0099-2240/78/0035-0124$02.00/0
APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 1978, p. 124-128

Vol. 35, No. 1
Printed in U.S.A.

Microbial Transformations of Styrene and [^{14}C]Styrene in Soil and Enrichment Cultures

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Received for publication 1 August 1977

Two different mechanisms were responsible for the disappearance of styrene in enrichment cultures: (i) a mixed population of microorganisms, capable of utilizing styrene as a sole carbon source, oxidized this substrate to phenylethanol and phenylacetic acid; (ii) the culture also mediated polymerization of the monomer to low-molecular-weight styrene oligomers. This chemical reaction probably occurred as the result of microbial degradation of butylcatechol, an antioxidant polymerization inhibitor present in commercial styrene. The resultant polymer material was subsequently metabolized. In soil incubation studies, $^{14}CO_2$ evolution from applied [8-^{14}C]styrene was used to estimate microbial degradation. Approximately 90% of the labeled carbon was evolved from a 0.2% addition, and about 75% was lost from the 0.5% application over a 16-week period.

There are numerous reviews on the microbial metabolism of both natural and synthetic aromatic hydrocarbons (6, 7, 12, 13). Most of the compounds for which degradative pathways have been documented are simple or alkyl-substituted aromatics. In a recent study, Omori et al. (22) tested a variety of alkenyl-substituted aromatics for utilization by soil microorganisms. Although methylstyrene has been shown to be metabolized (21), these investigators reported that utilization of styrene as the sole carbon source did not support the growth of a mixed population of microorganisms isolated from either garden or forest soils.

Microbial formation of styrene and styrene derivatives has been reported by several investigators. Decarboxylation of cinnamic acid to form styrene has been demonstrated with species of *Penicillium* (17), *Aspergillus* (5), and *Saccharomyces* (4). Harada and Mino (15) reported the formation of 4-hydroxystyrene from *p*-coumaric acid by the phytopathogenic fungus *Cladosporium phlei*.

The natural occurrence of styrene derivatives, resulting from the decay of vegetative material, has been proposed by Finkle (9) and Finkle et al. (10). Their concept is based on enzymatic decarboxylation reactions of *p*-hydroxycinnamic, ferulic, and caffeic acids to form styrene. Styrene derivatives, after ring hydroxylations and co-polymerizations, may be converted into soil humic acid.

The ease by which styrene forms free radicals accounts for the spontaneous polymerization reactions it undergoes (3). In the absence of stabilizers and inhibitors, a chain polymerization readily occurs. Although there are reports of the microbial degradation of stabilizers and other additives (23), no information on microbial degradation of the inhibitor 4-tertiary butylcatechol (TBC) has been described.

This report summarizes studies on: (i) the microbial metabolism of styrene, (ii) the formation of styrene oligomers in mixed culture, and (iii) the degradation of [8-^{14}C]styrene in soil systems.

(This work is part of a Ph.D. dissertation submitted by M. S. in partial fulfillment of the requirements for the Ph.D. degree.)

MATERIALS AND METHODS

Cultural. A mixed population of bacteria, capable of utilizing styrene as a sole carbon source, was obtained from landfill soil by the enrichment culture technique. The microorganisms were grown and subcultured in basal salts (BS) mineral medium (11) supplemented with 1% (wt/vol) styrene. Uninoculated controls contained BS plus 1% styrene to determine whether the polymerization of styrene was chemical or biological in origin. To ascertain whether the mixed cultures could utilize the antioxidant TBC, styrene-utilizing bacteria were inoculated into a BS medium containing 1% (vol/vol) antioxidant as the only carbon source. All incubations were carried out in duplicate at 28°C on a rotary platform shaker in the dark. Cell growth was monitored turbidimetrically at 525 nm in a Bausch & Lomb Spectronic 20 spectrophotometer after correcting for turbidity resulting from polymerized styrene. Cultures were filtered through a Whatman no. 1 filter that retained the polymer material but permitted the bacteria to pass through.

