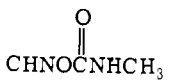
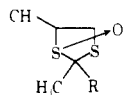
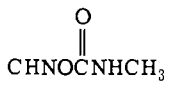
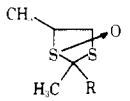
Thin-Layer Chromatography. Portions of the concentrated organic fractions were spotted on 0.5 mm TLC plates and chromatographed in chloroform–methanol (6:1, system A) and methylene chloride–acetonitrile (3:1, system B) solvent systems. The developed plates were observed under uv light and autoradiographed or scanned on a Varian Aerograph series 6000 radiochromatogram scanner. The spots were scraped and eluted with chloroform for quantitation and identification.

Acid and Enzyme Hydrolysis. A portion of the lyophilized aqueous fraction was redissolved in a small voume of water, made 0.5 *N* with HCl, and heated to 100 °C for 20 min to determine total conjugates. Other portions of the lyophilized aqueous fractions were incubated with β-glucosidase (Calbiochem, La Jolla, Calif.) in pH 4.8 sodium acetate buffer for 12 hr at 37 °C. Similar portions were also treated with sulfatase (Sigma, St. Louis, Mo.).

Autoradiography. Plants selected for autoradiography were separated into roots, large leaves, and the apex region (including the stem and several young leaves). The leaves were numbered according to their location on the plant for later comparison of similar leaves from different treat-

Department of Environmental Toxicology, University of California, Davis, California 95616.

Table I. Structure of Tirpate and Some Candidate Metabolites

No.	Compound	Parent group	R
I	Tirpate[a,b]		
II	Tirpate nitrile[a]		CN
III	Tirpate oxime[a]		HC=NOH
IV	Tirpate aldehyde[a]		CHO
V	Tirpate acid[a]		COOH
VI	Tirpate nitrile disulfone[a]		CN
VII	Tirpate disulfone[a]		
VIII	Tirpate monosulfoxide		
IX	Tirpate monosulfoxide nitrile		CN

[a] Standards supplied by 3M Agrichemical laboratories. Compounds VIII and IX were not available. [b] Asterisk denotes position of radiolabel.

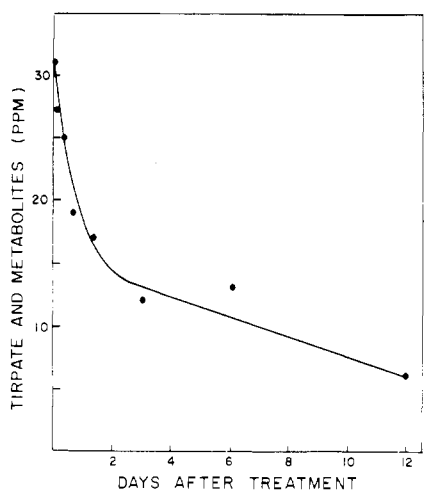


Figure 1. Concentration (parts per million) of Tirpate and metabolites in the plant following treatment.

ments. The plant materials were oven-dried at 70 °C in a press immediately after harvest. Further preparation of the plant materials for autoradiography was similar to that described by Crafts and Yamaguchi (1964). The X-ray film was exposed to the plant samples for 3 weeks.

Cleanup and Identification of Metabolites. The plant shoots treated with large quantities of Tirpate were combined and extracted in chloroform–acetonitrile as described above. The concentrated organic extract was separated by TLC (system A). The parent compound and major metabolites were eluted with chloroform, concentrated, and rechromatographed in system B. Major spots were again eluted with chloroform, brought to dryness, and dissolved in acetone. The extracts were analyzed by gas chromatography–mass spectrometry (GC–MS). The chromatographic column contained 2% OV-1 on Chromosorb W (60–80 mesh). The injector temperature was 220 °C, the separator temperature was 240 °C, and the ionization voltage was 70 eV; the column temperature was programmed from 150 to 230 °C.

