Derivatives of (+)-Limonene. Detection and Translocation of Quaternary Ammonium Plant Growth Retardants in Young Grapefruit and Bean Seedlings

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The translocation of two quaternary ammonium derivatives of (+)-limonene was studied in grapefruit, *Citrus paradisi* MacFayden, and beans, *Phaseolus vulgaris* L. Application in lanolin to the stems resulted in downward translocation to the roots, whereas application as a soil drench resulted in upward translocation to the leaves. Evidence is presented that these compounds are metabolized. A novel thin-layer chromatographic method for detecting these quaternary ammonium derivatives in plant tissue extracts is described.

A previous paper (*3*) describes the synthesis and growth-retardant activity of 10 new quaternary ammonium derivatives of (+)-limonene applied in lanolin to the stems of young bean plants. Because of the relatively high activity of several of these compounds, this work has been continued and extended in order to determine their fate in the plant system.

The translocation of *n*-alkyl quaternary ammonium fungicides in tomato plant cuttings has been investigated by Edgington and Dimond (*1*, *2*). Their results showed that as the alkyl group was lengthened from ethyl to dodecyl, the compounds lost mobility in soil and within plants. Those having 12 or more carbon atoms did not move systemically because of adsorption of the quaternary cation to the plant tissue. Salerno and Edgington (*4*) found that the movement of *n*-alkyl quaternary ammonium compounds in tomato xylem could be predicted from their mobility on paper chromatograms if aqueous solvent systems were used. They also conjectured that "the principles governing movement of quaternary ammonium compounds on paper and in xylem are applicable to other cationic compounds and other plants."

The purpose of this paper is to present the results of a qualitative study of the translocation of two selected quaternary ammonium derivatives of (+)-limonene in Duncan grapefruit (*Citrus paradisi* MacFayden) and Black Valentine beans (*Phaseolus vulgaris* L.).

Experimental

Test Compounds. The quaternary ammonium derivatives (quats.) selected for translocation studies were VIII (*trans-p*-menth-8-en-1-ol, 2-dimethylaminobenzyl chlorides) and X (*trans-p*-menth-8-en-1-ol, 2-dimethylamino-2,4-dichlorobenzyl chlorides) (*3*). As discussed previously, each of these is a mixture of the two possible trans isomers with respect to the hydroxyl and nitrogen substituents. Compound VIII was the first limonene quat. found to be an effective growth retardant in beans, and its dichloro derivative (X) was one of the most active of all the quats. tested.

Method of Application to Plants. Young Duncan grapefruit seedlings and Black Valentine bean plants were grown in steam-sterilized soil in 46-ounce containers or flats under greenhouse conditions. The test compounds were applied in either aqueous solution (3000 p.p.m.) as a soil drench or in lanolin (1%) containing 2.5% Tween-80 as the emulsifying agent (*3*). Soil applications were usually made at the rate of 100 ml. per can or 2000 ml. per flat. The lanolin mixtures were applied below the second node of bean plants and just below the growing tip of grapefruit seedlings.

Extraction of Plant Tissue. The frozen tissue (100 to 150 grams of roots, stems, or leaves) was thoroughly ground with water in a Waring Blendor and filtered. Samples (stems and leaves) containing chlorophyll were warmed briefly to about 70° C. and cooled prior to filtration in order to coagulate chlorophyll and speed up the filtering process. The filtrate was concentrated on a film evaporator (water bath temperature 50° C.) to approximately 100 ml. and refiltered. The clear filtrate was then evaporated to dryness and last traces of water were removed azeotropically first with 95% ethanol and then with absolute ethanol. The residue was triturated with absolute ethanol using a glass rod flattened on one end. The mixture was filtered and the filter cake washed once with ethanol. The filtrate was evaporated to dryness and the residue dissolved in about 50 ml. of water. This solution was washed three times with ether in a separatory funnel, and the washes were discarded. This removed all ultraviolet fluorescent impurities which interfered with detection of the quats. by thin-layer chromatography. The aqueous phase was then evaporated to dryness with the aid of absolute ethanol and the final residue made to the desired concentration in absolute ethanol.