Biochemical. For examination of the degradation products, the supernatant of 3 to 4 liters of culture after centrifugation (8,000 × *g* for 15 min) was acidified to pH 2 with 3 M H_3PO_4 and extracted three times with diethylether. The ether extract was dried over anhydrous sodium sulfate and concentrated in vacuo to a brownish, oily residue. The mixture was streaked across thin-layer silica gel (Mallinckrodt Silicar TLC-7GF) plates and developed in a solvent system of reagent grade benzene, hexane, and acetic acid (10:3:1, vol/vol/vol). Bands were located under short-wave ultraviolet light and eluted with ether. The fractions were rechromatographed in a solvent system of reagent grade chloroform and acetic acid (100:2, vol/vol), eluted with ether, and analyzed by mass spectroscopy. The insoluble polymer material was collected by filtration of the cultures, washed with distilled water, and evaporated to dryness. Subsequent saponification with 40% KOH removed associated lipid material.

Mass spectra were obtained from a Finnigan 3100 mass spectrometer. Polystyrene standards and solid, purified compounds were introduced through a solids probe inlet (70 eV, 50 to 250°C). Infrared spectra were obtained on a Perkin-Elmer 621 grating infrared spectrophotometer by pelleting about a 1- to 5-mg sample with 300 mg of KBr into clear disks.

Pure polystyrene standards of known molecular weights were purchased from Arro Laboratories (Joliet, Ill.) and Pressure Chemical Co. (Pittsburgh, Pa.). Styrene, phenylacetic acid, and 4-tertiary butylcatechol were obtained from Aldrich Chemical Co. (Milwaukee, Wis.).

[^{14}C]styrene decomposition in soil. Degradation of [8-^{14}C]styrene (New England Nuclear Corp., Boston, Mass.) was monitored in two types of soil: a landfill soil of heterogeneous composition (pH 6.9) and a neutral California agricultural top soil (pH 7.0) (Greenfield sandy loam). The major difference between the two soils was in the organic matter content: 0.35% organic matter content for the landfill soil versus 2.0% for the top soil. Freshly collected soil samples were air dried overnight, sieved through a 2-mm screen, and added to 250-ml Erlenmeyer flasks in 100-g portions. Labeled styrene, in an acetone solution (1.05 mg of [8-^{14}C]styrene–1 ml of acetone), and cold styrene to equal 200 and 500 mg, respectively, were thoroughly mixed into the dry soil. The activities for the two concentrations were: 200 mg = 2.42×10^6 dpm and 500 mg = 6.05×10^6 dpm. The mixtures were adjusted to 60% of the water-holding capacity with distilled water. Control flasks included: (i) equal samples of unamended nonsterile soil, (ii) soil supplemented with acetone in concentrations duplicating flasks receiving styrene-acetone treatments, and (iii) sterile soils amended with both 200 and 500 mg of [8-^{14}C]styrene. All flasks were connected to a closed aeration system (14). Air entering the reaction flasks was made free of CO_2 by passage through a KOH trap. All CO_2 and $^{14}CO_2$ evolving from the reaction flasks was adsorbed in 1 N KOH. At intervals of 1, 2, 4, 8, 12, and 16 weeks, the KOH tubes were exchanged. For total CO_2 determinations, 10-ml portions of KOH were added to 15 ml of 0.75 N $BaCl_2$ and titrated against 1 N HCl, using phenolphthalein as an indicator.

For determination of $^{14}CO_2$, 1- to 5-ml portions of KOH were acidified with 4 N HCl. The $^{14}CO_2$ released was trapped in 2.5 ml of NCS reagent (quaternary ammonium base-toluene solution; Amersham/Searle, Arlington Heights, Ill.) in a scintillation vial fitted with an absorption tower (14). After bubbling for 10 min, the tower contents were washed with 0.5% (wt/vol) 2,5-diphenyloxazole–toluene and the activity was determined with a Beckman liquid scintillation system (Beckman Instruments, Fullerton, Calif.). At the end of the incubation period, the soil preparations were air dried and finely ground. Residual ^{14}C activity was determined by combustion at 970°C in the presence of Cuprox metal catalyst (Coleman, Maywood, Ill.) and a pure stream of oxygen in a modified Coleman model 35 carbon-nitrogen analyzer. The $^{14}CO_2$ released was collected in 2 ml of NCS reagent in a modified scintillation vial.

