Effects of *Pseudomonas* Species on the Release of Bound ^{14}C Residues from Soil Treated with [^{14}C]Atrazine[†]

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The release of bound (nonextractable) ^{14}C residues from soil previously treated with [^{14}C]atrazine was investigated by incubation of the solvent-extracted soil with two species of *Pseudomonas* capable of metabolizing atrazine. The two species, 192 and 194, released bound ^{14}C residues from the soil. Addition of glucose, known to increase microbiological activities, to the incubated soil appeared to enhance the release of soil-bound ^{14}C residues, in particular in the presence of *Pseudomonas* species 192. The ^{14}C bound residues in soil, mainly present as the parent compound and its hydroxy and monodealkylated analogues, were released into the incubation mixture and were subsequently metabolized by the two species involving dechlorination and dealkylation.

INTRODUCTION

Many investigations have addressed the problem of bound pesticide residues formation in soil (Klein and Scheunert, 1982; Khan, 1982; Roberts, 1984; Führ, 1987; Calderbank, 1989), and the question of their potential release and biological availability has been reviewed in several papers (Lichtenstein, 1980; Führ, 1987, Khan and Dupont, 1987). In a recent study Racke and Lichtenstein (1985) demonstrated that soil-bound ^{14}C residues can be released, metabolized, and picked up by plants grown in these soils. Furthermore, it was observed that with an increase in soil microbial population a concomitant increase in the release of soil-bound ^{14}C residues occurred. In our earlier studies it was observed that significant amounts of bound [^{14}C]-prometryn and metabolites were released when the extracted soil was inoculated with a mixed microbial population derived from the untreated samples of the same soil (Khan and Ivarson, 1980). In a subsequent study it was shown that various physiological groups of soil microorganisms differed in their ability to release bound residues (Khan and Ivarson, 1982). As a continuation of these investigations we now report the results of our study concerned with the potential microbial release and subsequent degradation of bound ^{14}C residues from a soil previously treated with radiolabeled atrazine [2-chloro-4-(ethylamino)-6-(isopropylamino)-*s*-triazine]. The bacteria used in this study were species of *Pseudomonas* capable of metabolizing atrazine (Behki and Khan, 1986).

MATERIALS AND METHODS

Chemicals. All solvents were of pesticide grade and used as received. Uniformly ^{14}C-ring labeled atrazine, reference standards of atrazine, and metabolites were gifts from Ciba-Geigy Corp., Greensboro, NC.

Microorganisms. Two *Pseudomonas* species, 192 and 194, were used in this study. These species were isolated by a modified enrichment culture technique from a soil that had a long history of atrazine application (Behki and Khan, 1986).

Production of Soil-Bound ^{14}C Residues and Analysis. An organic soil (45.4% carbon, 2.4% nitrogen, 15.1% mineral matter, bulk density of 0.34, and pH of 5.2) was collected from plots located at the Ste. Clothilde experimental farm on a Humic Mesisol soil. The soil was never exposed to atrazine. Moist soil was incubated in an Erlenmeyer flask to which radiolabeled and cold atrazine was added to give a herbicide concentration of 25 μg/g and a total radioactivity count of 1.4 × 10^6 dpm/100 g. The flask was loosely stoppered with cotton wool and incubated at ≃22 °C in the dark for 1 year. Distilled water was added as necessary to maintain the initial soil moisture content (130%, oven dry weight basis) of the sample in each flask. The soil was then exhaustively extracted (×5) with methanol. Additional extractions of the soil with methanol did not result in the removal of any measurable ^{14}C. Residual methanol in the extracted soil was allowed to evaporate by air-drying. The control consisted of untreated soil processed in a similar manner. The total bound (unextractable) ^{14}C residues were determined by combustion of the air-dried extracted soil to $^{14}CO_2$ (Figure 1). A Lindberg tube furnace (Sola Basic S/B) was used for high-temperature distillation (HTD) of the solvent-extracted soil to release the bound ^{14}C residues as described earlier (Khan and Hamilton, 1980). The released ^{14}C material was collected in various traps. The first two traps contained chloroform and methanol, the third contained aqueous methanol (25% H_2O), and the last trap contained oxisorb to trap any released $^{14}CO_2$. The solution in all the traps were radioassayed for ^{14}C, and then the distillates in the first three traps were evaporated just to dryness. The material was then dissolved in a small volume of methanol, combined, and analyzed as described later (Figure 1).

