Penetration of Selectively Toxic Aromatic Hydrocarbons Into Crown Gall Tumor Cells

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ABSTRACT

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Crown gall tumor cells on tomato plants were killed selectively by a mixture of five aromatic hydrocarbons in paraffin oil (STO) without killing the adjacent stem cells. The selective toxicity is attributed to enhanced penetration of tissues and tumor cell walls rather than differential permeability of cellular membranes of diseased and healthy tissues. Tritiated 2,3-dimethylnaphthalene penetrated deeper into tumors than healthy stems and could be detected intracellularly only in tumor cells. Differential tissue penetration also was indicated by more rapid and greater nonspecific electrolyte leakage from excised treated tumors. When tissues were infused with STO containing Sudan IV dye, the intercellular spaces of treated stems remained filled with the colored STO for 3 weeks without damage, but tumors were rapidly penetrated and killed. Apparently, the STO was unable to penetrate the stem cells. A major difference between tumor and stem tissues was the absence of a differentiated epidermal layer over the tumors. This allowed rapid loss of water from tumors by evaporation, thus reducing aqueous barriers to incoming STO. Callose, a possible barrier to STO, was not detected in the cell walls of stems. Lipid materials that might aid penetration were not found in the cell walls of tumors. Once past the cell wall, some tumor cell tonoplasts were ruptured within 1.5 hours after treatment with STO. Membrane damage in treated tumor cells was detected much earlier (within 6 minutes) by measuring nonspecific electrolyte leakage from treated tissues.

In 1968, Schroth and Hildebrand (10) selectively killed crown gall tumor cells on tomato and woody plants with a mixture of aromatic hydrocarbons in oil or in an oil-water emulsion, and they suggested that the effectiveness of the treatment might have been related to differential permeability of the membranes of the tumor cells. Other work has shown that tumor cells accumulate solutes preferentially from dilute solutions, possibly through altered membrane permeability (13). Similarly, the selective toxicity of petroleum hydrocarbons used as herbicides has been attributed to differential membrane permeability in plant cells (12).

Schroth and Hildebrand (10) observed that the carrier oil containing Sudan IV dye as a marker penetrated tumors but moved poorly into healthy tomato stems, and suggested that selective toxicity may be caused by differential penetration of the toxic oils into the tumor tissues.

The present study was designed to determine the site of action of the aromatic hydrocarbons and whether selective toxicity to crown gall tumors on tomato stems resulted from differential permeability of tumor cell membranes and/or differential penetration of the tumor tissues. A preliminary report of these findings has been published (7, 8).

MATERIALS AND METHODS

Crown gall of tomato (*Lycopersicon esculentum* Mill., 'Bonny Best') was used as a model system for determining the action of selected hydrocarbons on tumor tissues. One-month-old plants were inoculated with a 24-hour culture of *Agrobacterium tumefaciens* (Smith and Townsend, 1907) Conn., 1942 and the tumors produced were treated when they were 2.0 to 2.5 cm in diameter.

A stock solution of a selectively toxic oil (STO) was prepared that contained 12.7% diphenylmethane (technical), 12.7% 1,2,3,4-tetrahydronaphthalene (practical grade), 12.7% dimethylnaphthalene (technical), 1.0% 2,4-xylenol (technical), and 1.0% *m*-cresol (practical) in nontoxic paraffin oil (Saybolt viscosity 125/135). Aliquots of the stock solution were diluted in paraffin oil to obtain the desired concentration. Paraffin oil or water was used on the control plants.

The STO solution was applied to the surfaces of tomato stems and tumors with a cotton swab or by infusing 1 ml of STO through a Pasteur pipet into a small hole made in the tumor or stem. Intact tomato stems and tumors were infused in vivo for 84 hours with 3.5% STO containing Sudan IV to bypass the epidermis and enhance penetration of the STO into the tissues. Infusing STO avoided potential interference with its penetration by exuded cellular sap from cut surfaces of the tissues.