RESULTS

Cultural. During the early log phase of the styrene enrichment cultures, an insoluble white material was observed on the surface of the cultures. Figure 1 relates the visible presence of the insoluble material to the growth curve for the organisms. The insoluble product, formed during the exponential growth period, gradually disappeared after 5 to 6 days and was accompanied by a decline in the culture pH. Uninoculated control flasks, containing mineral media and styrene amendment, did not produce the product when incubated under identical conditions for a maximum of 2 months.

Inoculation of TBC-BS medium with styrene-utilizing cells produced turbidity after incubation for 24 h. In addition, nonsterile TBC-BS medium, after microbial contamination upon exposure to air, also became turbid overnight.

Mass spectra of the crudely purified insoluble substance showed ion fragmentation patterns closely resembling standard polystyrene. Figure 2 shows a comparison of the fragmentation patterns for the isolated culture material, styrene, and standard 2,200-molecular-weight polysty-

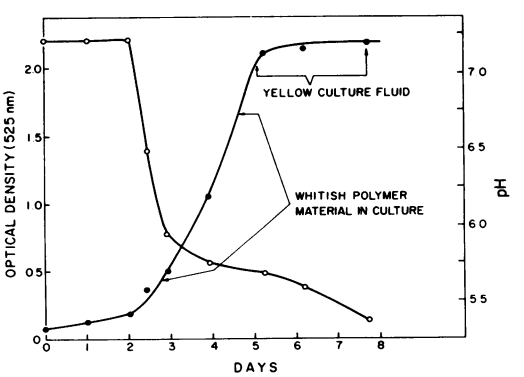

FIG. 1. *Relation of polymer culture material to growth curve and culture pH. Symbols:* ●, *growth curve;* ○, *culture pH.*

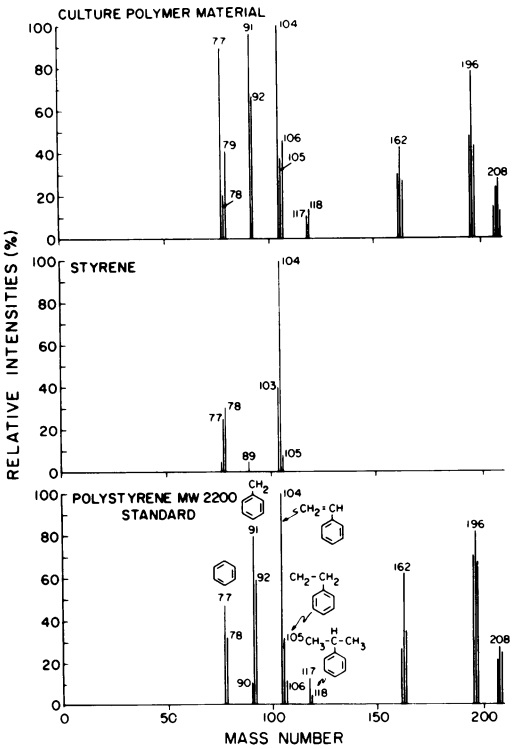

FIG. 2. *Comparison of mass spectra of culture polymer material, styrene, and polystyrene standard. MW, Molecular weight.*

rene. A base peak at 104 (monomer) was observed for all samples. The culture polymer material and standard polystyrene, however, also showed major peaks at 118, 162, 196, and 208 (dimer). These fragmentation patterns are in agreement with those reported by Beckewitz and Heusinger (1) for polystyrene.

A comparison of the infrared spectrum of the culture material with those for styrene and 2,200-molecular-weight polystyrene is shown in Fig. 3. Although the culture product is at best a crude preparation, it is possible to delineate several absorption characteristics. The primary similarity between the culture material and polystyrene that distinguishes these from styrene is a lack of absorption at 1,648 cm^{-1} for the vinyl group. Also evident in the spectra of polystyrene and the culture product is the CH vibration band at 2,845 cm^{-1}.

The culture material, in addition, was not soluble in most organic solvents. It was, however, soluble in dichloromethane and tetrahydrofuran.