Release of Bound ^{14}C Residues from Soil by *Pseudomonas* Isolates 192 and 194. Cells grown in BMN–glucose medium (Behki and Khan, 1986) were centrifuged and resuspended in BMN medium. Aliquots (10 mL/100 g) containing *Pseudomonas* isolates 192 and 194 were added to inoculate the solvent-extracted soil in flasks containing only bound ^{14}C residues described above. Control samples comprised solvent-extracted soil containing bound ^{14}C residues which received only BMN medium but were not inoculated with the test bacteria. Sterilized distilled water was added to bring the moisture content to 70% of field capacity, which was maintained during the incubation. All incubation experiments were run in triplicate, and average values are reported. After the incubation period of 14, 28, 42, and 56 days, flasks were removed and exhaustively extracted with methanol. At the end of 56 days glucose was added to the remaining incubated samples to stimulate microbial activity, and the experiment was continued for another 28 days. At the end of the experiment samples were exhaustively extracted with methanol. The methanol extracts from the above experiments were radioassayed for ^{14}C, concentrated to a small volume, and analyzed by GC (Figure 1).

Determination of Radioactivity. Combustion of dried solvent-extracted soil (total bound ^{14}C) was done in a Packard sample oxidizer, Model 306, to produce $^{14}CO_2$. The latter was absorbed in and admixed with an appropriate volume of Carbosorb and Permafluor V. Aliquots of HTD distillates and various solutions or extracts were analyzed by liquid scintillation counting (LSC).

Derivatization. Aliquots of HTD distillates, various solutions, or extracts in methanol (1 mL) were transferred into a 15-mL screw-cap centrifuge tube, and an excess of freshly prepared

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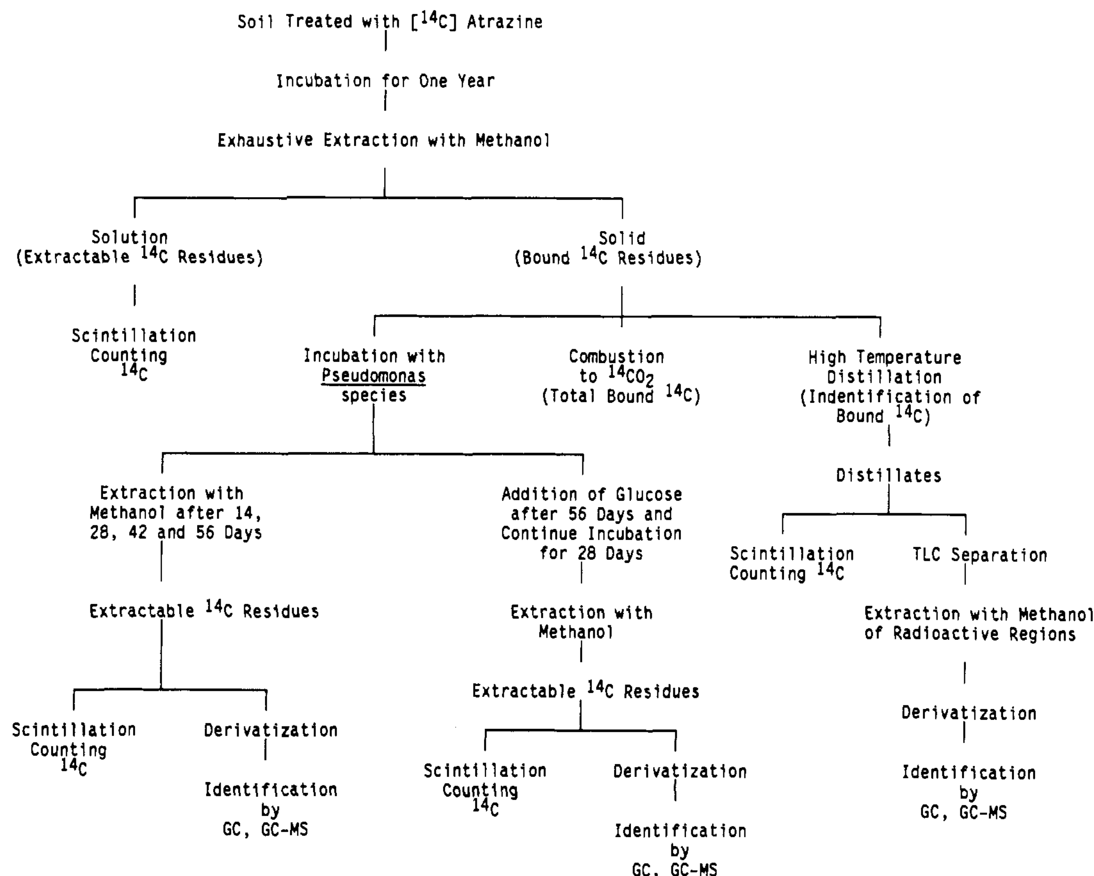