Electron microscopy and radiography.—Selectively toxic oils (3.5%) was painted

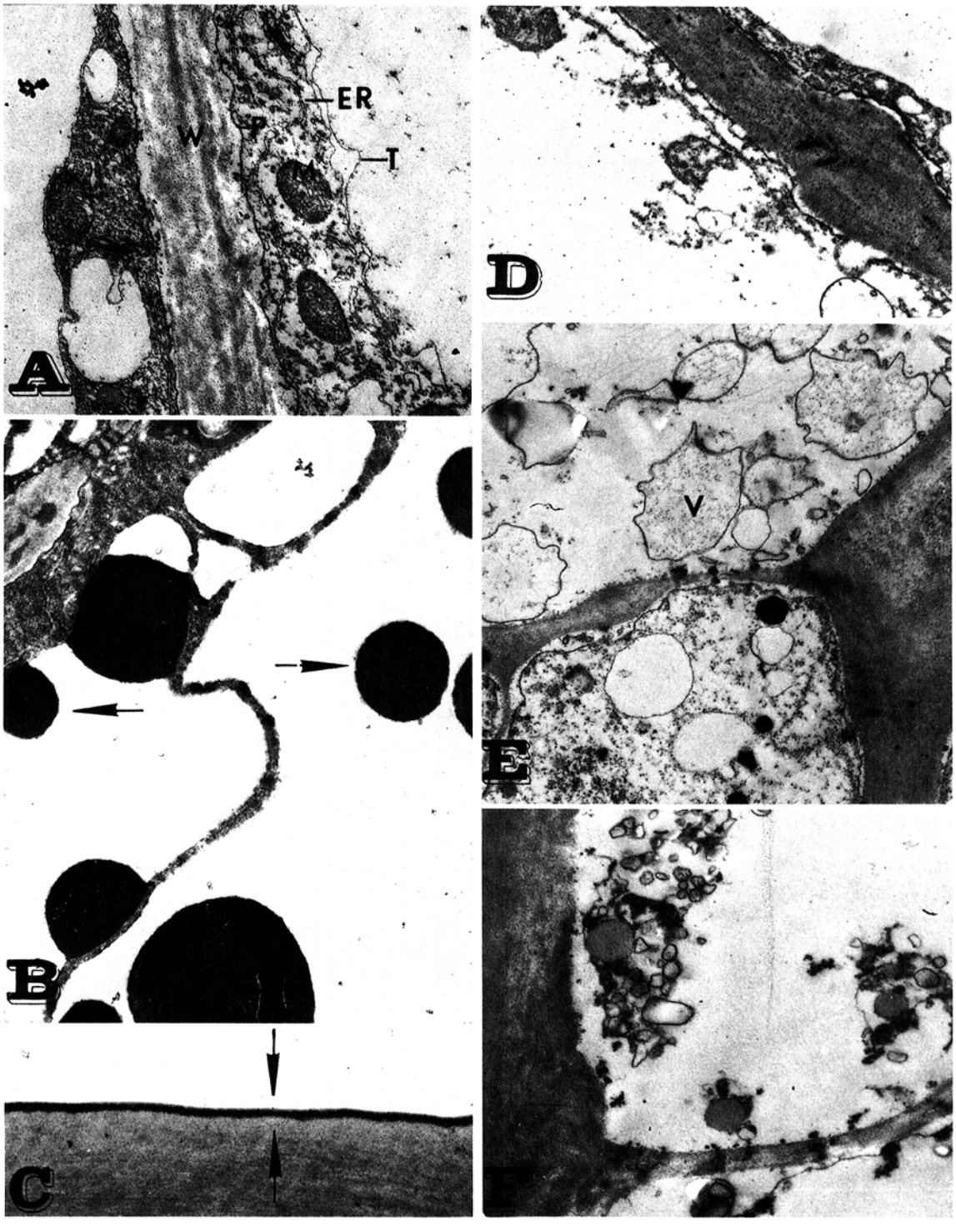

Fig. 1-(A to F) (× 11,400). Portions of cells from crown gall tumors on tomato stems that either were untreated or treated in vivo with 3.5% selectively toxic oil (STO). **A)** Untreated tumor cell. W = cell wall, P = plasmalemma, ER = endoplasmic reticulum bearing ribosomes, M = mitochondria, and T = tonoplast. **B)** Untreated tumor cell with electron-dense bodies (arrows). **C)** Cell wall of stem cell with densely stained cuticle layer (arrows). **D)** Tumor cells 2.5 hours after application of STO. Organelles in the lower cell are distorted, the tonoplast is missing, and the plasmalemma (although still intact) is pulling away from the cell wall in places. **E)** Tumor cell 6 hours after application of STO showing loss of cellular organization and membrane vesicle formation (V) in the upper cell. Vesicles also are forming within the groundplasm of the adjacent, more normal-appearing cell. **F)** Tumor cell contents completely disorganized 24 hours after application of STO.

