Behavior and Fate of Ethoxylated Alkylphenol Nonionic Surfactant in Barley Plants

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Nonionic surfactant (Triton X-100) had transient effects on photosynthetic O_2 evolution when dilute solutions were taken up through the cut ends of excised barley leaves. The [^{14}C]hexaethoxylate or [^{14}C]nonaethoxylate of *p-tert*-octylphenol was metabolized rapidly following absorption by either excised or intact leaves. There was limited translocation of surfactants or metabolites from the treated leaf areas of intact plants. Formation of volatile or insoluble ^{14}C-labeled products was not significant within the time periods studied. Some nonpolar metabolites, including lower ethoxylates, were formed but polar ^{14}C-labeled products accumulated. Acid hydrolysis of the polar products released about 30% of their ^{14}C as the parent surfactant. The results indicated that up to 20% of the surfactant taken up was oxygenated in the *tert*-octyl moiety and that these metabolites occurred mostly in polar conjugates. Metabolites were isolated and purified by adsorption and reversed-phase chromatography and were characterized by mass spectrometry.

Surfactants occur in many pesticide formulations and are added to pesticidal spray mixtures applied to growing plants. Their influence on herbicide effectiveness has been discussed by Holly (1976). Under certain conditions the phytotoxicity of various surfactants has been demonstrated (Furmidge, 1959; Parr and Norman, 1964), and there have been reports that plant enzymes are sensitive to surfactants in vitro (Frear et al., 1969; Weidner and Burchartz, 1978). Little is known about the fate of these various surfactants in plants. Radiolabeled surfactants studied previously in plants, with or without pesticides, were of undefined structure or were inherently heterogeneous so that the behavior or fate of any single surface active component could not be observed (Smith and Foy, 1966; Valoras et al., 1974).

The individual ethylene oxide (EO) adducts of some nonionic surfactants have been resolved chromatographically (Kelly and Greenwald, 1958; Bürger, 1963). Homogeneous, specifically ^{14}C-labeled nonionic surfactants with six EO (6EO) and nine EO (9EO) units and well-defined hydrophobic moieties have been synthesized (Tanaka and Wien, 1976; Tanaka et al., 1976). Analytical methods and reference materials were available (Stolzenberg et al., 1971) for the ^{14}C-labeled surfactants derived from *p*-(1,1,3,3-tetramethylbutyl)phenol, i.e., *p-tert*-octylphenol (*t*-octPhOH). This report describes the uptake, effect, and metabolism of these materials in leaf tissue of young barley plants (*Hordeum vulgare* L.).

MATERIALS AND METHODS

Analytical Methods. Nitrogen carrier gas and OV-1 silicone liquid phase were used in gas chromatographic (GC) analyses (Stolzenberg et al., 1971). The purity of many reference materials was verified by GC. A stream splitter was used to trap materials for ^{14}C determinations or mass spectrometric (MS) analyses.

Thin-layer chromatography (TLC) was performed on plates with 0.25-mm silica gel HF (E. Merck) developed in the following solvents: (S-1) water-saturated 2-butanone, (S-2) benzene–acetone, 3:1, or (S-3) chloroform–methanol–water, 65:25:4 (v/v/v). Reference compounds were visualized under UV light or with I_2 vapor. Reversed-phase TLC (RP-TLC) plates with a 0.25-mm layer of hydrocarbon-impregnated fluorescent silica (Analtech, Newark, DE) were developed in water containing 50–80% methanol (Stolzenberg, 1982). Reference materials were visualized under UV light. For rechromatography on silica the hydrocarbons eluted from the RP-TLC gel were moved beyond the R_f zones of interest by preliminary development(s) with toluene.

Radioactivity was quantified by liquid scintillation counting (LSC) with external standardization. Radioactive zones on TLC plates were detected with a radiochromatogram scanner (Packard Model 7201) or by autoradiography.

A 3.9 mm i.d. × 30 cm μBondapak C_{18} column (Waters Associates) was used for reversed-phase high-performance liquid chromatography (HPLC) of the surfactants and some of their metabolites using a 25-min linear gradient of 50–100% methanol in water (Mansager et al., 1979) at 2.0 mL/min (ca. 2000 psig). Detection was by UV spectrophotometry (280 nm) or by LSC of aliquots from successive 2-mL fractions. The parent surfactant, *t*-octPh6EO, was retained ca. 19 min; similar results were reported by Otsuki and Shiraishi (1979). The final cleanup of most metabolites was by HPLC. Reversed-phase adsorption cartridges (Sep-PAK C_{18}, Waters Associates) were used to extract surfactants and their metabolites from aqueous samples (Riggin and Howard, 1979). The cartridges were washed with 3–10 mL of water and the adsorbed materials were eluted with 3–10 mL of methanol. The ^{14}C-labeled components were expected to be removed from the aqueous samples since nonionic surfactants with various EO contents were retained on reversed-phase columns (Turner et al., 1976; Henke, 1978) as were glycosides with relatively large aglycon moieties (Erni and Frei, 1977; Seitz and Wingard, 1978).

Poly(ethylene glycol) (PEG) are present in most commercial EO-containing surfactants (Bürger, 1963). Reference materials and aliquots of ^{14}C-labeled samples were characterized by extracting their saline solutions with ethyl acetate (for nonionic surfactants) and then with chloroform (for PEG) by the method of Weibull (1960).

Electron impact MS (EI-MS) data were obtained at 70 eV with a Varian-MAT CH-5-DF instrument with a probe for solid sample insertion and an SS-200 data system.

Surfactants. Nonradioactive Triton X-100 (Rohm and Haas), a heterogeneous 9.5 mol (average) EO adduct derived from *t*-octPhOH, was purchased commercially. The chromatographic methods for isolating the individual EO adducts have been described (Kelly and Greenwald, 1958; Bürger, 1963; Stolzenberg et al., 1971). Homogeneous radiolabeled surfactants were synthesized by first reacting *t*-octPhOH with chloro[*carboxy*-^{14}C]acetic acid (Tanaka

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and Wien, 1976). Consequently, only the second carbon of the first EO group attached to *t*-octPhOH was labeled (sp act. 8 mCi/mmol) in the hexaethoxylate ([1EO-^{14}C]-*t*-octPh6EO) and the nonaethoxylate ([1EO-^{14}C]-*t*-octPh9EO). These surfactants were repurified by TLC (S-1). The individual EO adducts in a small amount of [*phenyl*-UL-^{14}C]Triton X-100 (sp act. ca. 0.9 mCi/g, Rohm and Haas) were separated by TLC (S-1). After autoradiography fractions were eluted and rechromatographed to yield small quantities of the [*phenyl*-^{14}C]-*t*-octPh6EO and [*phenyl*-^{14}C]-*t*-octPh9EO surfactants.