Detection of Quats. by Thin-Layer Chromatography (TLC). While investigating various coating materials on glass TLC plates, an unexpected decomposition of compounds VIII and X to their common amine precursors (2-dimethylamino-Δ8(9)-*p*-menthen-1-ols) was observed on aluminum oxide G (Brinkmann Instruments, Inc.) (pH ≈ 7.5). This was verified as follows: An absolute ethanol solution containing 30 mg. of X was streaked on an 8- × 8-inch TLC plate coated with aluminum oxide G. After drying at room temperature, the plate was developed using benzene-ethanol (97 + 3) and was again dried at room temperature. The aluminum oxide in the area 1/4 inch below the solvent front and 2 inches above the base line was scraped off and eluted with methanol. Evaporation of

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the methanol filtrate gave 6.1 mg. of strongly basic, amorphous residue. The infrared absorption of this material was identical with that of the mixed trans isomers of 2-dimethylamino-Δ8(9)-*p*-menthen-1-ol from which X was prepared. Since compound X is a mixture of two isomeric quats., its degradation would be expected to give two isomeric trans amines. The aluminum oxide at the origin, where the solution was streaked, was then removed from the plate, eluted with methanol, filtered, and concentrated to give 7.5 mg. of neutral, amorphous residue. The infrared absorption of this material was identical to that of quat. X. The recovery of X and its amine precursors was poor because of adsorption on the aluminum oxide. This procedure, when repeated using quat. VIII, gave the same partial conversion to the tertiary amine precursors. This conversion was found to be nearly complete when the streaked plate was heated at 100° C. for 10 minutes.

The degradation of quats. VIII and X to their amino alcohol precursors was also observed when neutral alcohol solutions of these compounds were added to neutral suspensions of TLC alumina in ethanol. The pH of the mixtures increased rapidly to 8 or 9 and this change was accelerated by heat. In fact, the conversion to the amines was so rapid that the characteristic fishy odor of the amines was immediately detectable after mixing.

This degradation of VIII or X on alumina provided a novel, indirect method for determining the presence of these quats. or their closely related metabolites in plant tissue extracts. After spotting or streaking the extract, the TLC plate was heated at 100° C. for 10 minutes, cooled to room temperature, and developed using benzene-ethanol (97 + 3). The plate was dried at 100° C. for 5 minutes and sprayed (warm) with anisaldehyde spray reagent No. 9B (*5*). After heating for 10 minutes at 100° C., the two amine degradation products of VIII or X or their metabolites were visible as an upper blue-green spot ($R_f \approx 0.80$) and a lower lavender spot ($R_f \approx 0.70$). Dragendorff's spray reagent (*6*) could not differentiate between the two amine degradation products and produced two red-orange spots which slowly faded. The color change of the anisaldehyde-sprayed spots was also very characteristic. The upper blue-green spot gradually changed to blue and than became colorless, while the lower lavender spot gradually became pink and then colorless. The separation of the pure *trans*-1,2-*cis*-1,4 isomer of 2-dimethylamino-Δ8(9)-*p*-menthen-1-ol was reported previously (*3*). Cochromatography of this pure isomer with the mixed *trans*-2-dimethylamino-Δ8(9)-*p*-menthen-1-ols showed it to be the upper, blue-green spot. Therefore, the lavender spot is probably the other possible trans isomer.

To test this detection method, 10 mg. of X in 150 ml. of water was mixed in a blender with six entire bean plants. The mixture was allowed to stand at room temperature overnight. The extract obtained using the above procedure was chromatographed on an alumina TLC plate. The two characteristic spots of the amine degradation products of X were clearly visible.