over the surface of tumor and stem tissues to study its effect on cellular fine structure. Treated and untreated tissues were collected 15 minutes, 1.5, 2.5, 4, 6, 8, 10, 18, 24, and 48 hours after treatment and prepared for electron microscopy. The samples were cut into 10 blocks (0.7-1.5 mm^3) and fixed in 6% glutaraldehyde in 0.15 M phosphate buffer (pH 7.0), rinsed with the buffer, and postfixed in 2% osmium tetroxide in 0.2 M sodium cacodylate (pH 7.3). The tissues were dehydrated with ethanol, embedded in Araldite, polymerized in a vacuum oven at 60 C for 24-48 hours, and sectioned with a diamond knife on a Porter-Blum II ultramicrotome. The sections were stained 10-15 minutes with either 2% barium permanganate or lead citrate. The entire treatment and sampling procedure was repeated four times.

Samples of tumor and stem tissue for autoradiography were cut serially, at depths of 0-1.5, 1.5-3.0, and 3.0-4.5 mm beginning at the surface. The samples were diced separately and fixed at 4C in 5% glutaraldehyde in 0.125 M Sorenson's phosphate buffer at pH 7.0, rinsed twice with the buffer, and dehydrated with acetone. At the 70% acetone step, the tissues were stained with uranyl acetate. After dehydration, the tissues were embedded in an Araldite-Epon mixture, polymerized, and sectioned as above. Silver-colored sections were mounted on formvar-coated 48 μm (300-mesh) copper grids, stained with lead citrate, and coated with Ilford L4 emulsion. The coated grids were developed after a 2-week exposure, examined, and counts were made of developed silver grains over the cytoplasm, vacuole, and cell walls. Seven to 10 sections with three to five cells per section were counted for each sample. Background silver grains located over a randomly selected grid opening adjacent to the tissue section also were counted.

Electrolyte leakage from tumors and stems.—Three tumors and three stems were infused with 3.5% STO and 84 hours later each tumor and stem was cut from the plant and each was divided into three separate sections. The sections were rinsed thoroughly with a jet of distilled water and placed in 5 ml of distilled water in 25-ml Erlenmeyer flasks. The flasks were shaken on a wrist-action water-bath shaker at 100 oscillations per minute and 27 $\pm$ 1 C. Conductivity measurements were made periodically from zero time with a conductivity bridge with an electrode constant of 1.0. After the final conductivity measurement, the tissues and suspending liquid were frozen, thawed, and shaken in a water bath at 27 C for 50 minutes. The conductivity of the thawed suspension was taken as the total conductivity potential of the tissue, and all measurements were expressed as a percentage of this total.

Excised tumor and stem tissue samples (each weighing about 1 g) were cut into four pieces and treated for 5 minutes at 28 $\pm$ 1 C by immersing the tissue in STO, paraffin oil, or water. After treatment, the tissues were rinsed thoroughly with distilled water and conductivity measurements were made as described above. The conductivity experiments were repeated three times, and the data were expressed as a mean of all three experiments.

Tissue penetration by selectively toxic oil containing ^{3}H-dimethylnaphthalene.—Tritiated 2,3-dimethylnaphthalene (International Chemical and Nuclear Corporation,) was added as a second marker to STO containing 0.1% Sudan IV, to determine if the aromatic hydrocarbons penetrated the tissue simultaneously with the dyed paraffin oil carrier.

Purity of the tritium isotope was determined by thin-layer chromatography (6). Tritiated and nontritiated 2,3-dimethylnaphthalene were spotted on temperature-activated silica-gel G plates and developed by ascending chromatography in *n*-hexane. Ultraviolet light (254 nm) and iodine vapors were used to detect the presence of the test compound. In addition, 1-cm-wide strips of the silica-gel were scraped laterally off the developed plate and each strip was placed in separate liquid scintillation vials containing 10 ml of liquid toluene counting fluor. The vial contents were mixed thoroughly and counted in a liquid scintillation counter. Ten μCi of tritiated 2,3-dimethylnaphthalene were added to a 12% solution of 2,3-dimethylnaphthalene in paraffin oil and the mixture was painted on tumors and stems. The tissues were harvested 24 hours after treatment and the tumors were cut into four sections perpendicular to the direction of oil movement. Each section was cut into six 0.5-mm cubes, labeled according to position in the layer, and each cube was placed in a scintillation vial. Fifteen milliliters of a toluene-fluor solution were added to each vial and the tissue cubes were soaked for 24 hours before counts per minute (CPM) were determined. The specific activity was expressed as CPM per gram of fresh tissue.