Plant Materials. Barley was grown in vermiculite-filled trays or in 10-cm pots subirrigated with $^{1}/_{3}$ strength Hoagland's solution. The plants were used at the 2-2$^{1}/_{2}$ leaf stage (2 weeks old). For most experiments the first leaves (ca. 9 cm) were excised under water 1 h before treatment.

Excised Leaf Treatments. Excised leaves (ca. 1 g fresh weight) were treated by inserting the cut ends into vials containing 1 mL of surfactant solution (nonradioactive and/or ^{14}C labeled) at concentrations of 6–500 mg/L (ppm). Control tissues were treated with distilled water. The excised leaves were kept under fluorescent light and the vials were refilled periodically to replace water lost through transpiration. After ca. 5 h the leaves were removed and their cut ends were rinsed.

The excised leaves were assayed either after treatment or were placed into fresh water, kept under continuous illumination for up to 24 h, and then assayed. Selected leaves were rinsed, frozen, lyophilized, and subjected to autoradiography.

Photosynthesis Measurements. Excised leaves, treated as described, were rinsed and blotted. Sections (ca. 1 cm × 1 cm) were cut from the middle of each leaf blade. The sections (ca. 120 mg) were placed on the bottom of 100-mL cylindrical respirometer flasks, with their adaxial (upper) surface downward. Each flask contained 0.5 mL of 0.05 M potassium phosphate buffer, pH 7.0. A center well contained Pardee CO_2 buffer and a paper wick (Shimabukuro and Swanson, 1969). The flasks were equilibrated at 22 °C in a Gilson differential respirometer and were then illuminated from below. The O_2 evolution was measured at intervals over a period of 2 h. With plant tissues assayed at the end of a 5-h treatment period, the midpoint of the respirometry measurements was ca. 8 h after the leaf blades had been excised from the plants.

Foliar Applications. To ensure wetting of barley tissues grown in pots, we added Triton X-100 at 50–500 ppm to 6-ppm aqueous solutions of [1EO-^{14}C]-*t*-octPh6EO or [1EO-^{14}C]-*t*-octPh9EO. The surfactant solution was applied as droplets to the upper surface of the first or second leaves along the midrib. The 10–50 μL/leaf included up to 12.5 μg of surfactant. The varied wetting, spreading, and drying characteristics of these droplets prevented evaluations of ^{14}C uptake rate vs. EO chain length or total surfactant concentration. At various times an entire treated leaf was excised, immersed in methanol and/or water to remove the surface ^{14}C, and prepared for autoradiography or extracted for analysis.

Metabolites. Treated tissues were homogenized in methanol and the slurry was filtered. Extracts (50–300 mL) from excised leaves (1–20 g) or the surface washings and the methanol extracts of intact barley leaves were assayed by LSC. Residual ^{14}C-labeled surfactants and their metabolites in these samples were separated by TLC, and the radioactive zones located by autoradiography were scraped and assayed by LSC. The posttreatment solutions rinsed from the excised barley leaves after ca. 5 h and the water in which the leaves subsequently were held were assayed for ^{14}C by LSC. Some aqueous samples were adsorbed on Sep-PAK C_{18} cartridges and the methanol eluates were analyzed by TLC.

Aliquots of some methanolic tissue extracts were concentrated nearly to dryness. Water was added and the solution was extracted repeatedly with hexane and ethyl acetate. Residual ethyl acetate was removed from the aqueous phase and the water-soluble ^{14}C-labeled metabolites were subjected to adsorption on a Sep-PAK C_{18} cartridge and to TLC (S-1, S-3) or RP-TLC.

Metabolites recovered from TLC plates or eluted from the C_{18} cartridges were concentrated to dryness prior to hydrolysis attempts. They were taken up in ca. 1 mL of 1.2 N HCl and heated at 100 °C for ca. 4 h in closed tubes. The ^{14}C-labeled products were then separated by TLC (S-1). Metabolites were also hydrolyzed with β-glucosidase buffered at pH 5.25 (Frear and Swanson, 1972).

RESULTS AND DISCUSSION

Surfactant Uptake by Excised Leaves. The fresh weight of excised barley leaves remained constant for over 24 h after treatment with water or Triton X-100. More than 70% of the ^{14}C-labeled surfactant was taken up within 5 h, and similar rates were assumed for all major components of Triton X-100. Then 1-mL treating solutions at 500 ppm transferred at least 325 μg of surfactant/g of fresh tissue weight (ppm) into the excised leaves.

The ^{14}C was uniformly distributed throughout the length of the leaf blade after 5 h of uptake. Only the treated, cut ends and the tissues adjacent to the midvein and to the four main veins of each leaf showed a slight accumulation. This distribution of ^{14}C did not change appreciably during the subsequent holding period in water, and less than 15% of the ^{14}C was released out into the water. Thus, tissue sections taken from the middle of the leaf blade for measuring photosynthetic O_2 evolution contained surfactant at representative concentrations.

Effect of Triton X-100 on Photosynthesis. Sections from excised leaves that had been held in water for 25–30 h evolved oxygen at nearly the same rate as sections from freshly excised leaves (Table I). The data on photosynthetic oxygen evolution indicated further that surfactant-treated leaf tissues were viable. Leaf tissues treated with 250 ppm of Triton X-100 solution had partially inhibited O_2 evolution rates after ca. 5 h. Partial recovery of photosynthesis occurred while the surfactant-treated excised leaves were held in water (Table I). In contrast, treatment with 10 μM (ca. 2 ppm) atrazine [2-chloro-4-(ethylamino)-6-(isopropylamino)-*s*-triazine], an inhibitor of the Hill reaction in photosynthesis, decreased O_2 evolution with no apparent recovery (Table I). Similar results with atrazine were observed in pea leaf disks (Shimabukuro and Swanson, 1969). Isolated spinach chloroplasts were photosynthetically inactive in buffered 100 μM (ca. 60 ppm) Triton X-100 (Deamer and Crofts, 1967).

Surfactant Metabolism in Excised Leaves. The nearly quantitative recovery of ^{14}C in the methanolic extracts indicated that neither insoluble nor volatile materials were formed in appreciable quantities in 24 h. TLC analyses (Figure 1, S-1, one ascent) of the extracts showed that excised barley leaves metabolized the surfactants even at high concentrations (Table II). The parent compounds [S-1, R_f 0.64 (6EO) and 0.31 (9EO)] were converted to both less polar (R_f >0.7) and more polar (R_f <0.15) products. At lower concentrations, both ^{14}C-labeled surfactants were metabolized rapidly. Similar trends were reported for the microbial degradability of ethoxylated alkylphenol surfactants (Patterson et al., 1968; Sturm, 1973).