Figure 1. Schematic diagram for the analysis of bound ^{14}C residues from the [^{14}C]atrazine-treated soil incubated with *Pseudomonas* 192 and 194.

ethereal diazomethane solution (prepared from Diazald, Aldrich, Milwaukee, WI) was added until the yellow color persisted. The tube was tightly closed, and the content was allowed to stand at room temperature for 3 h with occasional shaking. The solvent was then evaporated to just dryness in a stream of dry air. The residue was dissolved in 1 mL of methanol or hexane and analyzed by gas chromatography (GC).

Chromatographic Analysis. The combined methanol solution from the HTD distillates was subjected to preparative thin-layer chromatography (TLC) using 20 × 20 cm precoated silica gel plates which were then developed by using the solvent system chloroform/methanol (97:3). The TLC plates were photographed by a Berthold Beta camera LB292 to detect radioactive regions. Each radioactive zone was scraped off the plate, extracted with methanol, and analyzed by LSC to determine the total extractable ^{14}C. The extractable material was then further analyzed as shown in Figure 1.

Gas chromatography was performed by using a Varian Model 6000 chromatograph fitted with a thermionic specific detector. A silica Megabore column (15 m × 0.52 mm i.d.) coated with Carbowax 20 (0.25 μm) was used. The oven temperature was programmed at 1.5 °C/min from 190 to 200 °C. The detector and injector port temperatures were 290 and 200 °C, respectively. Helium was used as a carrier gas at a flow rate of 20 mL/min. Aliquots (1–2 μL) of calibration solutions containing various amounts of reference standards were injected so that the peak did not exceed full-scale deflection. Immediately, the column temperature was programmed as described above. A calibration curve for each reference standard was constructed by plotting quantity injected against peak height. The quantity of each compound in the sample represented by GC peak was obtained by its peak height using the calibration curve. The calibration solutions were injected daily to ensure proper cali bration. Under the GC conditions described, atrazine, deethylatrazine, deisopropylatrazine, methoxy derivatives of hydroxyatrazine, deethylhydroxyatrazine, and deisopropylatrazine, and ammeline showed peaks at retention times of 5.9, 10.8, 13.3, 4.4, 7.1, 8.6, and 24.6 min, respectively. The identities of the compounds were confirmed by comparing the GC retention times with those of authentic samples, cochromatography, and finally by gas chromatography–mass spectrometry (GC–MS). A high-resolution mass spectrometer, Model VG2AB-2F, connected to a Varian GC Model 3700 was used. The mass spectra were recorded at 70 eV.

RESULTS AND DISCUSSION

The soil contained 54% (nonextractable) bound ^{14}C of the total applied radioactivity following an incubation period of 1 year. HTD of the extracted soil showed that the amount of radioactivity in the combined solution for the first three traps (chloroform–methanol–aqueous methanol) was 79% of the total bound ^{14}C residues. The last trap (oxisorb) contained about 17% ^{14}C of the total bound ^{14}C residues, indicating thermal decomposition during HTD. It was also observed that about 3–4% ^{14}C was still present in the burned soil material left in the quartz tube.

