Overall, these data show that microbial degradation contributes to the decomposition of EPN in soil and that the phenyl ring was broken to liberate $^{14}CO_2$. The remaining metabolites were adsorbed to the soil as compounds which release PPA or were possibly incorporated into the humic acid fraction as lignin-like compounds.

CONCLUSIONS

EPN has been shown to rapidly disappear when applied to cotton. Breakdown of residual EPN in both cotton plants and soil proceeds through hydrolysis and oxidation to phenylphosphonic acid which ultimately is converted to CO_2 through soil microbial action. The observed rate of breakdown in soil was fastest in the field, especially in a warm climate such as Mississippi where the half-life of the intact compound was found to be about 2 weeks.

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Hydrolytic Release of Tightly Complexed 4-Chloroaniline from Soil Humic Acids: An Analytical Method

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Research was conducted to develop a method to release and quantitate "bound" or tightly complexed 4-chloroaniline (4-CA) from soil humic acids. An evaluation of several techniques to release tightly complexed [^{14}C]-4-CA from soil humic acids resulted in the development of an analytical method using alkaline hydrolysis and quantitation of the released 4-CA as its heptafluorobutyryl derivative detected by electron-capture gas–liquid chromatography (EC-GLC). Soil humic acids were treated with [^{14}C]-4-CA at 477 ppm. Two percent of the applied radioactivity was uncomplexed, 10% was extractable into benzene and acetone (loosely complexed), and 88% remained tightly complexed. The alkaline hydrolysis released 80% of the applied radioactivity (91% of tightly complexed residue), with 46% of the applied 4-CA reproducibly quantitated as its N-(heptafluorobutyryl)-4-chloroaniline derivative. Other extraction methods such as acid hydrolysis, methylation, heptafluorobutyrylation, sonication, or pyrolysis were not as efficient in releasing tightly complexed 4-CA.

Substituted chloroanilines are derived from several pesticides but especially phenylureas, phenylcarbamates, and anilide herbicides. It has been shown, for example, that herbicide-derived 4-chloroaniline (4-CA) and 3,4-dichloroaniline are bound abundantly and tenaciously to soil and particularly to soil humic acids (Hsu and Bartha, 1974a, 1976). A soil-bound residue has been defined as the chemically unidentified pesticide residue or its degradation product remaining in fulvic acid, humic acid, and humin soil fractions after exhaustive sequential extraction with nonpolar and polar solvents (Kaufman et al., 1976). A precise definition of a bound residue has yet to be wholly agreed upon; therefore, the term tightly complexed will be used to describe residues recalcitrant to extraction: this removes any ambiguity and connotation of covalent bonding.

Several toxicological risks have been evaluated for chlorinated anilines: phytotoxicity (Hoffman and Allen, 1960), carcinogenicity (Neish et al., 1964; Hall, 1979), mammalian toxicity (Bartha and Prammer, 1967; Weisburger and Weisburger, 1966), and microbial toxicity (Corke and Thompson, 1970). Analysis of tightly complexed chloroaniline soil residues is of paramount importance because of their innate toxicity and because of their potential to accumulate in soil organic matter, possibly affecting soil physical and biochemical properties such as water–holding capacity, soil structure, and soil organisms and the processes of nitrification, ammonificiation, and

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cellulose decomposition. Very slow mineralization rates of bound 3,4-dichloroaniline (Hsu and Bartha, 1974b) support concerns regarding the potential perniciousness of tightly complexed residues.

A method of analysis for tightly complexed 3-chloroaniline and 3,4-dichloroaniline in plant lignin was developed recently by Balba et al. (1979). This technique, developed to release the chloroanilines, was based on the use of pyrolysis. Similar methodology was investigated in this laboratory to release tightly complexed humic acid–4-chloroaniline, the results of which are reported in Worobey and Webster (1982). Khan and Hamilton (1980) succeeded in releasing tightly complexed prometryn from soil via pyrolysis; it thus appears that the chemical nature of the compound is of importance in effecting its release from soil. The work described in this study involves 4-CA. Experiments were conducted to release, detect, and quantitate intact tightly complexed 4-CA in soil humic acids. The development of a sensitive analytical method for tightly complexed 4-CA in soil will enable the environmental soil burden to be evaluated.