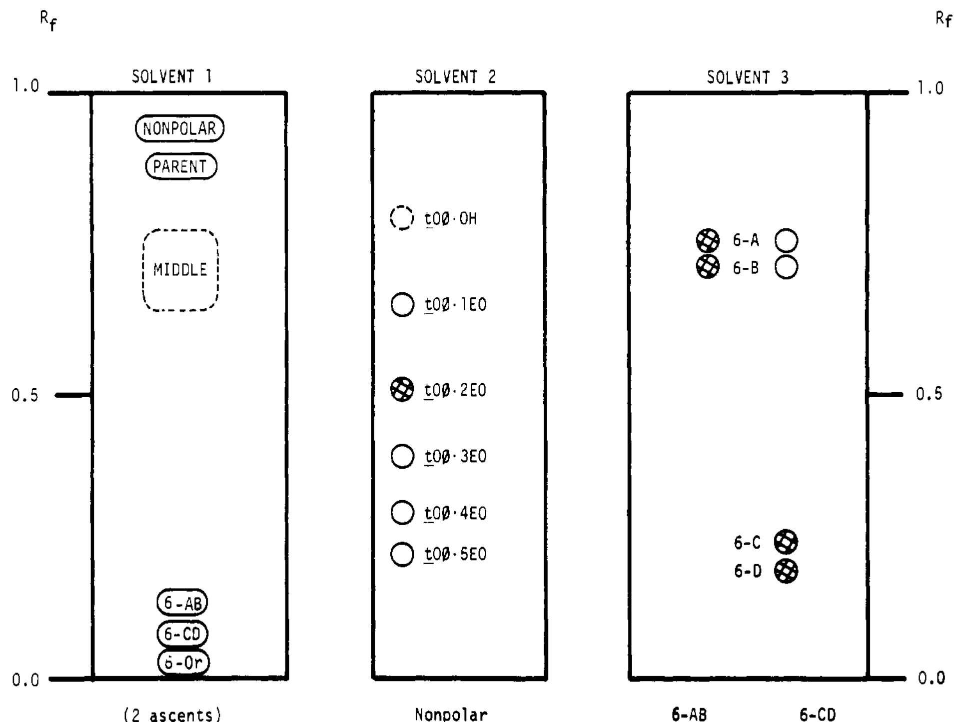


Figure 1. Representation of silica thin-layer chromatogram with metabolites of [^{14}C]-*t*-octPh6EO extracted from barley tissues with methanol: left, 2× in S-1, separation of the methanol solubles; center, S-2, resolution of the nonpolar metabolites (*t*-octPhOH was not formed; *t*-octPh2EO was the major component); right, S-3, separation of some polar metabolites.

Table I. Photosynthetic Oxygen Evolution by Tissue Sections from First Leaves of Barley Excised and Treated through Their Cut Ends

treating solution, ppm	expt	after treatment (4-5 h) rate[a]	after treatment (4-5 h) rel %	then in H_2O 17-19 h rate[a]	then in H_2O 17-19 h rel %
control (0)	1			82 ± 6[b]	100
X-100 (100)				80 ± 7	98
X-100 (500)				44 ± 12	54
control (0)	2	79 ± 13	100	85 ± 7	100
X-100 (250)		66 ± 10	83	87 ± 6	102
control (0)	3	111 ± 15	100	94 ± 11[c]	100
X-100 (250)		62 ± 10	56	67 ± 12	71
control (0)	4	44 ± 2	100	42 ± 8	100
X-100 (250)		14 ± 3	31	26 ± 2	62
atrazine (2)		14 ± 3	31	6 ± 2	13
control (0)	5	74 ± 9	100	61 ± 8	100
X-100 (250)		48 ± 6	65	47 ± 8	77
atrazine (2)		13 ± 4	17	14 ± 5	23
control (0)	6	80 ± 7	100	71 ± 11	100
X-100 (250)		44 ± 6	55	54 ± 7	76
atrazine (2)		12 ± 2	15	13 ± 3	18

[a] As microliters of O_2 evolved per gram fresh weight per minute. [b,c] Control sections from untreated leaves freshly excised (not aged ca. 24 h) from barley plants yielded rates of (*b*) 81 ± 11 and (*c*) 91 ± 6 when tested at the same time.

Excised barley leaves treated with two surfactant concentrations yielded the time-course metabolism data in Table III. At 6 ppm almost 40% of the *t*-octPh6EO was metabolized in 5 h, and after 13.5 h (8.5-h holding period) only 7% remained as the parent compound. Polar products accumulated at both concentrations. The time required for the excised tissues to metabolize most of the parent surfactant (Tables II and III) approximated that for the recovery of photosynthetic activity (Table I).

Residual Treating Solution. No significant microbial or photolytic degradation of *t*-octPh6EO occurred in the treating solutions. Over 95% of the ^{14}C in the posttreatment solutions plus the rinsings from the excised leaves (at 5 h) and the ^{14}C in the water in which the leaves were held subsequently (ca. 16 h) was retained on Sep-PAK C_{18} cartridges. The ^{14}C was eluted quantitatively with methanol. Over 99% and 95%, respectively, of the radioactivity was the parent compound in the posttreatment and holding solutions. The remaining 5% in the holding solution was predominantly less polar than the parent compound. Chromatography by two-dimensional TLC (S-1 and S-2; see Figure 1) indicated that the less polar compounds were a series of lower ethoxylates (Stolzenberg et al., 1971).

Nonpolar Metabolites in Tissues. Figure 2 gives a schematic of the fractionation and purification of larger amounts of the methanol-extractable ^{14}C-labeled materials, and Figure 1 represents the separations achieved by TLC on silica. Multiple ascents (Figure 1, S-1) resolved partially the polar metabolites, but metabolites less polar than the parent surfactant(s) coalesced near the solvent front. This mixture of several components (Figure 1, S-2) contained up to 20% of the ^{14}C in excised barley tissues. They were predominantly lower ethoxylates, *t*-octPh1EO through *t*-octPh5EO, as indicated by comparison with reference materials (Kelly and Greenwald, 1958; Stolzenberg et al., 1971) by GC, TLC, and/or HPLC (Figures 1 and 2; Table IV). Small amounts of other unidentified nonpolar metabolites were present in the extracts. The diethoxylate, *t*-octPh2EO, was the major nonpolar metabolite from either *t*-octPh6EO or *t*-octPh9EO. This [^{14}C]diethoxylate, present as less than 0.2% impurity in the radiolabeled surfactants used to treat excised leaves, was isolated as a product amounting to 2.7% of the absorbed dose of ^{14}C. The diethoxylate was not formed in aqueous treatment solutions when they were aged for 1–2 days without plant tissues. Rudling and Solyom (1974) characterized the corresponding diethoxylate as the only identifiable product after microbial degradation of EO adducts of a branched nonylphenol.