The HTD distillate required cleanup before analysis by GC. The concentrated distillate in methanol was subjected to TLC separation as described later. A photograph of the TLC plate by a Berthold Beta camera showed radioactive regions at R_f 0.80, 0.60, 0.35, and 0.00. The radioactive regions on TLC plate were scraped off the plate, extracted with methanol, and radioassayed for ^{14}C. The radioactivity of the extracts at R_f 0.80, 0.60, 0.35, and 0.00 amounted to 34%, 20%, 10% and 31%, respectively, of the total ^{14}C in the distillate. GC analysis of extracts showed major peaks at 5.9 (R_f 0.80), 10.8 (R_f 0.60), and 13.3 min (R_f 0.35), which were identified by comparison with the retention times (Rt) of authentic reference standards as atrazine, deethylatrazine, and deisopropylatrazine, respectively. GC analysis of the methylated extract (R_f 0.00) showed a major peak at 4.4 min, which was identical with the retention time of the methoxy derivative of hy-

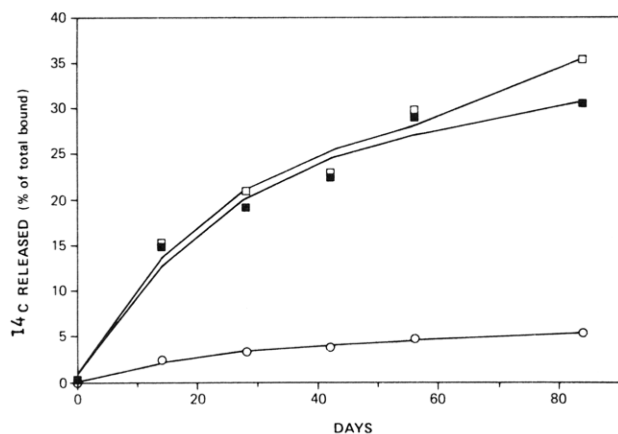

Figure 2. Effects of *Pseudomonas* 192 and 194 on the release of bound ^{14}C residues from soil. (□) Strain 192; (■) strain 194; (○) control BMN medium.

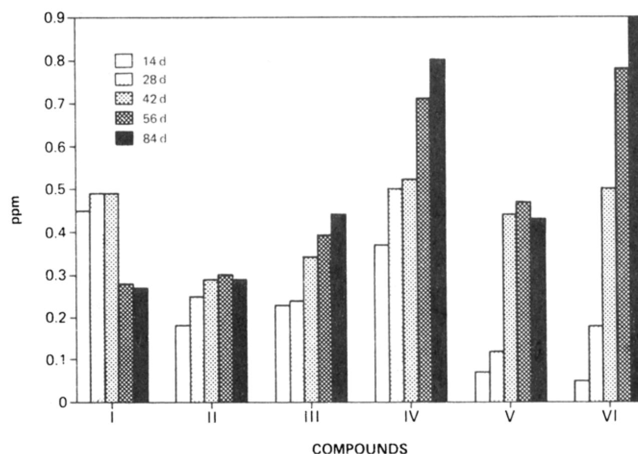

Figure 3. Compounds released from soil containing bound ^{14}C residues with *Pseudomonas* 192. (I) Atrazine; (II) hydroxyatrazine; (III) deethylatrazine; (IV) deisopropylatrazine; (V) deethylhydroxyatrazine; (VI) deisopropylhydroxyatrazine.