Styrene degradation products. Thin-layer chromatography of the ether extract from the styrene cultures showed several bands under ultraviolet light. The dominant band (R_f = 5.1) yielded two distinct components when rechromatographed. Product 1 showed major fragmentation ion peaks at mass numbers 136, 120, 92, and 77. For product 2, major peaks were observed at 122, 106, 92, and 72. Based on cochromatography with standard phenylacetic acid and mass spectral patterns of authentic phenylacetic acid, product 1 was identified as phenylacetic acid. Mass spectral properties of product 2 indicate that this compound could be phenylethanol.

[8-^{14}C]styrene decomposition in soil. The loss of labeled carbon recovered as $^{14}CO_2$ was used to estimate the percent decomposition in soils over a 16-week period. No $^{14}CO_2$ was detected in control flasks of sterile soils supplemented with [8-^{14}C]styrene, unamended nonsterile soils, or acetone-containing soils. Figure 4 shows the percentage of added [8-^{14}C]styrene that was evolved as $^{14}CO_2$ at two concentrations for both soils. With the 200-mg application, 95% of the labeled carbon was evolved as $^{14}CO_2$ by the landfill soil, while 87% was lost from the Greenfield sandy loam at the same substrate concentration. It was noted that a longer lag period and overall slower rate of decomposition occurred at both substrate concentrations in the landfill soil as compared with the agricultural soil. This phenomenon is likely related to the low organic matter content and consequently initially lower microbial numbers in the landfill soil. At higher substrate concentrations (500 mg), significantly less $^{14}CO_2$, on a percent basis, was evolved from both soils.

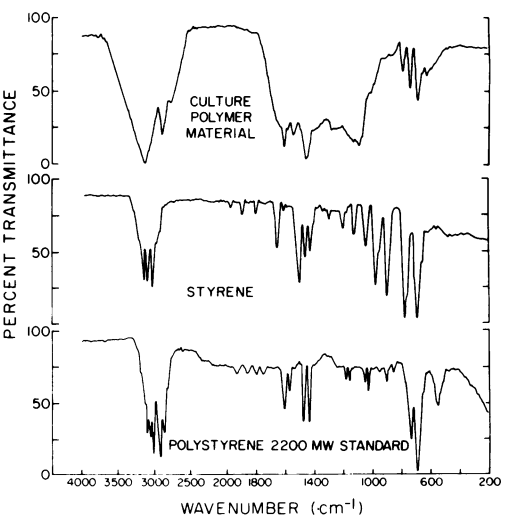

FIG. 3. *Comparison of infrared spectra of culture polymer material, styrene, and polystyrene standard. MW, Molecular weight.*

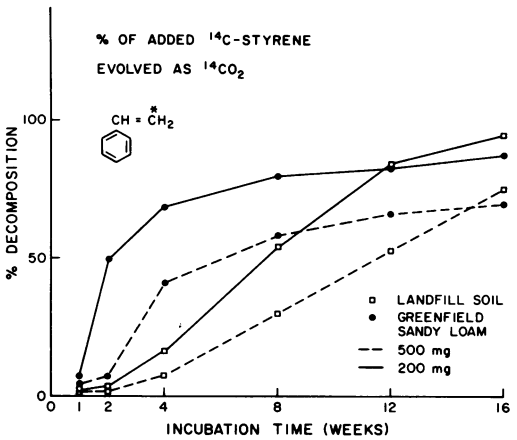

FIG. 4. *Decomposition of [8-^{14}C]styrene in soil systems.*

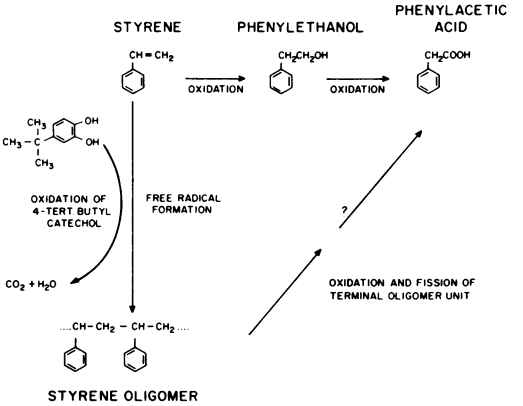

FIG. 5. *Proposed mechanism for the disappearance of styrene in liquid cultures.*

DISCUSSION

Although Omori et al. (22) reported no growth on styrene by microorganisms isolated from garden and forest soils, a mixed population capable of utilizing this substrate quickly developed from both the landfill and agricultural soils used in this investigation.

