beled [^{14}C]trifluralin was in a nonextractable form.

The trifluralin ring system does not appear to undergo extensive fission in the soil (Probst et al., 1975), but as mentioned, the fate of trifluralin in soils is very complex and a considerable number of transformation products have been implicated during its degradation (Probst et al., 1975; Golab et al., 1979). It has also been suggested Golab et al., 1979) that certain transformation products of trifluralin may become complexed directly with soil organic matter and thus be rendered nonextractable.

Perhaps with time, more of the unidentified soil transformation products from the present study would have become complexed or otherwise bound to the soil, thus decreasing their amounts in the soil and increasing the quantities of nonextractable residues to the levels reported by Golab et al. (1979).

Of concern is whether after repeated annual applications of herbicides bound residues have phytotoxic significance and are likely to adversely affect soil fertility. The findings from long-term studies conducted at the Agriculture Canada Experimental Farm at Indian Head, Saskatchewan, have indicated that crop fertility, as determined by wheat yields, has not been impaired by 24 repeated annual applications of 2,4-D [cf. Smith (1982)]. Similar long-term field studies carried out at the Weed Research Organization near Oxford, England, with repeated annual applications of triallate have shown no adverse effects on soil fertility as determined by crop yields, organic carbon, and soil pH measurements (Fryer, 1981). Such field data for dicamba and trifluralin have not been reported.

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Registry No. 1, 1918-00-9; [*ring*-U-^{14}C]-1, 89300-29-8; 2, 94-75-7; [*ring*-U-^{14}C]-2, 89300-30-1; 3, 2303-17-5; [2-^{14}C]-3, 89232-81-5; 4, 1582-09-8; [*ring*-U-^{14}C]-4, 89300-31-2.

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Biodegradation of *o*-Phenylphenol in River Water and Activated Sludge

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The compound *o*-phenylphenol (OPP) is widely used in disinfectant formulations and as a fungicide in the fruit packing industry. The biodegradation of [^{14}C]OPP, labeled on the phenolic ring, was examined in both river water and activated sludge. The rates of disappearance of [^{14}C]OPP, appearance of degradation products, and $^{14}CO_2$ formation were monitored. The data showed that a 50% reduction in [^{14}C]OPP concentration from its initial value occurred in approximately 1 week in river water, 24 h in nonacclimated sludge, and 3 h in acclimated sludge. The conversion of [^{14}C]OPP to $^{14}CO_2$ was found to be 50–65% after 16 days in the river water and 48 h in the activated sludge.

The compound *o*-phenylphenol (OPP) is widely used in disinfectant formulations and as a fungicide in the fruit packing industry. The biodegradation of OPP has been studied by several groups. Results of the simple biochemical oxygen demand (BOD) test for OPP indicate rapid and extensive biodegradation (Simmons et al., 1977). Voets et al. (1976) report that OPP (40 mg/L) was 100% degraded in an activated sludge confirmatory test as described by the Organization for Economic Cooperation and Developement (OECD). They also observed 100% deg-

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radation of OPP in tests designed to simulate aerobic and anaerobic conditions in lake water.

Pauli and Frank (1972) observed complete biodegradation of OPP in municipal wastewater treatment plant effluent within 2 days at loadings of 30 and 100 mg/L. Other workers reported slow degradation of OPP in various experiments designed to simulate biological wastewater treatment (Luessen, 1975; Maggio et al., 1976a,b; Shefer and Ammann, 1974). The latter experiments used concentrations of about 100 mg/L of OPP or greater, which may have inhibited biological activity.

The present study was conducted to determine the rate of biodegradation of OPP in river water and activated sludge at concentrations expected to be found in the environment. The OPP concentrations used were lower than those employed by previous investigators.

MATERIALS AND METHODS

River Water Study. River water was obtained from the Tittabawassee River in Midland, MI, at a site located upstream from municipal and industrial waste discharges. The river water was filtered through Whatman 114V filter paper and collected in polyethylene jugs.