Instrumentation. The polytron homogenizer (Type PT3500, Brinkmann Instrument, Inc., Westbury, N.Y.) with a sawtooth cutting blade was used to homogenize the plant material. Aliquots of radiolabeled fractions were assayed in a Model 2425 Packard Tri-Carb liquid scintillation spectrometer. The scintillation fluid contained 0.55% PPO (2,5-diphenyloxazole) in toluene–ethylene glycol monomethyl ether (2:1). Pre-coated TLC plates (silica gel, F-254) were purchased from Brinkmann Instruments Inc., Westbury, N.Y. TLC plates were scanned on a Varian Aerograph Series 6000 radiochromatogram scanner equipped with a dot printer. Mass spectra were obtained with a Varian Aerograph Series 1400 gas chromatograph coupled to a Finnigan Model 3000 Peak Identifier.

RESULTS AND DISCUSSION

The plants were harvested immediately after treatment (zero time) and after 3, 9.5, 16.5, and 34 hr, and 3, 6, and 12 days. Measurements of the radioactivity in the crude homogenate revealed that the plants took up an average of 82% of the applied radiolabel. This level was relatively constant for all treatments. An additional 14% of the label remained in the treatment flasks and chase solutions; thus, 96% of the applied [^{14}C]Tirpate was recovered. The concentration of Tirpate and its metabolites in the shoot declined from 31 ppm at the time of the initial sampling to 6 ppm at the final harvest (Figure 1). The level of radiolabel found at harvest as a percent of the total applied remained relatively constant; thus, the decline shown in Figure 1 represents primarily dilution from plant growth.

The roots and shoots were separated at harvest. Table II shows the distribution of the [^{14}C]Tirpate between the shoot and the root at various times following the treatment. Ninety-three percent of the radiolabel was found in the leaves and stem at the initial sampling time immediately after the 2-hr treatment period (zero time) indicating that most of the label was rapidly translocated from the root to the shoot. The percent distributed of [^{14}C]Tirpate in the shoot increased slightly thereafter reaching 98% after 34 hr. The radiolabel in the root decreased concomitantly from 6.9% initially, to 1.7% at the final harvest.

Autoradiography. Autoradiography of the plants also showed that the [^{14}C]Tirpate was rapidly taken up and

Table II. Distribution of Label in the Root and Shoot

Time	% ^{14}C in roots[a]	% ^{14}C in shoots[a]
0	6.9	93
3 hr	5.2	95
9.5 hr	7.3	93
16.5 hr	4.6	95
34 hr	2.3	98
3 days	2.6	97
6 days	1.7	98
12 days	1.7	98

[a] Percentages based upon the total uptake.

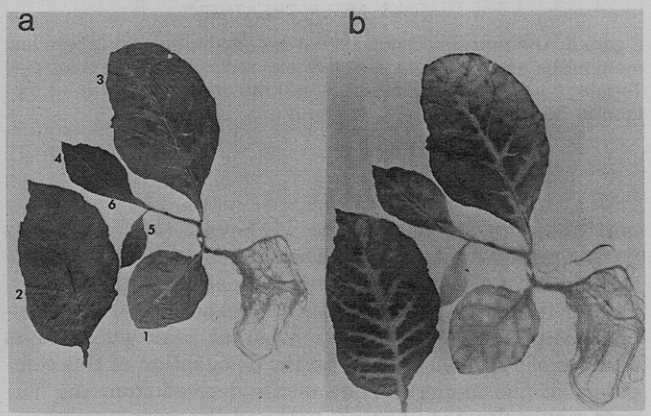


Figure 2. A 5-week old tobacco plant harvested immediately following treatment with [^{14}C]Tirpate: (a) pressed specimen; (b) autoradiograph of same specimen.

translocated into the leaves. Moreover, Figure 2 shows that the label was distributed throughout the plant by the end of the 2-hr treatment period. At this time the label was slightly more concentrated in the actively transpiring leaves (leaves 2, 3, and 4) than in the developing leaves (5 and 6) or the older leaf (leaf 1).