MATERIALS AND METHODS

Chemicals. Solvents were pesticide grade, distilled in glass (Caledon Co.). Boron trifluoride–methanol, 10–14% (w/v), N-methyl-N'-nitrosoguanidine used to generate diazomethane (caution: potential carcinogen, mutagen, and irritant) according to the method of Stanley (1966), heptafluorobutyric anhydride, and [^{12}C]-4-chloroaniline (recrystallized 3 times from hexane mp 71–72 °C) were obtained from Aldrich Chemical Co. 4-Chloro[U-^{14}C]-aniline hydrochloride (California Bionuclear Corp., 11.2 mCi/mm) was dissolved in water, basified, and extracted with benzene to yield the free base. TLC confirmation was based on R_f determined by autoradiography and iodine vapor reaction. N-(Heptafluorobutyryl)-4-chloro[^{12}C]-aniline (4-CA-HFB) primary standard was synthesized by addition of excess heptafluorobutyric anhydride to 4-CA in benzene; recrystallization from hexane–methanol: mp 93–94 °C; mass spectral confirmation $M^+ = 323$.

Soil. A Brandon Caroll clay–loam (Black Chernozem) was used. The first 30 cm was sampled from a farm with no previous (known) history of pesticide treatment. Analysis: 9.3% organic matter, 30% sand, 38% silt, 32% clay, and 0.6% $CaCO_3$ (pH 5.7). Cation analysis (mequiv/100 g): Ca^{2+}, 30.3; Mg^{2+}, 7.0; K^+, 0.1; Na^+, trace; H^+, 2.8; total, 40.2.

Apparatus. *Gas–Liquid Chromatography (GLC).* A Varian Model 2440 equipped with a tritium foil electron-capture (EC) detector was used. Operating conditions were as follows: injector temperature, 180 °C; column temperature, 150 °C; detector temperature, 210 °C; nitrogen carrier gas flow rate, 45 mL/min. The GLC column was 1.7 m × 4 mm i.d. Pyrex packed with 3% OV-17 on 130–140-mesh Anakrom A/B. Radioassay was performed by using a Searle Model 6880 Mark III liquid scintillation spectrometer with a low level activity program (E^2/B) for dpm computation. Radioactivity in solid residues were determined as $^{14}CO_2$ by combustion in a Packard-Tricarb Model 306 biological oxidizer followed by liquid scintillation counting of the trapped combustate. A sonic dismembrator (Artek Systems Corp.) with titanium microtip was used for ultrasonic extraction.

Isolation of Humic Acids from Soil. The soil (air-dried for 68 h and stored at –40 °C; prior to use it was put through a 3-mm sieve) was washed with 1.0 N HCl to remove carbonates, followed by distilled water (ca. pH 5) to remove excess HCl. Humic substances were extracted with 0.5 N NaOH under nitrogen after purging the solution
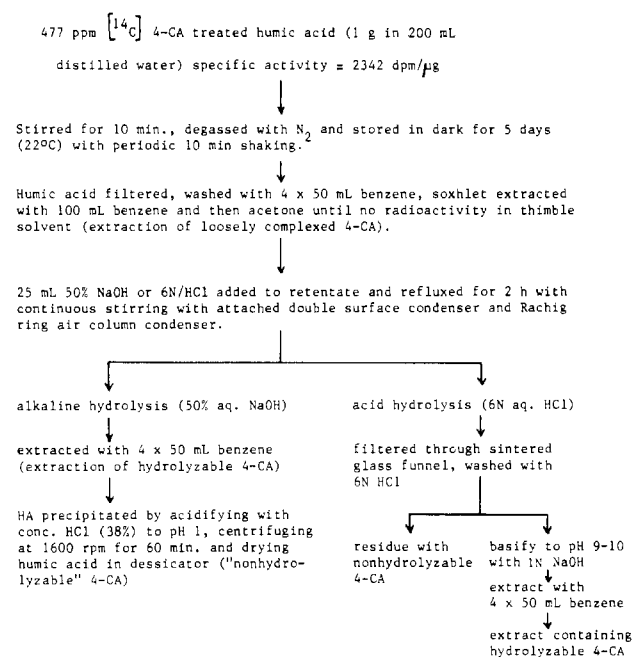

Figure 1. Treatment of humic acids and hydrolyses.

with nitrogen. Centrifugation (1000 rpm for 30 min) removed the insoluble material, humin, and acidification of the supernatant with HCl caused flocculation of the humic acid fraction; the fulvic acid fraction remained in solution. All procedures were performed with nitrogen-purged systems. The humic acid (HA) precipitate was recovered by centrifuging (1000 rpm for 60 min) and washed thoroughly with distilled water to remove excess HCl (0.1 N $AgNO_3$ test for chloride ion and wash water at pH 6.7). Humic acid recovered by this method accounted for 16% of the total organic matter (determined as total organic carbon). Approximately 50% of the isolated humic acid was accounted for as inorganic components, i.e., 50% ash, determined at 600 °C for 3 h in a muffle furnace.