The [^{14}C]OPP was dissolved in 5 mL of acetone and deposited on the inside surface of a gallon glass jar. After the acetone was allowed to evaporate, 2 L of river water was added with mixing. Dilutions were prepared by adding 200 and 20 mL of the river water fortified with [^{14}C]OPP to 1800 and 1980 mL of river water, respectively. Liquid scintillation counting determined the [^{14}C]OPP concentrations to be 123, 12.3, and 1.22 μg/L, respectively. Aliquots of the river water solutions (100 mL) were pipetted into 160-mL serum bottles. The bottle tops were covered with aluminum foil and Teflon-faced rubber septa and sealed with aluminum crimp seals. Control solutions were prepared by adding 1 mL of a 1% $HgCl_2$ solution to appropriate bottles to inhibit biological activity. Solutions were incubated at 20 ± 0.5 °C in the dark on a rotary shaker at 200 rpm.

Test solutions were analyzed for [^{14}C]OPP and degradation products periodically. A test bottle and a carbon dioxide trap were connected with syringe needles and glass tubing. A 0.2-mL aliquot of 0.5 M $NaHCO_3$ solution was injected into the sample bottle to obtain at least 0.001 M HCO_3^-. The $^{14}CO_2$ was released by adding 2.3 mmol of H_2SO_4 and purging with 15 mL/min nitrogen for 30 min. Carbon dioxide was trapped in a 30/70 (volume/volume) mixture of monoethanolamine and 2-methoxyethanol. Preliminary studies with [^{14}C]$BaCO_3$ demonstrated that the purging conditions employed trapped more than 90% of the $^{14}CO_2$. Following the nitrogen purge, the test solutions were extracted twice with 10-mL portions of methylene chloride and the extracts were combined and weighed. The extracts were concentrated with heating in a Kontes concentration tube fitted with a Kontes evaporative concentrator column. The concentrated extracts were weighed and aliquots injected onto a high-performance liquid chromatography (HPLC) analytical system to determine the [^{14}C]OPP and breakdown products.

Activated Sludge Studies. The biodegradation of OPP was examined in both nonacclimated and acclimated activated sludge obtained from the East Lansing, MI, domestic wastewater treatment plant. The mixed liquor was placed in a semicontinuous activated sludge apparatus that contained a total volume of 2 L. The closed system had an air flow to provide mixing and aeration. As the air flow exited the system, water vapor was accumulated in a 0 °C trap, volatile organics were accumulated in a −55 °C trap, and $^{14}CO_2$ was accumulated in a monoethanolamine/2-methoxyethanol (30/70) trap. The traps were arranged in series. Sludge solids were adjusted to 2500 mg/L and 13 mL of a nutrient concentrate solution was added. The nutrient concentrate was prepared according to the method of the Soap and Detergent Association (Snow, 1965) and consisted of, per liter, the following: $(NH_4)_2SO_4$, 2.5 g; K_2HPO_4, 13 g; glucose, 13 g; Bacto nutrient broth, 13 g; Bacto beef extract, 13 g.

The [^{14}C]OPP was dissolved in a small amount of 0.1 N NaOH solution and injected through a rubber septum into the activated sludge apparatus. The initial concentration of [^{14}C]OPP was determined to be 9.6 mg/L. At periodic intervals, 70-mL aliquots of activated sludge mixed liquor were obtained from a sample port and acidified to minimize subsequent biological activity. The CO_2 traps were replaced with fresh solutions at the same time. Two 10-mL methylene chloride extractions were made of the mixed liquor sample, combined, and concentrated in a manner similar to that described above for the river water extracts. Components present in the extracts were separated by thin-layer chromatography (TLC) as described below.

To determine the fate of OPP in acclimated sludge, the system was operated on a 24-h fill and draw cycle as described in the Soap and Detergent Association confirmatory test (Snow, 1965). Each day the solids were allowed to settle, and 1.33 L of the supernatant liquid was removed and replaced with tap water containing 10 mg/L nonlabeled OPP and 13 mL of nutrient solution. On day 7, [^{14}C]OPP was added at a concentration determined to be 10.4 mg/L in the mixed liquor. The experimental procedure from this point was the same as for the nonacclimated sludge study.

Analytical Methods. The [^{14}C]OPP and degradation products for the river water study were quantified by HPLC using a LiChrosorb RP-8 reverse-phase column (250 mm × 4.6 mm). Extracts were injected onto the column and eluted with a solvent system composed of 59.5% methanol, 39.5% H_2O, and 1.0% glacial acetic acid. The solvent flow rate was 2.0 mL/min. The sample volume was 50 μL. The retention time of OPP was 6.2 min under the analysis conditions. Fractions eluting from the column were collected at 35-s intervals and analyzed for radioactivity as described below.