The radiolabeled Tirpate was not actively retranslocated into the new leaves and apical region that grew subsequent to the initial uptake and translocation into the shoot. Figure 3 clearly illustrates that most of the label remained in the older leaves. An older leaf from a plant sampled 6 days after treatment was heavily labeled (Figure 3c,d) whereas younger leaves from the same plant having grown mostly since the treatment period were considerably less heavily labeled (Figure 3a,b). The radiolabel in plants sampled at 12 days was distributed similarly to those described above.

The aqueous plant extracts (supernatants) were initially partitioned against chloroform–acetonitrile (3:1) as described in the Experimental Section. Figure 4 shows the changes in the partitioning characteristics of ^{14}C-labeled compounds at different times following treatment. (Data are expressed as the percent of the total in each sample.) Ninety-three percent of the labeled compound partitioned into the organic solvent at zero time and the amount of organosolubles decreased steadily thereafter to 15% at 12 days. Therefore, 85% of the compound found in the shoot was metabolized to a more polar, water-soluble material in the 12-day period following treatment. An untreated plant was homogenized with [^{14}C]Tirpate and extracted similarly. Ninety-nine percent of the label partitioned into the organic phase (Figure 4) showing, along with further evidence from TLC, that there were no changes in the parent compound from homogenization and extraction per se.

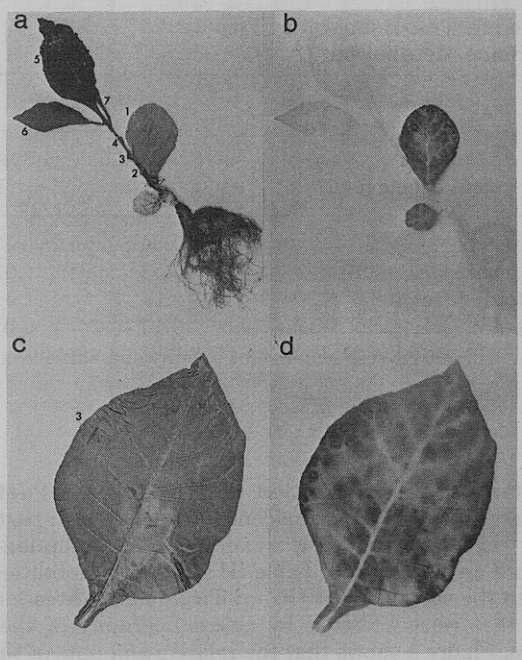


Figure 3. A tobacco plant at 6 days post-treatment: (a) plant with leaves 2, 3, and 4 removed; (b) autoradiograph of plant; (c) leaf 3; and (d) autoradiograph of leaf 3. The leaves were removed to facilitate drying. Leaf 4 (not shown) contained intermediate levels of the radiolabel.

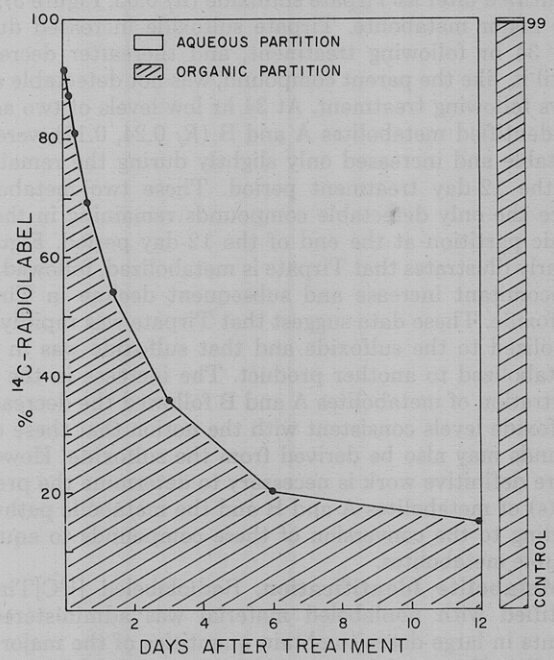


Figure 4. Distribution of ^{14}C radiolabel in the aqueous and organic partitions at different times following treatment with [^{14}C]Tirpate. The distribution from an untreated control fortified with [^{14}C]Tirpate is shown at the right.

