Hydrolyzable and Nonhydrolyzable 3,4-Dichloroaniline–Humus Complexes and Their Respective Rates of Biodegradation

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Up to 90% of the 3,4-dichloroaniline released during the biodegradation of several phenylamide herbicides becomes unextractable by solvents due to binding to the soil organic matter. Bond stabilities and model reactions with monomeric constituents of humic substances suggest covalent binding of the residue by at least two distinct mechanisms. A 190-day laboratory experiment with radiolabeled 3,4-dichloroaniline demonstrated that the hydrolyzable and hence analytically accessible portion of the humus-bound residue declines with time, while the nonhydrolyzable portion does not, or does so at a much slower rate. The shifting ratio of hydrolyzable vs. nonhydrolyzable residue complexes frustrates current attempts to assess the true 3,4-dichloroaniline burden of agricultural soils that have experienced previous treatments by corresponding phenylamide herbicides. The practical significance of the humus-bound residues as potential sources of crop contamination is discussed.

Pesticide residues stabilized by binding or conjugation and the problems associated with their monitoring are important novel concerns for the residue chemist (ACS Workshop, 1976). The partial biodegradation of many phenylamide herbicides results in release of chloroaniline moieties (Herrett, 1969; Geissbühler, 1969; Bartha and Pramer, 1970). Some of the liberated chloroanilines are transformed in part to solvent-extractable azo compounds, but up to 90% of the liberated chloroaniline moieties are bound to soil (Chisaka and Kearney, 1970; Bartha, 1971). In Nixon Sandy loam, roughly one-half of the bound residue is liberated by hydrolysis under acidic as well as alkaline conditions. The balance of the bound residue is liberated only on combustion, and ^{14}C-labeled chloroanilines were required to demonstrate the existence of such nonhydrolyzable residues (Hsu and Bartha, 1974a). Microorganisms were shown to mineralize (degrade to inorganic end products) humus-bound chloroanilines at a slow rate (Bartha, 1971; Hsu and Bartha, 1974b), but it remained to be determined whether or not both the hydrolyzable and nonhydrolyzable residues were degraded and, if so, at what respective rates. The practical implications of this question are the following. If the hydrolyzable chloroaniline residues are mineralized, but the nonhydrolyzable ones are not, or are so at a much slower rate, agricultural soils could carry substantial chloroaniline residues that are not detectable with presently available analytical techniques. On the other hand, if the mineralization rates of hydrolyzable and nonhydrolyzable residues are comparable, the chloroaniline residues liberated with hydrolytic treatment could be used, with appropriate correction, to estimate the total chloroaniline burden of the soil. In an effort to answer the outlined question and decide whether or not currently available analytical techniques can be used for determination of soil-bound chloroaniline residues in agricultural soils, we have undertaken to compare the respective mineralization rates of hydrolyzable and nonhydrolyzable 3,4-dichloroaniline (DCA) residues in natural soil as well as in culture solution. An attempt was also made to elucidate the mechanism for the nonhydrolyzable binding of aniline residues to humic compounds.

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EXPERIMENTAL SECTION

Degradation of DCA–Humus Complexes in Soil. A fresh sample of Nixon Sandy loam (pH 5.8, organic matter by ignition 6.0%, humic acid 2.3%, fulvic acid 1.3%) was collected in the month of April from a field of the Agricultural Experiment Station, New Brunswick, N.J. Without air-drying, the sample was passed through a sieve with 3-mm diameter openings. Fresh soil samples, equivalent to 50 g of dry weight (dried at 105°C overnight), were added into 500-ml Erlenmeyer flasks, each containing 0.25 mg (5 ppm on a dry soil basis) of uniformly ^{14}C-ring-labeled DCA dissolved in sufficient water to adjust the water content of the soil to 60% of the holding capacity. The radiolabeled DCA was purchased from Amersham/Searle, Des Plaines, Ill., and had a radiochemical purity, as determined by thin-layer chromatography and scanning, of 99%. The amount of radioactivity received by each flask was 1.581 μCi. The flasks were tightly closed with rubber stoppers, and were incubated at 28°C in the dark. Twice weekly, the flasks were opened under a hood for aeration.