This indirect TLC method for identifying the two quats. (VIII and X) or their metabolites proved to be very versatile. The sugars and other highly polar impurities, having the same solubility characteristics, did not move from the base line, while the amines formed by degradation of the quats. moved upward and could be detected in amounts as small as 1 μg. In all cases, extracts of tissue from treated and control plants were cochromatographed with the appropriate quat. for qualitative identification.

Results and Discussion

Using the method of Salerno and Edgington (*4*) to predict the mobility of quaternary ammonium cations in plant xylem, eight of the quats. previously prepared from (+)-limonene (*3*) were chromatographed on Whatman No. 3 paper with water as the developing solvent. The compounds tested and the R_f values obtained are listed in Table I. The R_f values of the 8(9) unsaturated series (VIII, IX, X) and the 8(9) saturated series (XII, XIII, XIV) show a slight but consistent decrease with successive additions of chlorine atoms to the benzene ring.

If this analogy between R_f value and mobility is valid for these limonene derivatives, they should move readily within plant xylem. Such mobility was demonstrated qualitatively for the two quats. tested (VIII and X). Both VIII and X are C_{19} derivatives of (+)-limonene having molecular weights of 323 and 394, respectively. Their relatively large size did not appear to affect their mobility within plant xylem. Obviously, no analogy can be made between these limonene quats. and the *n*-alkyl quats. studied by Edgington (*1*).

Translocation in Black Valentine Beans. Both VIII and X were applied to young bean plants in lanolin as described. The plants were harvested after 7 days and separated into leaves and petioles, upper stems, lower stems, and roots. Analysis by TLC showed trace amounts of the quats. in the leaves and none detectable in both stem samples. The major portion was present in the roots. These results were repeated several times and translocation of VIII or X was always

Table I. Paper Chromatography of (+)-Limonene Quats.

	Compound	R_f
VIII	Mixed *trans*-*p*-menth-8-en-1-ol, 2-dimethylaminomethiodides	0.89
VIII	Mixed *trans*-*p*-menth-8-en-1-ol, 2-dimethylaminobenzyl chlorides	0.94
IX	Mixed *trans*-*p*-meth-8-en-1-ol, 2-dimethylamino-*p*-chlorobenzyl chlorides	0.89
X	Mixed *trans*-*p*-menth-8-en-1-ol, 2-dimethylamino-2,4-dichlorobenzyl chlorides	0.86
XI	Mixed *trans*-*p*-menth-8-en-1-ol, 2-piperidyl methiodides	0.93
XII	Mixed *trans*-1-*p*-menthanol, 2-dimethylaminobenzyl chlorides	0.92
XIII	Mixed *trans*-1-*p*-methanol, 2-dimethylamino-*p*-chlorobenzyl chlorides	0.85
XIV	Mixed *trans*-1-*p*-methanol, 2-dimethylamino-2,4-dichlorobenzyl chlorides	0.80

downward to the plant root system. A tracing of a typical TLC chromatogram obtained using tissue extracts from bean plants treated with compound X is shown in Figure 1. Control extracts are not included because they were blank in all cases. The single spots obtained for both stem extracts were dark blue. Only a charred residue was visible at the origin where samples were spotted.

In one experiment, extraction of the roots from 45 bean plants treated with lanolin containing 1% quat. X yielded 10 mg. of amorphous residue. This was separated by TLC on alumina into two fractions, a pale yellow band on the solvent front and a band 1 inch below the front, designated bands 1 and 2, respectively. The infrared absorption curves of the eluates were different from that of the *trans*-amino alcohol precursors