Preparation and Hydrolyses of Tightly Complexed 4-Chloroaniline–Humic Acid Samples. Humic acids were treated with 4-chloroaniline (477 μg/g; 2342 dpm/μg) (Figure 1) and added to distilled water (200 mL), and the solution equilibrated at room temperature for 5 days after which the undissolved humic acid was filtered off by using a Buchner funnel under vacuum. Two percent of the original radioactivity remained in the filtrate. After removal of extractable (10% radioactivity loosely complexed) radioactivity, the residue containing 88% radioactivty tightly complexed was hydrolyzed under flux for 2 h with base or acid, and the Rachig ring column was washed with distilled water into the hydrolysate. The recovered residues were termed "nonhydrolyzable", and the filtrate radioactivity (extracted by using C_6H_6 prior to precipitation of the HA for base hydrolysis) after hydrolysis was termed the "hydrolyzable" fraction. Specific activities were determined by oxidation of dried residues to $^{14}CO_2$ in a Packard biological oxidizer as follows: nonhydrolyzed HA, 1124 dpm/mg; base hydrolyzed HA, 104 dpm/mg; specific activity based on 4-CA, 2340 dpm/mg.

Other Extraction Methods for Nonextractable Tightly Complexed HA–4-CA. (1) *Boron Trifluoride–Methanol (BF_3–CH_3OH) (10–14%); Heptafluorobutyric Anhydride (HFBA; 20% in Acetone).* BF_3–CH_3OH and HFBA were investigated as derivatization reagents for humic acids to release and/or solubilize complexed 4-CA. Humic acids containing nonhydrolyzed and base-hydrolyzed tightly complexed radioactivity were refluxed for 1 h with each solvent. After being cooled, the solution

was filtered through a sintered glass funnel and the humic acid was washed thoroughly with the same solvent used for extraction. The filtered residue was collected and dried in a desiccator under vacuum.

(2) *Ultrasonic (Microprobe) Extraction with Distilled Water, pH 9.2.* Ten milliliters of distilled water in a 15-mL glass-stoppered centrifuge tube plus approximately 20 mg of nonhydrolyzed humic acid with tightly complexed 4-CA (HA–4-CA) was sonicated by using the microprobe for 30 min at maximum power with an ice bath surrounding the centrifuge tube to dissipate heat. After sonication, the extract was adjusted to pH 12 and extracted with 3 × 5 mL of hexane. Aliquots of the hexane extract were analyzed in a liquid scintillation spectrometer.

(3) *Diazomethane Treatment of Humic Acid Followed by Hexane Extraction and Methanol Extraction.* Diazomethane treatment was used to investigate the release of bound 4-CA via methylation of the humic acid carboxyl groups to destroy the tertiary structure and release 4-CA if present as an inclusion complex. Freshly prepared diazomethane in diethyl ether was added to HA (477 ppm containing nonextractable bound residues) until the yellow color remained for at least 1 h. The excess diazomethane was destroyed with acetic acid and the ether evaporated off in the fume hood. For the hexane extraction, 4 mL of hexane was added, shaken briefly, and filtered through a 13-mm Swinney (0.5 μm) filter adapter and the entire filtrate was counted. The retentate was reextracted with methanol and the filtrate was counted.

Quantitation of Released 4-CA. To enhance the electron capturing properties and hence the EC detector sensitivity of 4-CA, we prepared heptafluorobutyryl derivative. Heptafluorobutyric anhydride (0.5 mL) was added to the final benzene extract and allowed to stand at room temperature for 10 min in a glass-stoppered 100-mL round-bottom flask. The benzene solution was concentrated to 1 mL in a water bath (50 °C) under a low flow of nitrogen. Hexane was added to the desired volume and an aliquot of the sample was injected. Depending on the method of release employed, the heptafluorobutyryl derivative was quantitated by EC-GLC analysis or, if the chromatogram was unsatisfactory, further purified by addition of 0.5–1.0 mL of hexane-washed distilled water, and aliquots were injected from the hexane layer.