After appropriate time intervals (18, 63, 124, and 190 days), duplicate soil samples were exhaustively extracted (Figure 1) with five portions of acetone (total volume 200 ml). A 1-ml aliquot of the combined extracts was introduced into a counting vial. Ten milliliters of Aquasol (New England Nuclear, Boston, Mass.) was added and the radioactivity was counted at 90% efficiency using a Beckman Model LS-230 liquid scintillation counter (Fullerton, Calif.). Two-gram amounts of the solvent-extracted soil were subjected to wet combustion (Allison et al., 1965) and the liberated $^{14}CO_2$ was trapped in 250 ml of 0.1 N NaOH. The radioactivity of the trapping solution was counted as described before with the same counting efficiency. Another two-gram amount of the same soil sample was subjected to alkaline hydrolysis (20 ml of 50% w/v NaOH for 2.5 hr in a reflux unit) and the liberated DCA was steam-distilled for 2 hr. The soil residue was filtered from the alkaline solution and subjected to wet combustion, as described earlier. The radioactivity of the steam distillate, of the alkaline solution, and of the $^{14}CO_2$ from the humin fraction was counted. The hydrolysis and distillation times were optimized in a series of preliminary determinations. An initial problem of low recovery was overcome by the meticulous sealing of all joints of the digestion and distillation apparatus with silicon compounds.

Degradation of "Intact" and "Residual" DCA–

Table I. Proportions of Hydrolyzable and Nonhydrolyzable Humic Complexes in DCA-Treated Soil at Various Times of Incubation[a]

Days	Acetone extract (A)	Hydrolyzable residue in distillate (B)	Nonhydrolyzable residue			Total Recovery (A + B + C + D)
			In alkaline solution (C)	In soil residue (D)	Total (C + D)	
18	8.94 (±0.03)	36.65 (±1.30)	33.59 (±0.56)	8.29 (±0.02)	41.87	87.46
63	4.82 (±0.02)	29.96 (±0.04)	38.52 (±0.48)	9.10 (±0.43)	47.62	82.40
124	4.01 (±0.03)	24.70 (±0.32)	42.85 (±0.36)	7.94 (±0.38)	50.79	79.50
190	3.20 (*b*)	23.84 (*b*)	40.41 (*b*)	9.43 (*b*)	49.84	76.88

[a] All data in percent of the originally applied radioactivity. The numbers in parentheses indicate the range of duplicate determinations. Capital letters in parentheses refer to the corresponding fractions in Figure 1. [b] Single determination only.

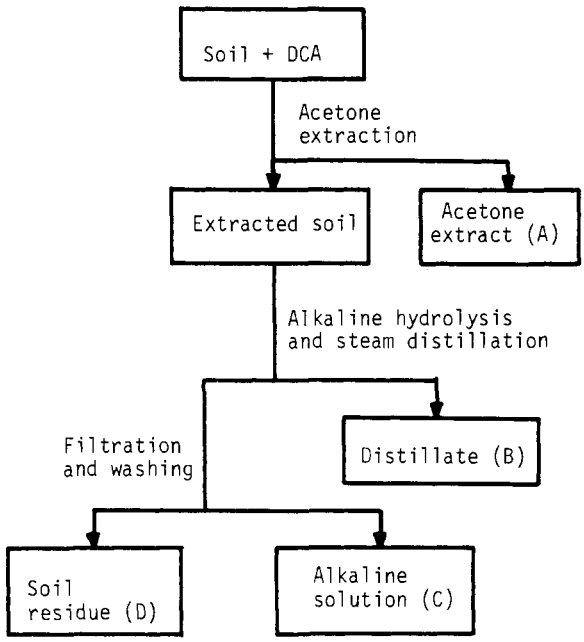

Figure 1. Flow diagram of the fractionation of DCA-treated soil.