Surfactant uptake and the chromatographic profile of the metabolites from excised barley leaves were similar

Table II. Analyses of ^{14}C-Labeled Materials (TLC, S-1) in Methanol Extracts of Excised First Leaves Treated with 6 ppm of [1EO-^{14}C]-*t*-octPh6EO or [1EO-^{14}C]-*t*-octPh9EO and Various Amounts of Triton X-100 (Nonradioactive) for 6 h and Transferred to H_2O for 10 h

^{14}C-labeled surfactant (M_r)	surfactant in tissue, ppm[a]	distribution, %, of ^{14}C in zones of increasing R_f[b]: polar (≃origin)	above origin	parent[c]	below front	nonpolar (≃front)
t-octPh6EO (470)	3	72	6	8		14
	160	25	5	57		14
	350	19	6	60		15
t-octPh9EO (602)	3	21		67	1	11
	140	11		76	4	10
	320	9		79	4	9

[a] Based on ^{14}C assays: difference between total micrograms of surfactant available to barley tissue (ca. 1 g) and amount remaining in treating solution (1 mL) after 6 h. [b] Typically 4000 dpm/TLC assay; overspotted with parent compound and later visualized under UV light; zones scraped for LSC. [c] At R_f 0.64 (6EO) or 0.31 (9EO) after one ascent.

Table III. Analyses at Different Time Periods of ^{14}C-Labeled Materials (TLC, S-1) in Methanol Extracts of Excised First Leaves Treated with [1EO-^{14}C]-*t*-octPh6EO for 5 h and Held in Fresh Water

treating solution, ppm[a]	holding time, h	distribution, %, of ^{14}C in zones of increasing R_f (origin to front)[b]: polar	middle	parent[c]	nonpolar
6	0	17	8	63	12
	3	41	12	30	17
	8.5	70	7	7	16
156[d]	0	7	7	80	6
	3	18	6	64	12
	8.5	26	6	53	15

[a] Transpiration uptake of the original 1 mL give tissue loadings of ca. 2.5 ppm (from 6 ppm) and 87 ppm (from 156 ppm). [b] Typically 4000 dpm/TLC assay; scraped zones quantified by LSC. [c] Typically R_f 0.64 on HF silica. [d] Included 150 ppm of nonradioactive Triton X-100.

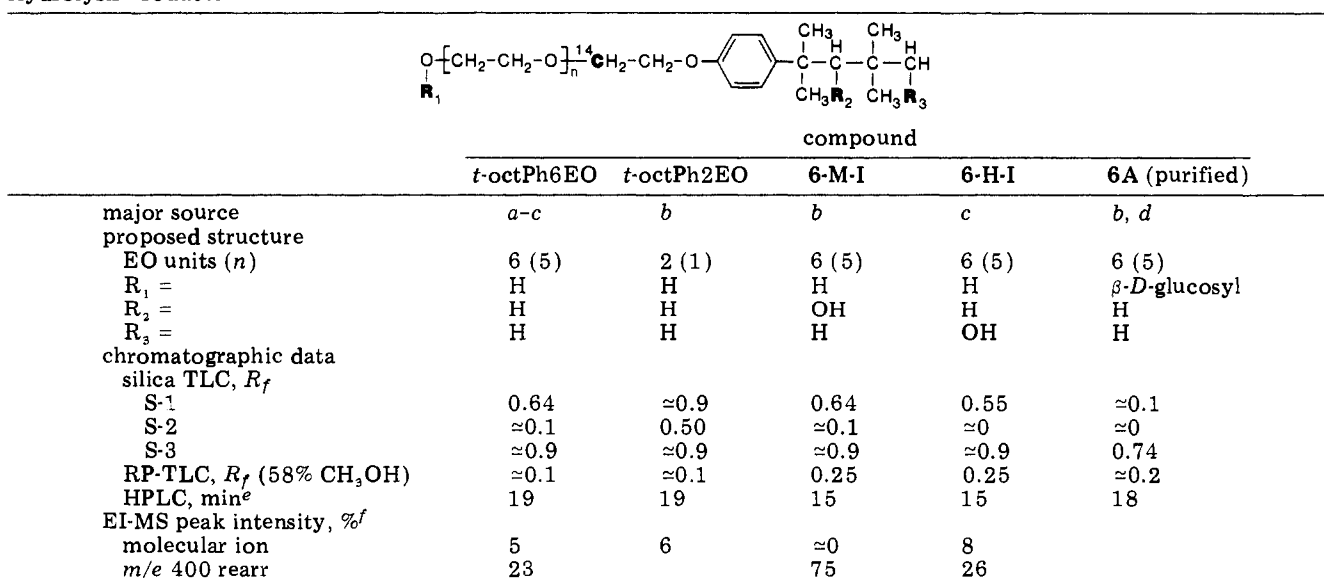
with either [*phenyl*-^{14}C]-*t*-octPh6EO or [1EO-^{14}C]-*t*-octPh6EO. This was also true when the tissues were treated with [*phenyl*-^{14}C]-*t*-octPh9EO or [1EO-^{14}C]-*t*-octPh9EO. The free alkylphenol, [phenyl-^{14}C]-*t*-octPhOH (Figure 1; R_f 0.79, S-2), was not detected as a nonpolar metabolite from either of the phenyl-^{14}C-labeled surfactants. This showed that none of the major metabolites was formed by scission of the entire EO chain from the phenyl moiety of the surfactants.

Metabolites of Intermediate Polarity. The quantity of unmetabolized *t*-octPh6EO or *t*-octPh9EO in extracts from treated excised leaves varied with the surfactant concentration in the treating solutions (Table II) and with time (Table III). Solvent partitioning and reversed-phase chromatography (Figure 2 and Table IV) showed that extracts from tissues treated with *t*-octPh6EO contained a metabolite (designated **6-M-I**) that cochromatographed with *t*-octPh6EO on silica (R_f 0.65, S-1). Their separation and the characterization of **6-M-I** are discussed later.

Only traces of materials slightly more polar than the parent surfactants (Figure 1, S-1, zone designated middle) were present in tissue extracts. This was the zone for the higher ethoxylates (Bürger, 1963) and the initial products of surfactant oxidation also were expected to be found here (Patterson et al., 1968).