The methylene chloride extracts from the activated sludge study were analyzed by TLC. The analyses were performed on Kontes Q1F silica plates (5 cm × 30 cm) with chloroform as the development solvent. The TLC plates were scraped in 0.5-cm sections and the sections were radioassayed. The location of the [^{14}C]OPP was determined by comparison of R_f values with nonlabeled OPP using ultraviolet visualization.

Radioactivity present in the methylene chloride extracts, sludge solids, water after extraction, CO_2 trap solutions, and HPLC eluent fractions was determined by liquid scintillation counting. Aliquots of the solutions were added to 10 mL of Aquasol scintillation cocktail (New England Nuclear Corp.) and analyzed by using a Packard Model 2425 liquid scintillation counter. Radioassay data were corrected for background and quenching.

Materials. The ^{14}C-labeled OPP was synthesized by Pathfinder Laboratories with uniform ^{14}C labeling on the phenolic ring. The compound had a specific activity of 13.34 mCi/mmol and was found to be 94% radiochemically pure by HPLC. The impurities consisted of 2–3% biphenyl or diphenyl oxide, 0.2% anisole, and 3% of an unknown compound. nonlabeled OPP [Dowicide 1 antimicrobial] was obtained from the Dow Chemical Co. and

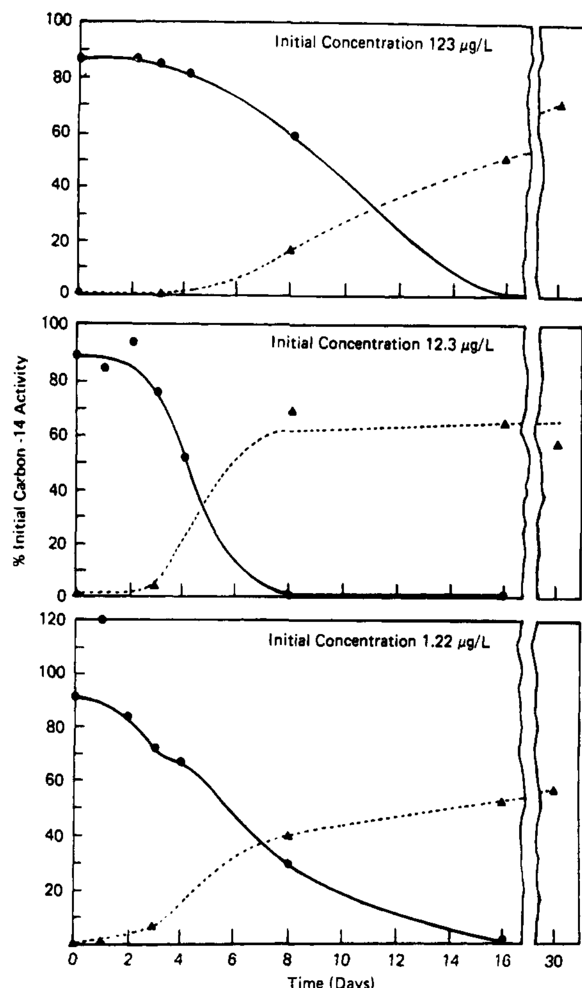

Figure 1. Distribution of carbon-14 activity in river water as a percentage of initial carbon-14 activity: (●) [14C]OPP; (▲) $^{14}CO_2$.

was found to be 99.8% pure. Nutrient broth and beef extract were obtained from Difco Laboratories, Detroit, MI. All other chemicals were of the highest purity commercially available and were used without further purification.

RESULTS AND DISCUSSION

Results of the river water study are presented in Figure 1. The distribution of carbon-14 in [14C]OPP and $^{14}CO_2$ as a percentage of initial carbon-14 activity is shown. Initial carbon-14 activity was the activity measured in the fortified river water samples prior to the extraction procedure. A 50% reduction in [14C]OPP concentration from its initial value occurred in about 1 week. After 16 days, the evolved $^{14}CO_2$ reached levels of 50%, 65%, and 50% of theoretical $^{14}CO_2$ production for the 1.22, 12.3, and 123 μg/L concentrations, respectively.