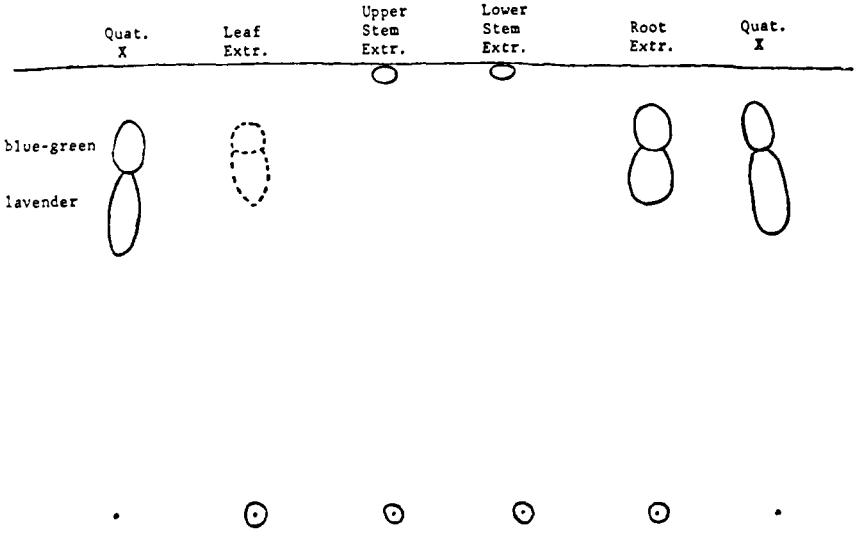

Figure 1. Thin-layer chromatogram of bean tissue extracts

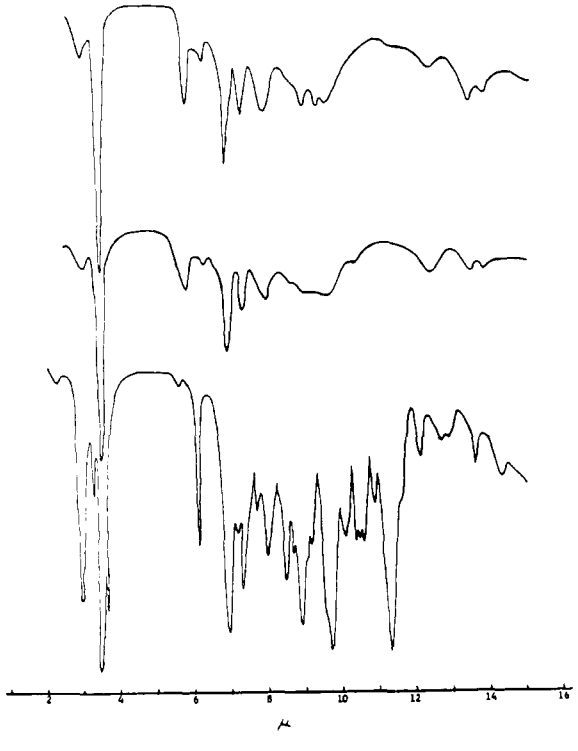

Figure 2. Infrared absorption curves of band 1 (upper), band 2 (middle), and *trans*-2-dimethylamino-Δ8(9)*p*-menthen-1-ols (lower)

of quat. X normally formed during TLC chromatography of X on alumina (lower curve, Figure 2). Structural changes in X must have occurred during the 7-day period in the bean plant. These curves are compared in Figure 2. The most striking changes are complete loss of the C=C absorption peak at 11.3 microns and a large decrease in amplitude of the hydroxyl band at 2.86 microns. This was observed repeatedly in other experiments. However, when these two fractions from the bean root extract were spotted on an alumina TLC plate, developed, and sprayed with anisaldehyde reagent, both gave a blue-green and a lavender spot. These had the same color and R_f values as the two spots obtained by chromatography of quat. X. This observation substantiates the well-known fact that cochromatography with a known compound and identification by spot color and R_f value is not always a criterion of identity. The observed spots are probably closely related metabolites of X formed in the plant.