The half-life of the compounds in the organic partition was estimated to be 3–4 days. The significance of this number as an estimate of Tirpate breakdown in plants under field conditions would largely depend on the rate of plant uptake and the environmental conditions. The uptake from a Florida sandy loam under similar environmental conditions to those described here (see Experimental Section) was much slower (unpublished), even when the compound was applied in liquid rather than the usual granular form.

Table III. TLC Behavior of Tirpate and Some Metabolites

Compound	R_f values System A[a]	System B[b]
Tirpate	0.64	0.46
Tirpate monosulfoxide	0.53	0.12
Metabolite A	0.24	<0.1
Metabolite B	0.17	<0.1

[a] 6:1, chloroform–methanol. [b] 3:1, methylene chloride–acetonitrile.

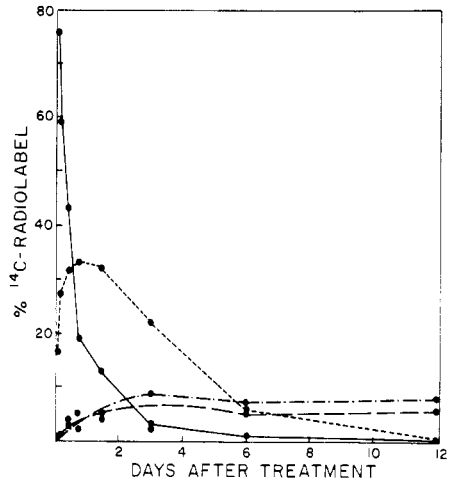


Figure 5. The temporal distribution of the organic soluble Tirpate and metabolites as a percentage of the total radiolabel in the plant: (—) Tirpate; (- - -) Tirpate sulfoxide; (– – –) metabolite A; and (–·–) metabolite B.

Changes in Composition of the Organic Partition. The amount of label in the concentrated organic partitions from each treatment was quantitated, and aliquots were spotted on TLC plates. Table III shows the mobilities relative to the solvent front (R_f) of Tirpate and three metabolites that were detected by autoradiography of the TLC plates. Figure 5 shows that the relative amounts of the four compounds, Tirpate, Tirpate monosulfoxide (identification to be discussed later), metabolite A, and metabolite B, changed during the treatment period. Tirpate (R_f 0.64) was the major detectable compound at the time of the initial harvest (zero time), but decreased with time relative to the other spots until at 12 days it was not detectable. The half-life of Tirpate in the plant was about 8–9 hr. A second spot, identified later as Tirpate sulfoxide (R_f 0.53, Figure 5), was the major metabolite. Tirpate sulfoxide increased during the 34 hr following treatment, and thereafter decreased until it, like the parent compound, was not detectable at 12 days following treatment. At 34 hr low levels of two as yet unidentified metabolites A and B (R_f 0.24, 0.17) were detectable and increased only slightly during the remainder of the 12-day treatment period. These two metabolites were the only detectable compounds remaining in the organic partition at the end of the 12-day period. Figure 5 clearly illustrates that Tirpate is metabolized, followed by a concomitant increase and subsequent decline in Tirpate sulfoxide. These data suggest that Tirpate was rapidly metabolized to the sulfoxide and that sulfoxide was in turn metabolized to another product. The increase in the concentration of metabolites A and B followed the decrease in sulfoxide levels consistent with the notion that these compounds may also be derived from the sulfoxide. However, more definitive work is necessary to determine the precursor(s) of metabolites A and B and the metabolic pathways leading to the conversion of these compounds to aqueous soluble metabolites.

Metabolite Identification. Radiolabeled [^{14}C]Tirpate fortified with nonlabeled material was administered to plants in large doses to obtain quantities of the major metabolite. Shoots of the treated plants contained 340 ppm of the compound as determined by radioassay. These large doses resulted in a characteristic necrosis along the leaf veins. No phytotoxic effects were visible, on plants treated with the lower levels of Tirpate as previously described.