Control samples to which $HgCl_2$ was added to inhibit biological activity were analyzed after 30 days of incubation. In the control samples, approximately 79% of the radioactivity was present as [14C]OPP. Extractable breakdown products accounted for about 8% of the radioactivity and radioactivity in the aqueous phase after extraction was less than 3%. The CO_2 traps accumulated less than 0.2% of the initial radioactivity in the control samples. Recoveries of total radioactivity ranged from 75 to 101% in the river water study.

The fate of OPP in activated sludge is shown in Figure 2. A 50% reduction in [14C]OPP concentration from its initial value was observed at 24 and 3 h in nonacclimated and acclimated sludge, respectively. The initial activity

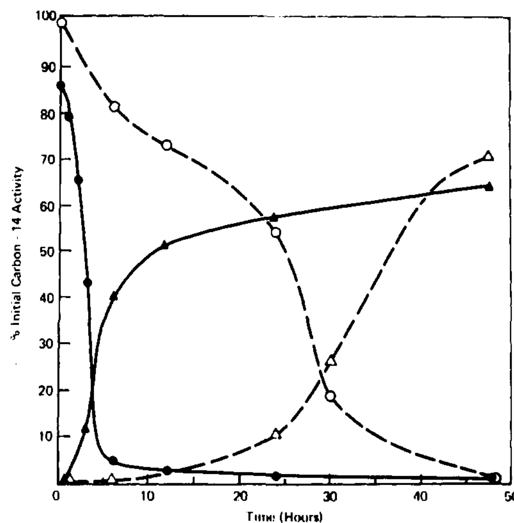

Figure 2. Distribution of carbon-14 activity in activated sludge as a percentage of initial carbon-14 activity: (O) [14C]OPP (nonacclimated); (△) $^{14}CO_2$ (nonacclimated); (●) [14C]OPP (acclimated); (▲) $^{14}CO_2$ (acclimated).

was a summation of the activity present at time 0 in the methylene chloride extracts and the sludge and water after extraction. $^{14}CO_2$ evolution after 48 h was approximately 65% of theoretical CO_2 production in the two experiments. Extractable degradation products accounted for less than 10% of the radioactivity. Radioactivity remaining in the aqueous phase after extraction reached levels of 24–35% after 48 h.

Recoveries of total radioactivity in the nonacclimated sludge experiment ranged from 90 to 117%, and in the acclimated sludge experiment the range was 78–100%. Radioactivity accumulated in the water vapor and volatile organic traps was negligible.

This study demonstrates that OPP is degradable at the low concentrations expected in the natural environment as represented in the river water experiment. The observation of minimal degradation of OPP in the control samples indicates that biodegradation is the predominant breakdown route. The biodegradation of OPP is enhanced in activated sludge, especially upon acclimation. The rate of disappearance of OPP was initially slow, followed by more rapid degradation, in both river water and nonacclimated activated sludge. This observation can be most easily explained as a result of a low initial population of microorganisms capable of metabolizing OPP. This population increased due to the supply of OPP as a nutrient by induction of the appropriate enzyme systems and/or by multiplication of the appropriate species (Spain et al., 1980). These processes apparently required about 24 h in the nonacclimated activated sludge and 3–4 days in the river water. The activated sludge acclimated to OPP did not demonstrate the initial OPP biodegradation lag period observed in the other experiments.

The rate of biodegradation in any natural environment will depend on temperature, microorganisms present, minerals, nutrient levels, oxygen concentration, etc. The antimicrobial effect of OPP will retard its own degradation when the compound is present in high concentrations. However, due to the common metabolic capabilities in natural microbial communities, OPP is expected to degrade in any aquatic environment.

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Species and Strain Comparison of the Metabolism of Gentian Violet by Liver Microsomes

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Comparative metabolism of hexamethylpararosaniline chloride, a poultry feed additive known as gentian violet, was examined in vitro in the presence of uninduced liver microsomes prepared from both sexes of four mouse strains, three rat strains, hamster, guinea pig, and chicken. Metabolites, isolated by solvent extraction and HPLC, were identified by comparison of reverse-phase HPLC retention times and electron impact mass spectral fragmentation patterns with those of standards prepared by unambiguous synthetic routes. The major metabolites were pentamethylpararosaniline and the isomeric *N,N,N′,N′*- and *N,N,N′,N″*-tetramethylpararosanilines. The pattern of demethylated metabolites was found to be comparable among the species with little difference between the sexes. The extent of demethylation was least with mouse microsomes, and formation of *N,N,N′,N′*-tetramethylpararosaniline by uninduced guinea pig microsomes was barely detectable.