Polar Metabolites in Excised Tissue. The polar metabolites in the tissue extracts (Tables II and III) were resolved partially by TLC (Figure 1, S-1, two ascents). Metabolite zones (designated 6-AB and 6-CD or 9-AB and 9-CD, respectively) detected by autoradiography were rechromatographed in a system used for separating glyco-

Table IV. Summary of Analytical Data and Structures Proposed for *t*-octPh6EO Surfactant, Some Metabolites, and Hydrolysis Products



	compound: *t*-octPh6EO	*t*-octPh2EO	6-M-I	6-H-I	6A (purified)
major source	*a–c*	*b*	*b*	*c*	*b, d*
proposed structure					
EO units (n)	6 (5)	2 (1)	6 (5)	6 (5)	6 (5)
R_1 =	H	H	H	H	β-*D*-glucosyl
R_2 =	H	H	OH	H	H
R_3 =	H	H	H	OH	H
chromatographic data					
silica TLC, R_f					
S-1	0.64	≃0.9	0.64	0.55	≃0.1
S-2	≃0.1	0.50	≃0.1	≃0	≃0
S-3	≃0.9	≃0.9	≃0.9	≃0.9	0.74
RP-TLC, R_f (58% CH_3OH)	≃0.1	≃0.1	0.25	0.25	≃0.2
HPLC, min[e]	19	19	15	15	18
EI-MS peak intensity, %[f]					
molecular ion	5	6	≃0	8	
m/e 400 rearr	23		75	26	

[a] Substrate for these experiments. [b] Found in extracts of treated barley tissues. [c] Acid hydrolysis of polar metabolites from tissue. [d] Other minor polar metabolites were removed during purification. [e] Gradient elution (see the text). [f] Relative to tropylium ion (m/e 399; for *t*-octPh2EO, m/e 223) normalized at 100%.

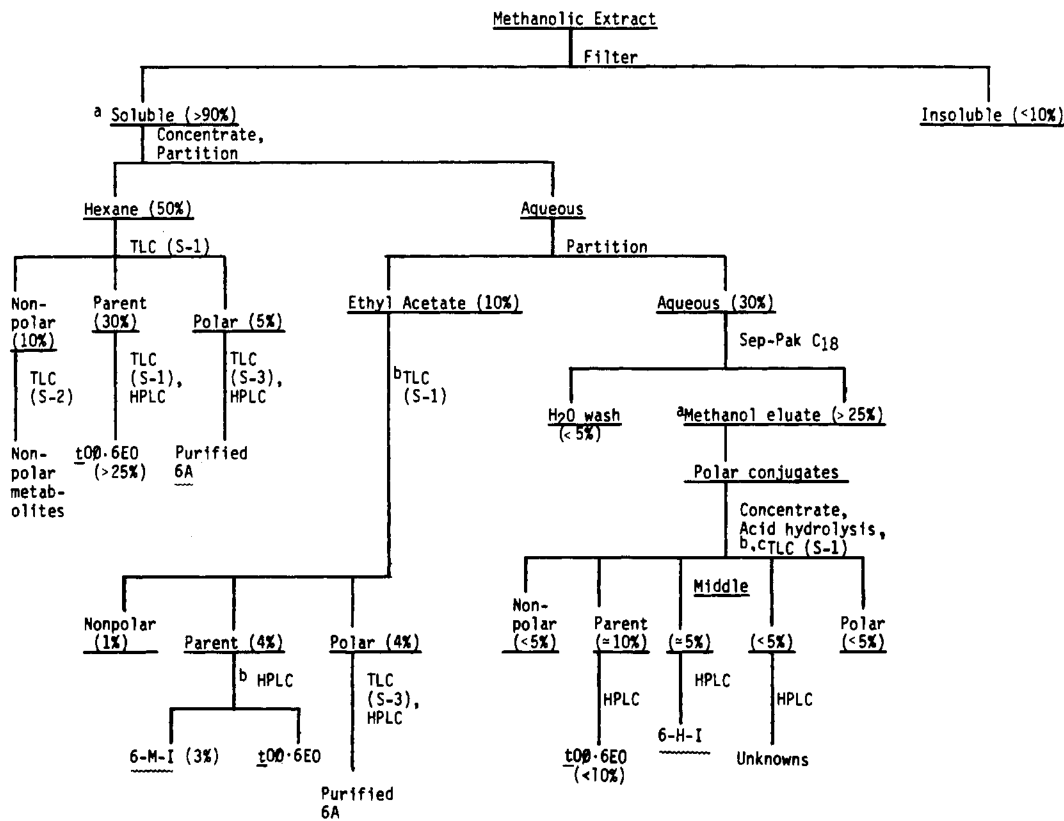


Figure 2. Scheme for the isolation of *t*-octPh6EO metabolites, conjugated metabolites, and their hydrolysis products in homogenates of excised barley leaves. Average yields from several isolations (percent ^{14}C recovered) following treatment in solutions with 50–90 μg of surfactant/g fresh weight; extracted after ca. 24 h. (a) Aliquots taken for TLC analyses. (b) Optionally, TLC (RP, 58%). (c) Optionally, absorption onto a Sep-PAK C_{18} cartridge.

sides (Frear and Swanson, 1972) and/or phospholipids. Zones 6-AB or 9-AB each yielded two partially resolved TLC zones (Figure 1, S-3; 6-A, R_f 0.74, and 6-B, R_f 0.70; 9-A, R_f 0.82, and 9-B, R_f 0.79). Similarly, zones 6-CD or 9-CD each yielded two zones in system S-3 (6-C, R_f 0.20, and 6-D, R_f 0.15; 9-C, R_f 0.25, and 9-D, R_f 0.20). Metabolites **6-CD** or **9-CD** and other ^{14}C-labeled product(s) near the origin (Figure 2, S-1; **6-Or** or **9-Or**) accumulated with time. The surfactant metabolites **6-CD** or **9-CD** were unstable, and the more mobile **6-AB** or **9-AB**, respectively, were found when TLC was repeated (Figure 1, S-3). By TLC of the individual zones it was found that **6-C** yielded **6-A** and that **9-C** yielded **9-A**. The decomposition products from **6-D** or **9-D** were identical chromatographically to **6-B** or **9-B**, respectively.