An external standard method of quantitation was performed by comparing sample peak heights with those of a heptafluorobutyryl-4-CA (authentic) standard solution; peak heights were within the dynamic linear range of the detector. The structure of the derivatized standard was confirmed to be the mono-HFB derivative of 4-CA by mass spectrometry.

RESULTS AND DISCUSSION

Hydrolysis Methodology. Acid or base hydrolysis of tightly complexed 4-CA in humic acid released 38 or 46% of the original radioactivity as 4-CA determined as its heptafluorobutyral derivative by using EC-GLC (Figure 2). A control humic acid was free of interferences at the R_T for 4-CA-HFB.

Ten percent of the applied [^{14}C]-4-CA was extractable ("loosely complexed") into benzene–acetone, and 88% of the radioactivity was nonextractable or "tightly complexed". Two percent of the radioactivity was found to be in the original filtrate obtained after incubation; this was termed "free" or nonadsorbed 4-CA.

Other Methods for Release of Tightly Complexed and Nonhydrolyzable HA–4-CA. *BF₃–Methanol Extraction.* Attempts to release tightly complexed 4-CA from nonhydrolyzed and base-hydrolyzed [^{14}C]-4-CA residues

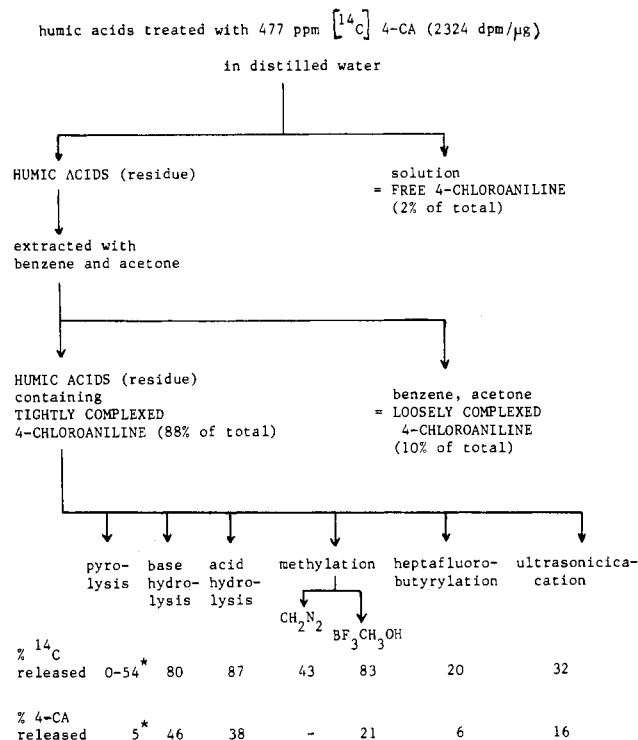| humic acids treated with 477 ppm [^{14}C] 4-CA (2324 dpm/μg)						

Figure 2. Extractability of tightly complexed 4-chloroaniline from treated soil humic acids. (*) These values are described in the following paper (Worobey and Webster, 1982).

	pyrolysis	base hydrolysis	acid hydrolysis	methylation	heptafluorobutyrylation	ultrasonicication
% ^{14}C released	0–54*	80	87	43	83	20	32
% 4-CA released	5*	46	38	–	21	6	16

yielded 82.7 and 77.2%, respectively, leaving 18 and 27.2% in the residues. Although a high percentage was extracted into benzene (66.4%) from a basified solution for the nonhydrolyzed HA, only 21% of this was detected as intact 4-CA as its HFB derivative. In the case of the base-hydrolyzed humic acid, 72.8% radioactivity was solubilized by the BF₃–CH₃OH, but the alteration in structure (1°; 2°; 3°; 4°) prevents release of 4-CA. The fact that 45% of the nonhydrolyzed residue was solubilized might support a mechanism of carboxyl (humic acid) methylation, followed by the breaking of intermolecular hydrogen bonding and hence solubilization of the residue.

Twenty Percent Heptafluorobutyric Anhydride in Acetone Extraction. Figure 2 shows that this method was not useful for extraction of 4-CA since only 20% of the radioactivity was solubilized by this reagent for either hydrolyzed or nonhydrolyzed HA–4-CA complexes. For the nonhydrolyzed HA–4-CA complex, 46% of the solids were solubilized, and approximately 90% of the radioactivity was still present in the solids after extraction. The effect of solubilization using HFBA may be analogous to that described for BF₃–CH₃OH; i.e., O-heptafluorobutyrylation may be occurring. The portion of humic acid solubilized (almost half) did not contain [^{14}C]-4-CA.