When VIII and X were applied to young bean plants as a soil drench, translocation was upward to the leaves. Trace amounts could be detected in the leaves after 1 hour and positive tests for both quats. were easily obtained after 3 hours. In one experiment, bean plants were harvested 23 hours after applying a soil drench of X. Extraction of the plant tops (260 grams fresh weight) and chromatography on TLC plates gave

10 mg. of residue. The infrared absorption of this material was very similar but not identical to that of the amino alcohol precursors of X. Considerable reduction in the amplitude of the C=C peak at 11.3 microns was already evident. Material isolated from the leaves 8 days after soil application had much the same infrared absorption as that obtained from roots 7 days after stem application of X in lanolin. In both cases, the C=C absorption peaks were no longer evident, indicating conversion to a metabolite.

Translocation in Duncan Grapefruit. The translocation of VIII and X in grapefruit seedlings was the same as that found in bean plants. Quat. X was applied in lanolin (1%) just below the growing tips of seedlings, and the plants were harvested after 2 months. The root extract contained the major portion of X which was detected by spot color and R_f value. The systemic nature of X was further verified by using a seedling with a split root system, each half-root being established in a separate container of soil. An aqueous solution of X was applied to the soil of one root system. After 2 months, the untreated root system was harvested and extracted. Upward and downward movement of quat. X was indicated by qualitative identification of this compound in the untreated root system.

Attempts to apply VIII or X as foliar sprays were not successful. Therefore, most applications were made as a soil drench. In one experiment, 30 1-year-old seedlings were treated with a soil drench of X. Positive identification of X was made by TLC in root and leaf samples after time intervals of 1, 2, 3, and 4 weeks. As in the case of bean plants, the infrared absorption of the residues isolated by TLC from both leaves and roots showed that the quats. were being chemically or enzymically changed within the plant. This change was again greater the longer the quats. remained in the plants. Therefore, all qualitative identifications of VIII or X above do not distinguish between the quats. *per se* and their metabolites. The infrared absorption curve of the residue isolated by TLC from a root extract of grapefruit seedlings watered with VIII was identical to that of the root residue obtained from beans treated with lanolin containing 1% X (band 1, Figure 2). Since VIII and X are identified by conversion to their common amino alcohol precursors on alumina TLC plates, this is not surprising. However, it does indicate that the same molecular change is taking place in the terpene moiety in both plant species. Mass spectrographic analysis of eluted TLC spots obtained by chromatographing extracts of leaves and roots indicated an increase of about 80 in molecular weight. This may be associated with addition to the 8(9) double bond and esterification of the 1-hydroxyl group. As in the case of the residues isolated from bean plants, all leaf and root samples from treated seedlings gave positive tests (spot color and R_f) for quat. VIII or X regardless of how much molecular change was indicated by infrared absorption. Additional tracings of chromatograms are not presented since the amino alcohols identified are the same in all cases (Figure 1).

The possibility that VIII and X could be degraded to their common amino alcohol precursors in the plant prior to extraction and identification cannot be avoided. Evidence that such degradation does not take place in the plant was obtained as follows: Quat. X was applied as a soil drench to young bean plants. The stems and leaves were harvested after 7 hours and extracted as described under Experimental. The crude extract residue was made to 30% in lanolin and applied to bean plants. The treated plants showed typical growth-retardant response and the first internode length was decreased about 50% compared to the control plants. This indicates that quat. X is present *per se* or in an altered form and has not been degraded to the amino alcohol precursors which have almost no growth-retardant activity (*3*). This bioassay procedure was repeated several times and similar growth-retardant activity was observed. Bioassays of extracts prepared from treated plants harvested after 24 hours produced a growth reduction of 15 to 20%. This again indicates progressive changes in X occurring in the plant and suggests detoxification to a less active metabolite.

The fact that several analogs of quats. VIII and X have been found to be inhibitors of pseudocholinesterase was mentioned in a previous publication (*3*). The infrared and mass spectrographic data indicate that VIII and X are metabolized in the same manner in both plant species studied. This molecular change may be the result of interaction with an enzyme system common to both plant species.

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