Stem tissues were treated similarly except that only three sections were cut at varying depths in the stem because of the smaller amount of tissue available. The outermost sample consisted of an epidermal scraping about 0.25 mm deep.

RESULTS

Influence of aromatic hydrocarbons on tumor cell structure.—Fine structure of cells in the surface layer ($\leqslant$ 3 mm deep) of tumors treated in vivo with 3.5% STO was noticeably affected, but not all cells responded similarly. Degenerating cells frequently were adjacent to normal-appearing cells. In some treated tumor cells the tonoplasts were ruptured 1.5 hours after treatment, the groundplasm was granular and diffuse, but the mitochondria and chloroplasts appeared to be normal. Two and one-half hours after treatment, the integrity of many tonoplasts was lost, the mitochondria were distorted, and the structure of ribosomes was indistinct (Fig. 1-D). The plasmalemma appeared to be intact at both sampling times. However, after 6-8 hours of treatment the fine structure of many tumor cells had deteriorated severely, cellular detail was lost, ribosomes were absent, bounding membranes were ruptured, numerous membrane vesicles had developed, and the groundplasm appeared to have become coagulated (Fig. 1-E). Even at this advanced stage of deterioration, the fine structure of some adjacent tumor cells appeared to be normal (Fig. 1-E).

Electron-opaque bodies (0.3 to 1.2 mm in diameter) were noted in serial sections of some tumor cells (Fig. 1-B) but were absent in normal stem cells. These bodies were in the groundplasm and the vacuole but smaller bodies often were associated with the tonoplast. The opaque bodies first were observed following treatment of the tumor with STO, and they were interpreted initially as products of the

treatment. However, similar opaque bodies subsequently were observed in older, larger untreated tumors. The morphology of the opaque bodies isolated by density-gradient equilibrium centrifugation from fractionated tumor cells (2, 11) was similar to those in sectioned tissue, and they had an apparent density of 1.23 to 1.26 (1). This is in the density range of proteins or protein-rich lipoproteins and is much too dense for lipid particulates. Opaque bodies were not observed in any of the density-gradient layers prepared from control stem tissue treated in the same way.

Macroscopically, the tumor surface was darkly discolored 8 hours after treatment and about 17 hours later this intensified to a blackened and shrivelled necrotic condition. The tumor tissues underlying the necrotic areas contained some plasmolyzed cells and the cellular contents of most cells were disorganized (Fig. 1-F).

Tumors treated with only the paraffin oil carrier, and healthy stems treated with STO or paraffin oil, remained healthy and failed to exhibit the modifications of fine structure typical of tumors treated with STO.

The influence of selectively toxic oil on nonspecific electrolyte leakage from tumors and stems.—The rate and magnitude of leakage from excised tumor tissues treated in vitro were significantly greater than that from excised stem tissues (Fig. 2). Most of the leakage occurred within the first 6 minutes (Fig. 2) following treatment. Rate and magnitude of leakage always was greater from treated tumors than from stem tissues regardless of the STO concentration (3.5 to 10%) or duration of treatment (2.5 to 15 minutes) with a fixed concentration of the STO.

The mean nonspecific electrolyte leakage from infused tumors was 86% (standard deviation = 8.8%) of the total possible, but only 17% (standard deviation = 1.7%) from infused healthy stems. No further damage to the infused stems had occurred after 3 weeks, even though the intercellular spaces of the treated stem were filled with STO. Apparently the STO did not penetrate the stem cells, or if it penetrated, the cells were less sensitive to the STO than were tumor cells. Electrolyte leakage from tumors and stems infused with water was 14% and 16%, respectively.