Gentian violet (**1a**, Figure 1) is an intensely colored, basic triphenylmethane dye that has had direct human application both topically as an antifungal agent and internally as an antihelminthic agent. Although still used, it has largely been superseded by more effective disinfectants. Its principal use now resides in agriculture, particularly as a mycostatic agent in poultry feeds. Reports of its genotoxic potential, reviewed by Combes and Haveland-Smith (1982), have led to some concern over residues that may be found in edible tissues of treated animals.

Little is known about the metabolic transformation of triphenylmethane dyes. Some recent work has measured the biliary excretion and tissue distribution of the acidic dyes Guinea Green B (Minegishi and Yamaha, 1974), Benzyl Violet 4B (Minegishi and Yamaha, 1977), and Brilliant Blue FCF (Phillips et al., 1980). Taylor (1977) applied a colorimetric method to determine total residue of gentian violet in chick tissues. None of these studies was designed to detect and identify metabolites derived from the dyes.

The work described here is a first step in elucidating the metabolism of a major group of dyes and describes the synthesis and isolation of in vitro metabolites of gentian violet. In addition, comparisons have been made among several rodent species to observe how closely mammalian and avian metabolism are related in this case.

EXPERIMENTAL SECTION

Materials and Synthetic Procedures. Chemicals and solvents of standard reagent and HPLC grades were obtained from commercial suppliers. Gentian violet (hexamethylpararosaniline chloride) was obtained as crystal violet from Aldrich Chemical Co. and contained >98% of a single component absorbing at 546 nm, as determined by HPLC.

Pentamethylpararosaniline Chloride, **1b**. The preparation of the pentamethyl homologue from **2a** (2.67 g, 10.3 mmol), *n*-butyllithium (2.2 M hexane solution, 6.3 mL, 13.9 mmol) and 4,4′-bis(dimethylamino)benzophenone (2.5 g, 9.3 mmol) was carried out essentially as described for the tetramethyl homologues: yield, 500 mg (16%) of **1b** as a dark green powder; purity >98% (HPLC, 300-nm detection); UV_{max} (98% CH_3CO_2H) 578 nm (ϵ 100 000); 60-MHz NMR (CD_3OD) δ 3.0 (s, 3, $NHCH_3$), 3.2 [s, 12, $N(CH_3)_2$], 6.67–7.25 (m, 12, aromatic).

N,N,N′,N′-Tetramethylpararosaniline Chloride, **1c**. Lithiation of *N,N*-bis(trimethylsilyl)-4-bromoaniline (1.07 g, 3.4 mmol), **2b** (Broser and Harrer, 1965), in ether was accomplished by addition of an excess of 2.2 M *n*-butyllithium (2.1 mL, 4.5 mmol) in hexane while maintaining the reaction temperature at 25 °C. After 10 min, 4,4′-bis(dimethylamino)benzophenone (0.89 g, 3.4 mmol) was added and the reaction mixture stirred for 3 h at room temperature. After the solvents were removed in vacuo, the residue was dissolved in 1 M NaOH and the dye base was extracted into ether. The ether solution was washed with water, dried over anhydrous Na_2SO_4, and evaporated. The residue was treated with 0.06 M HCl on a steam bath for 2 h. The resulting dye was precipitated by addition of NaCl to the cooled solution. The material was further purified by dissolving the precipitate in absolute ethanol followed by filtration. The dye (**1c**) was reprecipitated as a deep blue-green powder in 19% yield by addition of ether. Purity, as determined by HPLC with peak integration at 300 nm, was >98%: UV_{max} (98% CH_3CO_2H) 576 nm (ϵ 72 300); 60-MHz NMR (CD_3OD) δ 3.25 (s, 12, CH_3), 6.75–7.5 (m, 12, aromatic).

N,N,N′,N″-Tetramethylpararosaniline Chloride, **1d**. A mixture of *N*-methyl-4-bromoaniline (7.00 g, 37.6 mmol) and oil-free sodium hydride (1.35 g, 56.5 mmol) in 50 mL of tetrahydrofuran was warmed under argon until hydrogen evolution ceased. Chlorotrimethylsilane (7.2 mL, 56

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