These unresolved polar metabolites (e.g., **6-AB**, **6-CD**, and **6-Or**) were hydrolyzed nearly quantitatively by acid. Most of the numerous hydrolysis products had increased R_f values (S-1). The respective parent surfactants (*t*-octPh6EO or *t*-octPh9EO) accounted for up to 30% of the ^{14}C present after these polar metabolites were hydrolyzed. These parent surfactants were released primarily from the polar metabolites in TLC zones 6-A and 6-C or 9-A and 9-C. Several materials found in the middle TLC zone (Figure 1, S-1, one ascent) also accounted for up to 30% of the ^{14}C present after the polar metabolites were hydrolyzed. They were released primarily from the metabolites in TLC zones 6-B or 9-B rather than from zones 6-A or 9-A. One compound, designated **6-H-I** (S-1, R_f 0.55), was purified for MS analyses from the acid-hydrolyzed polar metabolites in TLC zone 6-B of extracts from tissues treated with *t*-octPh6EO surfactant. Figure 2 presents an alternate isolation of **6-H-I** and its characterization is presented in a later section. Acid hydrolyses of these polar metabolites also released more than 10 other ^{14}C-labeled products of varying TLC mobility and these remain largely unidentified. The free alkylphenol, *t*-octPhOH, was not detected after hydrolysis of metabolite(s) **9-AB** isolated from excised barley leaves treated with [*phenyl*-^{14}C]-*t*-octPh9EO.

Characterizations of **6-Or** or **9-Or** as intact metabolites were unsuccessful. These were even less mobile on TLC than some oligomeric PEG samples (R_f ca. 0.2, S-1, I_2 visualization), potential metabolites from the 1EO-^{14}C-labeled surfactants. The PEG samples (e.g., tetraethylene glycol, Aldrich Co.) did chromatograph on silica (S-1) similarly to the acid-labile, polar metabolites **6-AB**, **6-CD**, **9-AB**, and **9-CD**. After hydrolysis, however, the ^{14}C-labeled products released from the metabolites, including **6-H-I** described above, partitioned nearly quantitatively into ethyl acetate from saline and also had increased mobility on TLC. The PEG, in contrast, was not hydrolyzed and did not partition into ethyl acetate (Weibull, 1960). In other work, solvent partitioning was coupled with RP-TLC techniques to show that the polar, water-soluble metabolites from [EO-^{14}C]-*t*-octPh6EO could contain only traces of PEG, free or in acid-labile conjugates (Stolzenberg, 1982).

Mass Spectra of Ethoxylated Alkylphenol Surfactants. Figure 3 gives the EI-MS of [1EO-^{14}C]-*t*-octPh9EO. This polyether had a molecular ion ($M^{+}\cdot$) at m/e 602 and an intense ion at m/e 531 (M − 71) following loss of the neopentyl group, C_5H_{11}. Intense peaks for the corresponding polyethoxylated dimethyltropylium ions were observed for each EO adduct of *t*-octPhOH examined, and *t*-octPh6EO yielded m/e 470 for $M^{+}\cdot$ and m/e 399 for its M − 71 ion (Julia-Danes and Casanovas, 1979; Paulson et al., 1980). Similar fragmentations were reported in the EI-MS of nonylphenol EO adducts (Harless and Crabb, 1969; Huber et al., 1972). Each pure EO adduct of *t*-

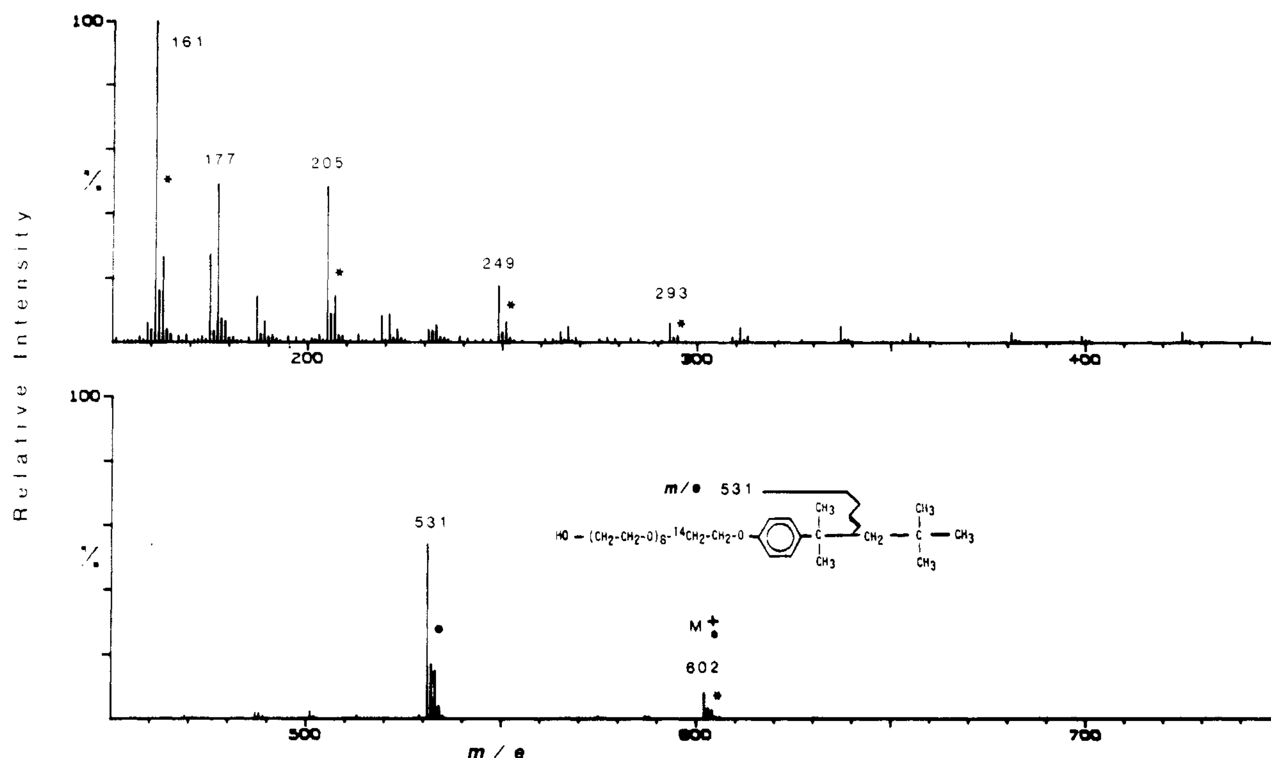


Figure 3. Mass spectrum of the [1EO-^{14}C]-*t*-octPh9EO; ions with appreciable ^{14}C-labeled satellites at P + 2 are designated by an asterisk.

octPhOH yielded ions of only minimal intensity by sequential losses of EO (44 amu) from the M − 71 ion, so that any significant peaks (Figure 3) at *m*/*e* 487 or 443 would have indicated contamination by surfactants with 8EO or 7EO, respectively. The *m*/*e* 293, 249, 205, and 161 fragments were attributed to sequential losses of EO from dehydrated polyethoxylated dimethyltropylium ions (Julia-Danes and Casanovas, 1979). For [1EO-^{14}C]-*t*-octPh9EO at high specific activity (Figure 3), this series of ions had satellites (P + 2) diagnostic of fragments still containing the radiocarbon atom. This assignment was confirmed also by an elemental composition of $C_{13}H_{17}O_2$ (Paulson et al., 1980) obtained from the precise mass measurement of the *m*/*e* 205 fragment from *t*-octPh6EO, showing that it was not an octylphenoxide ion formed by loss of all the EO groups. The intense *m*/*e* 135 ion from [1EO-^{14}C]-*t*-octPh9EO, a fragment common to *t*-octPhOH and each of its EO adducts, lacked a P + 2 satellite and it was attributed to a dimethylhydroxytropylium species.