It was found that two different mechanisms were responsible for the disappearance of styrene from the cultures. Figure 5 shows a diagrammatic representation of these two pathways. In the first mechanism, styrene appears to be oxidized to phenylethanol and phenylacetic acid. These initial reactions are similar to those reported by Leibman (18) in mammalian systems. The fate of phenylacetic acid was not investigated since several ring fission pathways for this intermediate have already been documented (16).

The second mechanism of the transformation of styrene in liquid culture seems to be the spontaneous polymerization of the monomer to low-molecular-weight oligomers. Based on mass spectral and infrared analyses, the similarity of the insoluble product formed in cultures supplemented solely with the monomer to that of standard styrene polymers suggests a microbially mediated polymerization reaction. Since it was shown that the antioxidant styrene polymerization inhibitor TBC is readily oxidized by microorganisms, it is feasible that once this substrate is microbially degraded, chemical polymerization of styrene can occur.

After the complete disappearance of styrene due to direct oxidation and/or polymerization, microbial growth continued to be supported. This was indicated by a loss of the polymer material in the mixed cultures. Further investigation of styrene polymer degradation by microorganisms will be reported in another paper.

The soil incubation studies revealed a greater percent release of $^{14}CO_2$ from [8-^{14}C]styrene at 200-mg concentrations than at 500 mg. The large amount of $^{14}CO_2$ evolved would, in itself, imply oxidation to phenylacetic acid at C8. After complete removal of the labeled C8 as $^{14}CO_2$, one might expect benzoic acid as an intermediate. Haider and Martin (14) have already demonstrated the rapid degradation of ring-labeled benzoic acid in soil incubation studies. It would appear that this is the major pathway operating in the two soils incubated with 200 mg of labeled styrene.

A smaller percentage of the applied [8-^{14}C] styrene was evolved as $^{14}CO_2$ from the 500-mg amended soils. Several explanations may account for this decreased activity. Assuming first that the initial oxidative steps yield phenylacetic acid, it is possible to envision ring hydroxylation of this intermediate before release of the labeled C8 as $^{14}CO_2$. Similar hydroxylation reactions of phenylacetic acid by *A. niger* have been reported by Bocks (2) and Faulkner and Woodcock (8). After ring hydroxylation of the phenylacetic acid molecule, it could be reactive enough to copolymerize into soil humic polymers. Analogous reactions have been demonstrated with hydroxybenzoic acids, hydroxyphenols, hydroxytoluenes, and other aromatic molecules (20). Similar reactivity with hydroxylated styrene intermediates could also be expected, as proposed by Finkle (9).

A second mechanism may involve the incorporation of the labeled part of the phenylacetic acid molecule into specific cellular constituents, such as fungal melanins. Such reactions may be expected after either: (i) initial transformations not involving ring cleavage (19) or (ii) ring cleavage prior to the loss of the labeled C8 as $^{14}CO_2$.

Also, the polymerization of styrene cannot be overlooked. Solomon (24) has demonstrated conclusively that certain clay mineral fractions of the soil can act as electron acceptors to catalyze the rapid polymerization of styrene. Although a mineral analysis of the soils used in this study was outside the scope of this investigation, clay minerals constitute some portion of nearly all soils.

In these three hypotheses, the labeled C8 of styrene is not immediately released as $^{14}CO_2$ but remains an integral part of the molecules. With higher concentrations of substrate, such mechanisms, particularly polymerization, could, in part, account for the lower percentage of $^{14}CO_2$ evolution from higher concentrations of styrene.

It is also possible that the higher concentration of styrene in the soil could have favored a population that metabolized the chemical more slowly or converted a greater percentage of the C into biomass or products.

It is possible that some styrene could volatilize from the soil and account for a portion of the ^{14}C reported as $^{14}CO_2$. Two observations, however, indicate that this was not a significant factor. As indicated above, $^{14}CO_2$ was not recovered from sterile soil controls. Furthermore, if styrene volatilization were significant, it would have been greatest during the early stages of incubation, when actually 1- to 2-week lag periods were observed during which little or no ^{14}C activity was recovered.

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