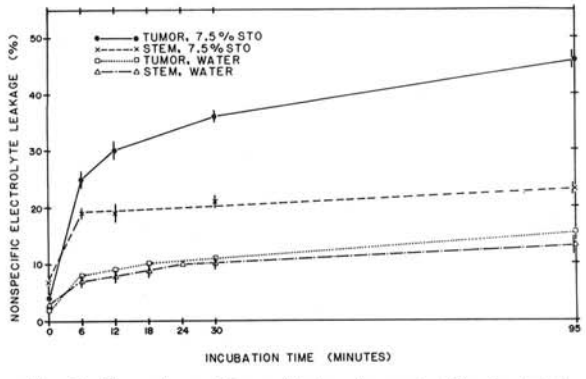

Fig. 2. The rate and magnitude of nonspecific electrolyte leakage from crown gall tumor and tomato stem segments treated in vitro for 5 minutes with 7.5% selectively toxic oil (STO) or distilled water. Electrolyte leakage is expressed as a percentage of the total possible leakage. Vertical bars through points on the curve represent the standard deviation about the means.

Anatomical and histological barriers to penetration by selectively toxic oil.—Tumors on tomato stems did not have a specialized epidermal cell layer comparable to the cutinized epidermis covering healthy stem tissues (Fig. 1-C). Instead, the outer parenchyma cells of tumors were indistinguishable from similar cells within the tumor. The epidermis may prevent loss of water from the stem's interior tissues, water that is essential for maintenance of a lipophobic barrier to STO penetration. To test this hypothesis, the rate of water loss from 3- to 4-g segments of stem and tumor tissues was determined gravimetrically. The tissue segments were weighed, the cut surfaces sealed with warm Vaseline (44 C), and the segments were reweighed. Four replicates each of tumor and stem tissues were kept at 22 ± 2 C, and the decrease in fresh weight was measured periodically for 72 hours.

Water loss from tumors was greater and more rapid than from stem segments (Fig. 3). After 60 hours the mean fresh weight loss of tumors was 80% and the stem weight loss was 15%. Total mean water content of the tumor and stem tissues was 91 and 92%, respectively. Thus, tumors without a protecting epidermis are more susceptible to water stress, and the adverse effect of STO on membrane permeability could greatly increase that stress.

Cell walls of tumors lacking a cutinized epidermal cell layer may contain more lipids than cell walls of stems. This would tend to lessen moisture stress from excessive evaporation and might aid STO movement through the intercellular spaces of tumors. To test for lipids in cell walls, blocks of fresh stem and tumor tissue were quick-

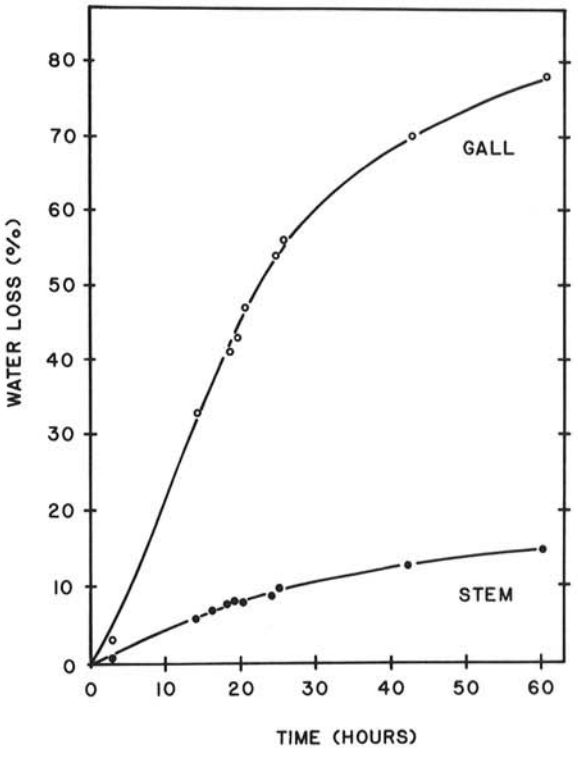

Fig. 3. Decrease in fresh weight, shown as percent water loss, of crown gall tumors and segments of noninoculated tomato stems.

frozen in a mixture of isopentane-methylcyclohexane cooled over liquid nitrogen (5) and sectioned on an International-Harris Model CTI cryostat (Harris Refrigeration Co., Needham, Massachusetts) at temperatures from −10 to −20 C. After being air-dried, the sections were stained with Sudan Black B in ethylene glycol to reveal the presence of lipids.

Callose in stem cell walls also was tested as a possible deterrent to oil penetration because of its impervious nature and rapidity of induction (3). Tissues treated with STO and untreated stem tissues were examined for callose by the aniline blue-fluorescence method (3).

Cell walls of tumor and stem tissues did not stain with Sudan Black B and callose was not detected. Therefore, the differential penetration of STO into stem and tumor tissues cannot be explained by enhanced capillarity along tumor cell walls containing lipids or by an impervious callose layer on the walls acting as a barrier to penetration.