droxyatrazine. The identities of these compounds were further confirmed by cochromatography with authentic standards and finally by GC–MS analysis. The GC–MS of the peaks with retention times of 5.9, 10.8, and 13.3 min showed molecular ions at m/e 215, 187, and 173, respectively, in addition to the chlorine isotopic peaks ($M^{\bullet +}$ + 2). Furthermore, the molecular ions decomposed with loss of CH_3 and $CH_3{\cdot}CH{=}CH_2$ to give m/e 200 and 173 (Rt 5.9 min), m/e 172 and 145 (Rt 10.8 min), and with a loss of CH_3 and C_2H_4 to give m/e 158 and 145 (Rt 13.3 min). The spectrum and further fragmentation were analogous to those of authentic atrazine (Rt 5.9 min), deethylatrazine (Rt 10.8 min), and deisopropylatrazine (Rt 13.3 min). The mass spectra represented by GC peak at retention time 4.4 min (R_f 0.00) exhibited a molecular ion at m/e 211 which decomposed with the $CH_3CH{=}CH_2$ ($M^{\bullet}$ – 42) to give m/e 196 and loss of both CH_3 ($M^{\bullet}$ – 15) and m/e 169 with further fragmentation analogous to that observed for the authentic 2-methoxy derivative of hydroxyatrazine. In view of the foregoing the major compounds identified as bound residues in the methanol-extracted soil were atrazine (3.7 μg/g), hydroxyatrazine (1.5 μg/g), deethylatrazine (2.1 μg/g), and deisopropylatrazine (1.1 μg/g). These compounds, when calculated equivalent to atrazine, represent 27%, 12%, 18%, and 10%, respectively, of the total bound residues in soil. In this study the data reported for hydroxyatrazine should only be regarded as qualitative. Preliminary experiments showed that the recoveries of hydroxyatrazine and its dealkylated analogues were low due to poor efficiency of the methylation (50–80% yield). Furthermore, some of the bound ^{14}C residues released in HTD distillates were likely lost during the working procedure prior to GC analysis under the experimental conditions described.

The extracted soil containing bound residues described above was incubated with *Pseudomonas* species 192 and 194. Cumulative amounts of methanol-extractable ^{14}C residues at different time intervals are shown in Figure 2. It was observed that small amounts of initially bound ^{14}C were also released during incubation from soil containing BMN medium without any test bacteria. However, substantially more ^{14}C residues were released from the incubated soil inoculated with *Pseudomonas* species 192 and 194 (Figure 2). Addition of glucose at the end of 56th day appears to increase the release of bound ^{14}C from soil, particularly by strain 192 (Figure 2). Glucose appears to increase the population and/or activities of bacteria, resulting in a concomitant increase in the release of soil-bound ^{14}C residues. Thus, at the end of the incubation period the amounts of the released (extractable) ^{14}C residues ranged from 30% to 35% of the initially bound ^{14}C.

The nature of the released ^{14}C residues in methanol extracts from the incubated soils described above was determined by GC and GC–MS. The extracts showed GC peaks confirming the presence of atrazine, deethylatrazine, and deisopropylatrazine as described earlier. Similarly, GC of the methylated extracts indicated the presence of the 2-methoxy derivative of hydroxyatrazine. However, two additional major peaks also appeared in the chromatograms of the methylated extracts at retention times of 7.1 and 8.6 min. These retention times were identical with those of authentic 2-methoxy derivatives of hydroxy analogues of the monodealkylated products. The mass spectra of compounds represented by GC peaks at retention times of 8.1 and 8.6 min exhibited molecular ions at m/e 183 and 169 and base peaks ($M^{\bullet +}$ – CH_3) at m/e 168 and 154, respectively. Further fragmentation pattern was analogous to the mass spectra obtained for 2-methoxy derivatives of deethylated atrazine (Rt 7.1 min) and deisopropylated atrazine (Rt 8.6 min). The identities of the compounds were further confirmed by cochromatography with the authentic reference standards. Thus, the methanol extracts of the soil containing bound ^{14}C residues and incubated with *Pseudomonas* species 192 and 194 contained compounds I–VI as shown in Figures 3 and 4. The amounts (Figures 3 and 4) were calculated from peak heights by using the calibration curves. Furthermore, 2-methoxy derivative values were converted to their respective 2-hydroxy analogues.