Using TLC (system A) two major spots were revealed, the first corresponding to the parent compound (R_f 0.64) and the second to the major metabolite (R_f 0.53). The relative distribution of compound was similar in this 36-hr treatment to that found at 34 hr at lower doses (Figure 5). After clean-up as described in the Experimental Section, the compound was eluted, concentrated, and identified by GC–MS. The oxime moiety is labile under the operating temperatures employed in gas chromatography, and the corresponding nitrile is observed (personal communication with the 3M Company). Tirpate extracted in the organic partition and eluted from the TLC plate (R_f 0.64) had a parent peak with a mass number of 159 and the spectrum was identical with that obtained from either authentic Tirpate or its nitrile (Table I; I and II respectively). The second compound (R_f 0.53) gave a parent peak with a mass number of 179, consistent with the designation of this compound as the monosulfoxide nitrile derived from the Tirpate monosulfoxide (Table I; VIII and IX, respectively).

Anticholinesterase Activity. Tirpate and Tirpate monosulfoxide, the major metabolite, were tested for anticholinesterase activity by the method of Winterlin et al. (1968) using aluminum oxide TLC developed with solvent system A. Both Tirpate and the Tirpate monosulfoxide were active anticholinesterases. This evidence suggests that the major metabolite was, in fact, the monosulfoxide and not the monosulfoxide nitrile since loss of the oxime carbamate moiety renders the compound inactive as an anticholinesterase.

Aqueous Soluble Materials. The nature of the conjugated metabolites in the lyophilized aqueous fraction was determined by acid and enzyme hydrolysis. Ninety-seven percent of the radiolabel was recovered following lyophilization; thus no compounds were lost in that procedure. Tirpate remained unchanged when treated with hydrolytic procedures as described in the Experimental Section, thus indicating that the parent compound was not labile under those conditions.

Table IV shows that about 50% of this extract was hydrolyzed by acid, although as the time after treatment increased to 3 days or more the amount hydrolyzed declined slightly. Subsequent extraction and TLC (system A) of the aglycones released by acid hydrolysis indicated that they were different than Tirpate or any of the three metabolites shown in Table III. With the possible exception of metabolite B, the R_f values of the major TLC spots were less than 0.1 in system A indicating that they were more polar than any of the compounds listed in Table I. These metabolites were not further characterized.

The enzymes glucosidase and sulfatase released only small amounts of conjugated compounds (Table IV). The data of Table IV are corrected for the percent hydrolysis which occurred at pH 5 and therefore the values represent only enzymatic hydrolysis. The enzyme-labile fractions were higher shortly after the treatment period, although they represented only 10% or less of the radiolabeled compounds of the aqueous partition and declined to nondetectable levels by 12 days. Subsequent TLC (system A) of the

Table IV. Percent Aqueous [^{14}C]Tirpate Metabolites and Effect of Acid, Glucosidase, and Sulfatase Treatment

		% hydrolyzed[b]		
Time	Aqueous[a]	Acid	Glucosidase	Sulfatase
0 hr	7.5	50	11	8
3 hr	13	51	7	11
9.5 hr	19	55	8	10
16.5 hr	31	53	8	5
34 hr	46	53	5	7
3 days	64	47	1	
6 days	80	45		
12 days	85	40		3

[a] Percent of total. [b] Percent of aqueous fraction hydrolyzed as detailed in the Experimental Section.

compounds released by sulfatase and glucosidase indicated that they were very polar. The sulfatase hydrolysate contained low levels of a compound with a similar R_f to the Tirpate monosulfoxide at 9, 16, and 34 hr following the treatment. The yields from enzyme hydrolysis were too low to permit further qualitative analysis.

ACKNOWLEDGMENT

The authors thank Harold Dreever for greenhouse assistance and Maria Kurilowich and Clay Reece for technical assistance with portions of this work.