Ultrasonic Extraction. Figure 2 showed that the use of ultrasound to supply energy for release of 4-CA from the nonhydrolyzed HA–4-CA complex was partially successful with a pH 9 aqueous solution but only 15% was detected as 4-CA-HFB.

Diazomethane Methylation and Extraction. Extraction with methanol and hexane (radioactivity extracted from tightly complexed HA–4-CA after methylation) accounted for 31 and 12% of the original radioactivity, approximately 43% radioactivity extracted in total. The hydrolysis procedure is safer (CH₂N₂ is explosive and carcinogenic) and released more total radioactivity. As much of the released radioactivity was determined as 4-CA as was released in total radioactivity from methylated HA (44%; Figure 2). This method is suggested at present to be the

one of choice for estimation of recalcitrant 4-CA and possibly other xenobiotics in soil humic acids.

Methodology Conclusions. Figure 2 summarized results pertaining to extractability of tightly complexed HA–4-CA residues. Hydrolysis with base or acid was shown to be the best procedure to release large amounts of 4-CA from soil humic acids (90%) with approximately 40–50% analyzable as intact 4-CA.

The quantitative method reported in this study is very sensitive. A 75-fg (75 × 10⁻¹⁵ g) injection of 4-CA, as its heptafluorobutyryl derivative, into an EC-GLC produced a standing current decrease equivalent to a 10% full-scale recorder deflection at an attenuation of 1 and signal:noise ratio of 3:1. Such high sensitivity should permit preconcentration of HA extracts to detect much lower levels of 4-CA.

Hydrolysis and quantitation techniques were reproducible and free from coextraction interferences; high sensitivity may permit one to dilute out interferences from other substrates, or if more elaborate cleanups are required or extensive subsampling employed, such sensitivity will facilitate, if not make possible, quantitation of very low concentrations. Material balances using hydrolysis were excellent; 94–100% radioactivity was accounted for. Figure 2 shows operational definitions of 4-CA interaction complexes with soil humic acid. The same scheme may be applicable to whole soil as well as other xenobiotics.

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Pyrolytic Release of Tightly Complexed 4-Chloroaniline from Soils and Soil Humic Acids

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The release of tightly complexed 4-chloroaniline (4-CA) from treated soil humic acids and whole soils by using pyrolysis in an atmosphere of helium was studied. Pyrolysis of soil humic acids containing tightly complexed 4-chloroaniline resulted in the release of up to 54% of the radioactivity with approximately 5% detected via radioassay or gas–liquid chromatography as the intact 4-CA. Three soils of varying organic matter and clay concentrations complexed 100% of applied 4-chloroaniline; extractable radioactivity increased and tightly complexed 4-chloroaniline decreased as organic matter concentration decreased and clay concentration increased. The quantity of radioactivity released by pyrolysis was greatest for soils with low organic matter and high clay contents; 22–73% of the radioactivity could be released with 3–16% extractable into benzene from a basified pyrolysate, the amount depending on the nature of the soil, the type of pyrolysis probe, and the concentration of the applied 4-chloroaniline.

Pesticides have the capability to complex abundantly and tenaciously to soils and especially to soil humic acids. Analytical techniques for the determination of tightly complexed ("bound") pesticides and/or their degradation products are not yet readily available. Worobey and Webster (1982) recently reported a sensitive quantitative method for the determination of 4-chloroaniline tightly complexed to a soil humic acid. Several methods were investigated, and alkaline hydrolysis under reflux yielded the greatest amounts of intact 4-chloroaniline quantified by electron-capture gas–liquid chromatography (EC-GLC)

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as its heptafluorobutyryl derivative.

During these studies, anaerobic pyrolysis was intensively investigated as an analytical technique to release tightly complexed 4-chloroaniline from soil and soil humic acids, since it has proven useful in the release of tightly complexed 3-chloroaniline and 3,4-dichloroaniline from plant lignins (Balba et al., 1979). While our studies were in progress, Khan and Hamilton (1980) reported the successful release of tightly complexed prometryn from an organic soil using a similar pyrolytic technique to that of Balba et al. (1979). Our work examines the effect of soil type, that is, of varying organic matter and clay content, on the amount of tightly complexed 4-chloroaniline formed and its subsequent release using various pyrolysis conditions and pyroprobes.

MATERIALS AND METHODS

Reagents. All solvents were pesticide grade (Caledon Co.); [¹²C]-4-chloroaniline (4-CA) was obtained from the