The reference lower ethoxylates of *t*-octPhOH also gave an $M^{+\cdot}$ and had intense ions for M − 71 and *m*/*e* 135. The dehydrated ion series which includes *m*/*e* 161 and 205 was of reduced intensity. This was observed when the nonpolar metabolites of *t*-octPh6EO or *t*-octPh9EO from excised barley leaves were analyzed. These lower ethoxylates gave spectra similar to that of *t*-octPh3EO in the report by Sheldon and Hites (1979) that described the EI-MS fragments below *m*/*e* 135 and also reported enhanced yields of the parent ion (M + 1) by chemical ionization (isobutane) MS.

Isolation of Metabolite 6-M-I. The partitioning steps shown in Figure 2 were adopted to isolate the polar metabolites from excised leaves treated with 50–90 μg of *t*-octPh6EO/g fresh weight. The major ^{14}C-labeled component in the hexane extract chromatographed with the reference 6EO surfactant in HPLC and TLC assays. However, the ethyl acetate extract contained both the ^{14}C-labeled surfactant and an abundant moderately polar (RP-TLC, 58% CH_3OH, R_f 0.25) material, designated **6-M-I**, that accounted for 2–6% of the total ^{14}C in the tissue. When relatively impure samples of **6-M-I** were analyzed by EI-MS, no $M^{+\cdot}$ was observed and the only significant ion was *m*/*e* 399. Presumably *t*-octPh6EO was not a contaminant, and **6-M-I** also yielded a hexaethoxylated dimethyltropylium ion (see Figure 3) by loss of a metabolized neopentyl fragment. The lack of an analogous ion at *m*/*e* 415 indicated that **6-M-I** did not have an oxygenated phenyl moiety or a 1-hydroxymethyl substituent on the butyl moiety. Final purification was by HPLC (elution ca. 15 min for **6-M-I** vs. 19 min for *t*-octPh6EO). The analytical data for **6-M-I** are compared with those for other metabolites in a later section (Table IV).

***O*-β-D-Glucoside of *t*-octPh6EO from TLC Zone 6-A.** The most abundant polar metabolite in the hexane and ethyl acetate extracts (Figure 2) was similar to metabolite zone 6-A (Figure 1) by TLC. It was isolated from either organic phase by repeated TLC (S-1, R_f ca. 0.1; S-3, R_f ca. 0.7) or by RP-TLC (Table IV); final purification by HPLC yielded a single component (by UV absorption and LSC of ^{14}C) eluting at ca. 18 min (Table IV). This metabolite (designated **6-A**) was identical by TLC analysis (S-1 or S-3) to the *O*-β-D-glucoside of *t*-octPh6EO produced in vitro by a soluble enzyme system isolated from etiolated pea epicotyl tissues (Frear et al., 1977). Purified samples of **6-A** from excised barley leaves were cleaved nearly quantitatively by acid hydrolysis to release *t*-octPh6EO and treatment with β-glucosidase caused over 90% hydrolysis to *t*-octPh6EO (TLC, S-1).

Isolation of Hydrolysis Product 6-H-I. The aqueous phase, after partitioning with hexane and ethyl acetate, contained up to 40% of the methanol-extractable ^{14}C as polar metabolites from *t*-octPh6EO (Figure 2). Adsorption from the aqueous phase onto a Sep-PAK C_{18} cartridge afforded nearly quantitative recovery of the ^{14}C in methanol. TLC analyses (S-3) indicated that although some of the zone 6-A metabolite(s) had partitioned into hexane and ethyl acetate, the metabolites in zones 6-B, 6-C, 6-D,

and 6-Or (Figure 1, S-1 or S-3) were recovered from the C_{18} cartridge unchanged. The more polar (and somewhat unstable) metabolites in zone 6-CD were more abundant than those in 6-AB when samples were analyzed within 1 day after extraction from the tissues. Since nearly all of the water-soluble metabolites of *t*-octPh6EO or *t*-octPh9EO were retained by Sep-PAK C_{18} cartridges, the hydrophobic *t*-octPh moiety probably remained intact for the time periods studied in excised leaves and the greatly increased polarity observed by TLC on silica was due to conjugation.

Acid hydrolysis of the mixed polar metabolites of *t*-octPh6EO converted most of them to less polar materials. Several components (TLC, S-1, R_f 0.45–0.65) accounted for the majority of the ^{14}C. The least polar and most abundant was identified as the parent surfactant, *t*-octPh6EO, by several chromatographic techniques (Table IV). The metabolite **6-M-I** was detected only in trace amounts among the hydrolysis products. In the subsequent chromatographic steps to purify **6-H-I** (R_f 0.55, S-1) several other minor ^{14}C-labeled products with similar chromatographic characteristics were removed.

Assignment of Metabolite Structures. The *t*-octPh6EO, **6-M-I**, and **6-H-I** were purified by HPLC (Table IV). Gradient HPLC elution trials with several commercially available phenyl-substituted linear and branched alkanols showed that changes in the carbon chain length or the hydroxyl content had a greater effect on retention than did structural isomerism. The results were similar by RP-TLC (Stolzenberg, 1982). Structural isomers, however, were resolved more readily by silica TLC. The HPLC data suggested that metabolite **6-M-I** and hydrolysis product **6-H-I** contained an additional hydroxyl group compared to *t*-octPh6EO.

The EI-MS were obtained for *t*-octPh6EO (extracted from excised leaves treated with [1EO-^{14}C]-*t*-octPh6EO diluted to low specific activity) and for purified samples of **6-M-I** and **6-H-I**. The m/e 45 or 89 fragment from the EO chain usually was the base peak (Sheldon and Hites, 1979), and the higher EO adducts had reduced intensities at m/e 133, 177, and 221 (see Figure 3). The m/e 399 ion typically had an intensity approximating that of the m/e 135 or 161 ions discussed earlier (Figure 3). This showed that the 6EO group remained intact and that the formation of hexaethoxylated dimethyltropylium ions from the three compounds was a favored process. None of the spectra had an m/e 415 ion of similar intensity to suggest an ethoxylated hydroxytropylium ion arising from ring-hydroxylated metabolites or a 1-hydroxymethyl substituent on the butyl moiety. The expected $M^{+\cdot}$ at 470 for *t*-octPh6EO was observed and a $M^{+\cdot}$ at 486 for **6-H-I** (Table IV) supported the proposed monohydroxylation. No $M^{+\cdot}$ was observed for **6-M-I**. This suggested that **6-M-I** had a secondary alcohol group in the neopentyl moiety in addition to the primary alcohol terminus on the EO chain, while **6-H-I** was a primary diol. Thus, a 4-hydroxy substituent (at R_3, Table IV) was assigned tentatively to **6-H-I**.