Humic Complexes in Culture Solution. The DCA–humic acid complex was prepared as described earlier (Hsu and Bartha, 1974b) and was purified by dissolution in alkali and re-precipitated at pH 1.0. The precipitate was removed by centrifugation, and was dissolved in a minimal amount of 1% (w/v) K_3PO_4 solution. The solution was diluted with water, was adjusted to pH 6.0 with H_3PO_4, and was freeze-dried. The powder was washed with anhydrous diethyl ether to remove DCA that was not covalently bound, was dried, and was used subsequently as "intact" (nonhydrolyzed) DCA–humic acid complex. This complex contained DCA bound in a hydrolyzable as well as in a nonhydrolyzable manner. A 2-g amount of the above material was subjected to alkaline hydrolysis (25 ml of 50% w/v NaOH for 3 hr in a reflux unit). The remaining complex was precipitated by adjusting the pH to 1.0 using concentrated HCl. The precipitate was removed by centrifugation. The supernatant was discarded and the pellet was purified and treated as described in the preparation of the intact DCA–humic acid complex. This material, containing DCA bound in the nonhydrolyzable manner only, was used in the subsequent experiment as the "residual" (prehydrolyzed) DCA–humic acid complex. The intact and residual DCA–humic acid complex was subjected to mineralization by a humic acid degrading soil fungus *Aspergillus versicolor*. The replacement culture technique, as described in an earlier publication (Hsu and Bartha, 1974b), was closely followed in this phase of the work. In brief, the fungus was pre-grown in a culture solution containing sucrose, minerals, and 0.1% (w/v) humic acid. The mycelial pellets were aseptically washed and transferred into a mineral solution containing, at 0.1% (w/v) concentration, radiolabeled DCA–humic acid complexes of the intact and residual type, respectively, as the only sources of carbon and energy. The replacement cultures were incubated with agitation in a stream of CO_2-free air at 28°C in a gas train apparatus. Each unit was connected to an alkaline and an acid trap containing 50 ml of 0.2 *N* NaOH and 0.25 *N* HCl, respectively. Periodically, the traps were changed. Total CO_2 was determined by titration of the residual alkalinity; $^{14}CO_2$ and other radioactive products were counted as described earlier. The data were plotted cumulatively.

Model Experiments for Covalent DCA Binding. 4-Methylcatechol was obtained from Aldrich (Milwaukee, Wis.), and *N*-(4-aminophenyl)-*p*-benzoquinone diimide (indamine) from Pfatz and Bauer (Flushing, N.Y.). Two 800-ml portions of 0.1 *M* phosphate buffer (pH 6.0), each containing 5 mg of radiolabeled DCA plus the 100-fold amount of cold carrier (4×10^{-3} mol of DCA, 4.68×10^7 cpm), were prepared. To each of these solutions one of the above compounds was added in equimolar amounts, and the flasks were incubated with shaking at 28°C for 4 days. The precipitates that formed in the initially clear solutions were filtered, washed with distilled water, and air-dried at 37°C. The products were weighed and measured amounts were dissolved in acetone and were counted for total incorporated radioactivity. Internal standardization was used to avoid counting errors due to the strongly colored solutions. Measured amounts of the products were subjected to alkaline hydrolysis by refluxing for 3 hr under nitrogen in 30 ml of 50% (w/v) NaOH. The hydrolysis was followed by steam distillation for 3 hr using 20 ml of 0.5 *N* HCl in the receiving flask to trap DCA. The nonvolatile radioactivity remaining in the hydrolysates and the volatile radioactivity of the steam distillates were counted and expressed as percentages of the total radioactivity of the products.

RESULTS AND DISCUSSION

Degradation of DCA–Humus Complexes in Soil. The binding of low levels of DCA (5 ppm) to soil is rapid (Hsu and Bartha, 1974a) and was expected to be complete prior to the first analysis at 18 days after treatment (Table I). Acetone extraction removed only physically adsorbed DCA (Hsu and Bartha, 1974a). This fraction (A) declined strongly between 18 and 63 days, but thereafter exhibited only very slow decrease. The covalently bound but hydrolyzable DCA (B) showed a similar pattern with a rapid initial and a slower subsequent decline. Nonhydrolyzable DCA residues (C + D) actually increased up to day 124 and stayed essentially level thereafter to the conclusion of the experiment at 190 days. The increase of this fraction (C + D) during the first 4 months of the experiment indicates that some physically bound and/or hydrolyzable DCA gradually shifted to the nonhydrolyzable form. The