Although the *Pseudomonas* species 192 and 194 were shown to be capable of utilizing atrazine as a sole source of carbon (Behki and Khan, 1986), it appears that organic matter in soil containing bound ^{14}C residues also served as alternative substrate for carbon source, resulting in increased microbial activities. Racke and Lichtenstein (1985) demonstrated a concomitant increase in the release of soil-bound ^{14}C residues by an increase in population of soil microorganisms. In this study initially the bound ^{14}C residues may be inaccessible to the *Pseudomonas* species. However, utilization of organic matter as substrate and its breakdown by the *Pseudomonas* species over a period of time accelerated the release of bound ^{14}C residues from soil. Some of the released ^{14}C residues were then metabolized by the *Pseudomonas* species.

Figures 3 and 4 show the compounds released (ex-

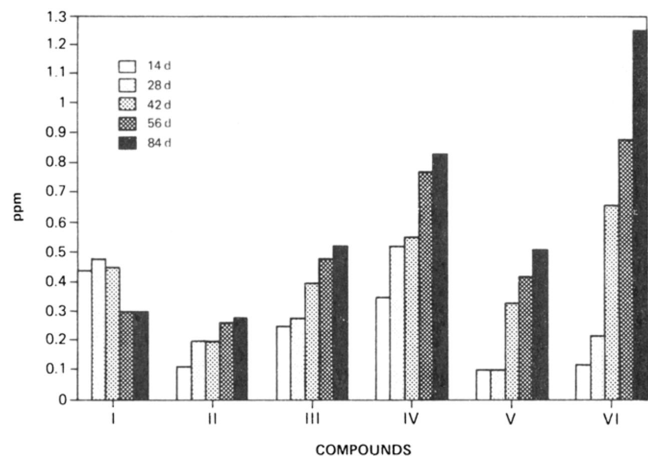

Figure 4. Compounds released from soil containing bound ^{14}C residues with *Pseudomonas* 194. (I) Atrazine; (II) hydroxyatrazine; (III) deethylatrazine; (IV) deisopropylatrazine; (V) deethylhydroxyatrazine; (VI) deisopropylhydroxyatrazine.

tractable with methanol) at different time intervals. An initial increase in the extractable ^{14}C residues of atrazine up to 28 days was followed by a decrease in concentration of the herbicide over the incubation period of 84 days. In contrast, the amounts of extractable ^{14}C residues of hydroxyatrazine, dealkylated atrazine, and their hydroxy analogues increased during incubation. It was demonstrated in an earlier study (Behki and Khan, 1986) that very little dechlorination of the parent compound atrazine occurred with species 192 and 194. It was suggested that the presence of both alkyl groups on atrazine may be inhibitory for bacterial dechlorination. Thus, it is apparent that the extractable hydroxyatrazine determined during the incubation period was mainly released from the bound form present in the soil rather than resulting from the dechlorination of the released parent compounds. Species 192 was relatively more effective than species 194 in releasing the bound hydroxyatrazine.

Analysis of soil extracts after different incubation periods revealed a relatively high proportion of deisopropylatrazine and its hydroxy analogue. The amounts of the extractable compounds present (Figures 3 and 4) cannot be solely attributed to the release of such residues from the bound form. Apparently, some of the released bound atrazine was also further metabolized preferentially by N-dealkylation of the isopropyl moiety by the two *Pseudomonas* species (Behki and Khan, 1986). Furthermore, in accordance with our earlier observations (Behki and Khan, 1986) the monodealkylated products were readily subjected to dechlorination, resulting in the formation of the respective hydroxy analogues (Figures 3 and 4).

The results of this study demonstrate that soil-bound ^{14}C residues can be released by *Pseudomonas* species previously shown to be capable of metabolizing atrazine. The organic matter in the soil containing bound ^{14}C residues was effectively used as an alternative substrate by the isolates. This resulted in a concomitant release of bound ^{14}C residues, which were subsequently metabolized by the *Pseudomonas* species 192 and 194. These species appear to possess a wide range of biosynthetic, degradative, and catabolic activities and substrate specificity.

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Registry No. Atrazine, 1912-24-9; hydroxyatrazine, 2163-68-0; deethylatrazine, 6190-65-4; deisopropylatrazine, 1007-28-9; deethylhydroxyatrazine, 19988-24-0; deisopropylhydroxyatrazine, 7313-54-4; glucose, 50-99-7.