LITERATURE CITED

Andrawes, N. R., Bagley, W. P., Herrett, R. A., *J. Agric. Food Chem.* **19**, 731 (1971).
Bartley, W. J., Andrawes, N. R., Chancey, E. L., Bagley, W. P., Spurr, H. W., *J. Agric. Food Chem.* **18**, 446 (1970).
Coppedge, J. R., Lindquist, D. A., Bull, D. L., Dorough, H. W., *J. Agric. Food Chem.* **15**, 902 (1967).
Crafts, A. S., Yamaguchi, S., *Calif. Agric. Exp. Stn. Ext. Serv.*, **Manual 35** (1964).
Huffaker, R. C., Radin, T., Kleinkopf, G. E., Cox, E. L., *Crop Sci.* **10**, 471 (1970).
Metcalf, R. L., Fukuto, T. R., Collins, C., Borck, K., Burk, J., Reynolds, H. T., Osman, M. F., *J. Agric. Food Chem.* **14**, 579 (1966).
Winterlin, W., Walker, G., Frank, H., *J. Agric. Food Chem.* **16**, 808 (1968).

Received for review February 3, 1975. Accepted July 23, 1975. The authors thank 3M Company for partial support of this research. J.E.H. held a National Institutes of Health Postdoctoral Traineeship (ES00125).

Cannabis Smoke Condensate. Identification of Some Acids, Bases, and Phenols

Louis A. Jones* and Robert S. Foote[1]

The acids, bases, and phenols were chemically separated from the smoke condensate of 2638 marijuana cigarettes and semiquantitatively analyzed by GC and GC–MS. The following compounds were identified: acids—hexanoic, heptanoic, octanoic, benzoic, salicylic, hexadecanoic, heptadecanoic, and octadecanoic; bases—dimethylamine, piperidine, pyridine, 2-methylpyridine, pyrrole, 3-(and/or 4-) methylpyridine, and dimethylpyridine; phenols—phenol, cresols, quaicol, catechol, hydroquinone, *p*-hydroxyacetophenone, scopoletin, and/or esculetin.

Despite the fact that smoking has been the preferred method of ingestion of *Cannabis* for decades, it is only in the last 6 or 7 years that attention has been directed to the determination of those compounds generated and/or transferred during the smoking process. Heretofore, the major thrust has been directed to the fate of the cannabinoids under the pyrolytic conditions of smoking (Mechoulan, 1973, an excellent review; Fish and Wilson, 1969) and only recently have reports appeared concerning the identification of other compounds present in the smoke condensate. Identified by gas chromatography–mass spectrometry (GC–MS) were long-chain hydrocarbons (Adams and Jones, 1973) while phytosterols were shown to be present in the smoke condensate of *Cannabis* cigarettes by gas chromatography (GC) (Adams and Jones, 1975). Other compounds identified include several acids and phenols (Fentiman et al., 1973) as well as carbazole, indole, and skatole (Zamir-ul Haq et al., 1974).

The latter two studies employed GC–MS for separation and identification and preliminary separations by chemical methods were employed in all studies reported. As part of our continuing study of *Cannabis* and its smoke condensate, we now wish to report the classical chemical separation, GC, and, in some cases, GC–MS identification of some acids, bases, and phenols isolated from *Cannabis* smoke condensate.

Department of Chemistry, North Carolina State University, Raleigh, North Carolina 27607.
[1] NIMH Postdoctoral Fellow, 1971–1973.

EXPERIMENTAL SECTION

The procedure for the preparation of cigarettes and method of smoking and collection of condensate has been previously described (Adams and Jones, 1973). The bases, phenols, and acids from the smoke condensate of 2638 cigarettes were extracted and analyzed as follows.

Bases. The methylene chloride–acetone solution (1:1, 3 l.) containing the smoke condensate was shaken with 10% HCl (1 l.). The aqueous acid solution was washed with ether (2 × 500 ml) and then continuously extracted with ether for several days. The ether washes and extracts were combined with the methylene chloride–acetone solution and reserved for the extraction of phenols and acids. The aqueous acid solution was cooled to 5–10°C, covered with a layer of ether (1 l.), and made alkaline with solid sodium hydroxide. The alkaline solution was extracted with several portions of ether and then continuously extracted for 5 days to remove final traces of amines. The ether extracts were re-extracted with 5% HCl (1.5 l.) and the acid solution was continuously extracted with ether until the extracts were colorless. The acid solution was then stripped on a