Tissue penetration by STO containing ^{3}H-dimethylnaphthalene.—Twenty-four hours after surface application, a decreasing concentration gradient of ^{3}H-labeled STO in both healthy stem and tumor tissues developed inward from the site of application (Fig. 4), even though sections of tissue much deeper were stained red by Sudan IV. With stems, 89% of the total radioactivity was retained in the epidermal area and only 7% penetrated to a depth of 1 mm. In contrast, 70% and 24% of the radioactivity was present 1 mm and 4 mm, respectively, below the site of application on the tumors. Thus, 2,3-dimethylnaphthalene penetrated further into tumors than into stem tissues.

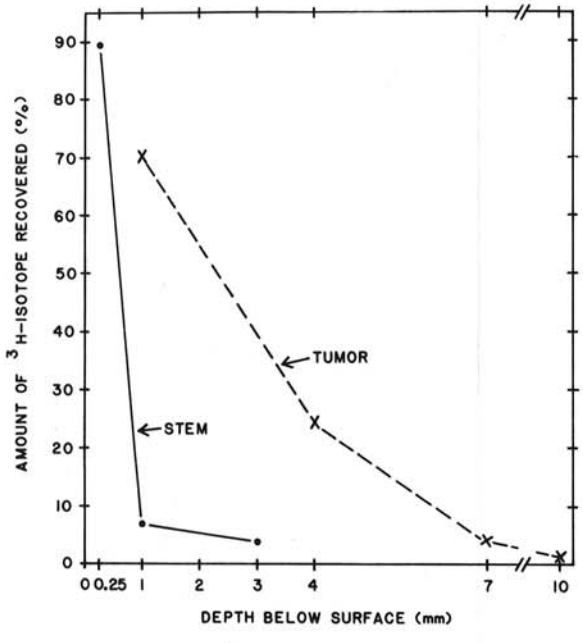

Fig. 4. Recovery of ^{3}H-2,3-dimethylnaphthalene at various depths below the surface 24 hours after painting intact tomato stems and crown gall tumors with the isotope. Each point represents a percentage of the total activity recovered from each tissue: 799,000 counts per minute (CPM) per gram of stem tissue and 463,333 CPM per gram of tumor tissue (fresh weight).

The isotope also penetrated cells in treated tumors as shown by high-resolution autoradiography. Intracellular penetration was greatest in cells near the tumor surface (0 to 1.5 mm deep) (Table 1). From 10 tissue sections averaging five cells per section, 115 silver grain tracks were positioned over the cytoplasm, 113 over the vacuole, 14 over the cell walls, and 12 were counted as background contamination over a formvar-coated grid opening. The tracks were nearly equally distributed between the cytoplasm and vacuole and could not be associated specifically with a cellular organelle. Isotope detection dropped off rapidly the further the sample was removed from the site of application. Five tissue sections averaging three cells per section from tissues 1.5 to 3.0 mm below the surface of the tumor had no silver tracks over the cytoplasm and cell wall, one track over the vacuole, and two background tracks. In contrast to the tumor, sections of treated stem tissue had very few intracellular tracks even at the stem surface. Seven sections of tissue averaging five cells per section had no tracks over the cytoplasm and cell walls, two tracks over the vacuole, and two background tracks. Thus, penetration of 2,3-dimethylnaphthalene into healthy tomato stem tissue is limited both at the inter- and intracellular level as contrasted to tumor tissue.

DISCUSSION

Tonoplasts of tumor cells were affected by STO treatments at a time when the membranes of stem cells were normal. Membrane disruption of tumor cells was not apparent until 1.5 hours after treatment, whereas accelerated electrolyte leakage from treated tissue occurred immediately after treatment. Disruption of the tonoplast would subject the groundplasm and organelles to the potentially harmful contents of the vacuole.

Possibly the opaque bodies commonly observed in tumor cell vacuoles and associated with the tonoplast are similar to lysosomes, and interaction with STO may release their enzymes to attack the tonoplast. The relatively high density and general appearance of the opaque bodies are comparable to the zymogen granules described and isolated by Siekevitz and Palade (11), who

TABLE 1. Location of ^{3}H-2,3-dimethylnaphthalene in tomato stem and tumor cells 24 hours after application as demonstrated by autoradiography

Sample depth (mm)	Total number of developed silver grains[a]			
	Cytoplasm	Vacuole	Cell wall	Background[c]
Tumor				
0.0-1.5	115[b]	113[b]	14	12
1.5-3.0	0	1	0	2
3.0-4.5	0	0	1	2
Stem				
0.0-1.5	0	2	0	2

[a]Counts were made from 7-10 sections of tissue each containing cross sections of three to five cells. The emulsion of the autoradiographs was developed after 2 weeks of exposure.