The ^{13}C contribution at m/e 400 in EI-MS would have been ca. 21% of the m/e 399 tropylium ion intensity, and this was observed for *t*-octPh6OE and for **6-H-I** (Table IV). This was in agreement with the structure of *t*-octPh6EO and supported the structure proposed for **6-H-I** since neither had a hydrogen on its γ carbon for rearrangement back onto the ring while losing the largest substituent on the α carbon as an unsaturated neutral fragment (Julia-Danes and Casanovas, 1979). The yields of rearranged ions from compounds with hydrogen(s) on a γ atom (e.g., carbon) were reported to increase sharply when hydrogen was present on a γ heteroatom (Gilpin, 1958). This was attributed to McLafferty-type rearrangements since a carbonyl-containing neutral fragment was lost. The abundant m/e 400 ions from **6-M-I** suggested that a 2-hydroxy group was present (at R_2, Table IV). The reasons against assigning a 1-hydroxymethyl substituent, which would also have a hydrogen on a γ heteroatom, in any of these metabolites and for regarding **6-M-I** as a secondary alcohol already were given.

Surfactant Metabolism in Intact Leaves. These analogues of Triton X-100, [1EO-^{14}C]-*t*-octPh6EO or [1EO-^{14}C]-*t*-octPh9EO, were absorbed from the upper surface of leaves of intact barley plants. Water or methanol rinses of these leaves removed less than 30% of the ^{14}C after 1 day and less than 20% after 6–8 days. The parent surfactant was the major ^{14}C-labeled component in the leaf rinses (TLC, S-1). Autoradiography showed that most of the ^{14}C remained in the treated region, but up to 10% was translocated acropetally toward the leaf tips. No appreciable basipetal translocation into other parts of the plant was observed.

The ^{14}C-labeled surfactant was metabolized although there was slight injury to treated leaf surfaces by the high concentration of Triton X-100 included in the foliar applications. About 50% of the ^{14}C in methanol extracts from surface-rinsed leaves was parent surfactant after 1 day and less than 20% after 6 days. Nonpolar metabolites (TLC, S-1) were not present in the quantities found in extracts of treated excised leaves. Polar metabolites accumulated and their distribution was similar, qualitatively, to that in excised leaves (TLC, S-3). The major acid hydrolysis products of the polar water-soluble metabolites in intact leaves treated with [1EO-^{14}C]-*t*-octPh6EO were similar chromatographically to *t*-octPh6EO and **6-H-I** that were isolated from excised leaves (Figure 2).

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Structural Effects on the Microbial Diazotization of Anilines

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Thirty-six aniline derivatives were examined for microbial converstion to diazonium salts by using a 2-naphthol trapping technique. The reaction is general except for anilines which are either 2,6 disubstituted or which are substituted with strongly electron withdrawing groups. For five representative anilines, the rates of biological and chemical diazotization at pH 6.3 are identical and are not substituent dependent. This shows that the organisms act only to convert nitrate to nitrite and that the actual diazotization step occurs without biological assistance. At this pH, formation of the active nitrosating agent is rate limited. Yields of azonaphthols are lower for the biological system than for the chemical system, and it is proposed that cellular metabolites interfere by trapping some of the diazonium ions to give as yet unidentified byproducts.

Substituted anilines are formed during the microbial degradation of a variety of aniline-based herbicides applied to soils and are subject to many subsequent transformations. Chloro- and methyl-substituted anilines are converted to azobenzenes (Bartha and Pramer, 1967; Bartha, 1968; Chisaka and Kearney, 1970). The compound 3,4-dichloroaniline is transformed to such compounds as 1,3-bis(3,4-dichlorophenyl)triazene (Plimmer et al., 1970), and 3,3′,4′-trichloro-4-(3,4-dichloroanilido)azobenzene (Linke, 1970). The bacterium *Bacillus firmus* forms 4-chloroacetanilide, 4-chloropropionanilide, and 7-chloro-2-amino-3*H*-3-hydroxyphenoxazine from 4-chloroaniline (Engelhardt et al., 1977).

Plimmer et al. (1970) have proposed that 1,3-bis(3,4-dichlorophenyl)triazene formation in soil may involve the reaction of 3,4-dichloroaniline with soil nitrite to form the diazonium ion, which then couples with unreacted 3,4-dichloroaniline. The same mechanism was proposed by Minard et al. (1977) to occur in the transformation of anilines to triazenes by a *Paracoccus* sp. Corke et al. (1979) proposed that the diazonium ion was the key intermediate in the formation not only of the triazenes but also of azobenzenes and biphenyls from aniline derivatives. This was shown by trapping the diazonium ion (whose formation was initiated by the bacterial reduction of nitrate to nitrite) with 2-naphthol. In the presence of 2-naphthol, the production of biphenyl, azobenzene, and triazene was substantially reduced in favor of the coupling product between the diazonium ion and 2-naphthol. This pathway for forming the azo compound is different from the peroxidase mechanism advocated by Bartha et al. (1968), although it must be noted that these latter observations were made under very different experimental conditions.

This paper summarizes studies on the effects of ring substitution of anilines on the formation of coupled azonaphthols and comparisons of rates of formation of these compounds in chemical and microbial systems.

MATERIALS AND METHODS

Culture and Growth Medium. *Escherichia coli* B (No. 263), obtained from the culture collection of the Department of Microbiology, University of Guelph, was used in all experiments. The basal medium was as follows (grams per liter of distilled water): $MgSO_4{\cdot}7H_2O$, 0.2, $CaSO_4{\cdot}2H_2O$, 0.05, KH_2PO_4, 3.65, Na_2HPO_4, 5.7, and Difco yeast extract, 1.0, adjusted to a final pH of 6.9 after autoclaving. The complete growth medium was formulated by the addition of required volumes of filter-sterilized solutions of glucose and sodium nitrate to yield final concentrations of 1% (w/v) and 100 μg of NO_3–N mL^{-1}, respectively.

Chemicals. A total of 36 anilines was used in this study and these are listed in Table I. Liquid anilines were purified by vacuum distillation from zinc dust. Solid anilines were chromatographed on alumina columns and

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