[b]Counts ranged from 0-10 developed grains per cell.

[c]The number of developed grains in a 48 μm (300-mesh) copper grid opening adjacent to the tissue section.

equated the zymogen granules to lysosomes because of their high content of hydrolytic enzymes. On the other hand, the opaque bodies could be spherosomes since the presence of OsO_4-staining bodies (9) and spherosomes with phosphatase activity (4) have been observed in crown gall tumor cells. However, the spherosomes which supposedly were present only in the groundplasm also were found in meristematic cells. Our observations indicate that the opaque bodies were absent from meristematic cells and commonly located in the vacuole of tumor cells.

The absence of ultrastructural changes in stem cells treated with 3.5% STO could have resulted from differences in permeability between cellular membranes of tumor and stem cells, detoxification mechanisms within stem cells or tissues, or differential penetration of the STO into stem and tumor tissues. The latter may be the case because STO applied to the stem surface is rapidly dispersed over the lipophilic cuticle, a structure missing from the tumor. Once inside the epidermis, hydrated thick-walled collenchyma and vascular tissues present another barrier to STO penetration. However, when these barriers are bypassed by infusing the STO, the intercellular spaces of the infused stem are permeated thoroughly with the STO for several days without causing injury. The lack of cellular damage and poor penetration of ^{3}H-2,3-dimethylnaphthalene into stem cells strongly suggest that STO passage is inhibited across the cell walls of the stem. The increased rate and magnitude of nonspecific electrolyte leakage from treated tumor tissue compared with that from stem tissues further demonstrates the differences in these tissues that may account for the selective action of STO.

LITERATURE CITED

1. ANONYMOUS. 1967. A handbook of data for biological and physical scientists. 2nd ed. Instruments Specialties Co., Inc., Lincoln, Nebraska. 32 p.
2. ERDOS, E. G., L. L. TAUGE, and I. MIWA. 1968. Kallikrein in granules of the submaxillary gland. Biochem. Pharmacol. 17:667-674.
3. ESCHRICH, W., and H. B. CURRIER. 1964. Identification of callose by its diachrome and fluorochrome reactions. Stain Technol. 39:303-307.
4. HOLCOMB, G. E., A. C. HILDEBRANDT, and R. F. EVERT. 1967. Staining and acid phosphatase reactions of spherosomes in plant tissue culture cells. Am. J. Bot. 54:1204-1209.
5. JENSEN, W. A. 1962. Botanical histochemistry. W. H. Freeman, San Francisco. 408 p.
6. KUCHARCZYK, N., J. FOHL, and J. VYMETAL. 1963. Dunnschichtchromatographie von Aromatischen Kohlenwasserstoffen und einigen heterocyclischen Verbindungen. J. Chromatogr. 11:55-61.
7. MOORE, L. W., and M. N. SCHROTH. 1967. Effect of aromatic hydrocarbons on tomato crown gall cells. Phytopathology 57:823 (Abstr.).
8. MOORE, L. W., and M. N. SCHROTH. 1968. Effect of aromatic hydrocarbons on the permeability of crown gall cells. Phytopathology 58:1061 (Abstr.).
9. RIKER, A. J. 1927. Cytological studies of crown gall tissue. Am. J. Bot. 14:25-37.
10. SCHROTH, M. N., and D. C. HILDEBRAND. 1968. A chemotherapeutic treatment for selectively eradicating crown gall and olive knot neoplasms. Phytopathology 58:848-854.
11. SIEKEVITZ, P., and G. E. PALADE. 1958. A cytochemical study on the pancreas of the guinea pig. I. Isolation and enzymatic activities of cell fractions. J. Biophys. Biochem. Cytol. 4:203-218.
12. VAN OVERBEEK, J., and R. BLONDEAU. 1954. Mode of action of phytotoxic oils. Weeds 3:55-65.
13. WOOD, H. N. 1967. Plant carcinogensis. Progr. Exp. Tumor Res. 9:286-311.