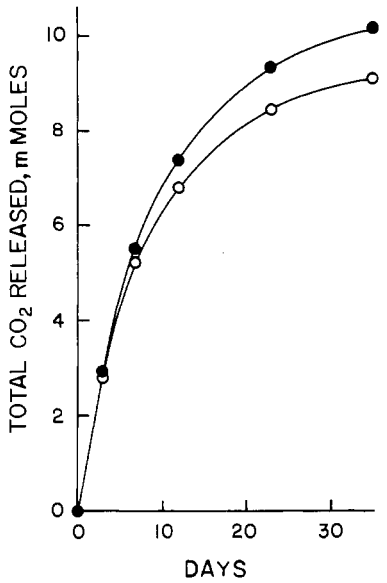

Figure 2. Total CO_2 evolution from cultures of *Aspergillus versicolor* utilizing intact (solid circles) and residual (open circles) radiolabeled DCA-humic acid complexes.

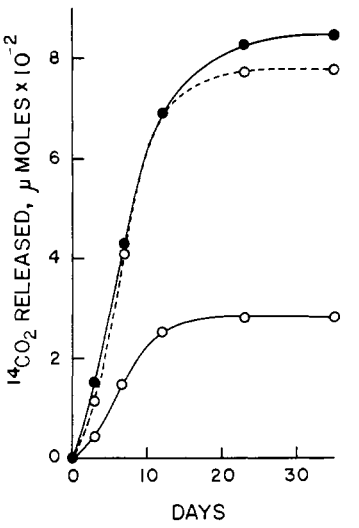

Figure 3. $^{14}CO_2$ evolution from cultures of *Aspergillus versicolor* utilizing intact (solid circles) and residual (open circles) radiolabeled DCA-humic acid complexes. When correction was made for the lower specific radioactivity of the residual complex, the resulting curve (open circles, broken line) closely resembles radiocarbon evolution from the intact material.

total recovery (A + B + C + D) declined during this time, indicating that some of the DCA residues were mineralized to CO_2 (Hsu and Bartha, 1974b) but this process either affected only the physically adsorbed and/or hydrolyzable DCA, or any mineralization of the nonhydrolyzable DCA was offset by a shift of the previous two residue forms to the latter one. Short of radiotracers, to date no analytical technique capable of detecting nonhydrolyzable chloroaniline residues in soil has been devised. As to the hydrolyzable chloroaniline–humus complexes, from our radiotracer studies reported here and in an earlier publication (Hsu and Bartha, 1974a) the outlines of an analytical procedure have emerged. After exhaustive extraction by acetone–benzene or other suitable solvents to remove undegraded herbicide and physically bound chloroaniline, the soil sample is to be subjected to hydrolysis and steam distillation as outlined in Figure 1. The distillate is to be adjusted to alkaline pH and extracted by diethyl ether or another suitable solvent (Hsu and Bartha, 1974a). The dried and concentrated extract is to be analyzed by gas chromatography with electron capture detection, e.g. as described by Kearney et al. (1970).

The scheme proposed above is reasonably straightforward and appears suitable for routine analysis of soil samples. From our results summarized in Table I we must conclude, however, that analysis restricted to the hydrolyzable portion of the DCA–humus complex will present a misleading picture as to the total bound DCA burden of the soil, since the hydrolyzable DCA complex decreases in concentration with time while the nonhydrolyzable one does not, or at best does so at a much slower rate.

Degradation of Intact and Residual DCA–Humic Acid Complex in Culture Solution. Faced with the apparent stability of the nonhydrolyzable DCA–humus complex in natural soil, we have undertaken to examine whether or not these residues are inherently recalcitrant to biodegradation.

When offered as the only source of carbon and energy to a replacement culture of *A. versicolor*, a humus-degrading soil fungus, the intact DCA–humic acid complex supported an only marginally higher total CO_2 evolution than the residual complex (Figure 2). Hence, we concluded that the pretreatment did not decrease the general availability of the humic acid to *A. versicolor* significantly.

A marked difference was observed in $^{14}CO_2$ release from the intact and residual complexes (Figure 3), but this difference was largely due to the fact that the removal of the hydrolyzable DCA from the DCA–humic acid complex had lowered the specific radioactivity of the complex from 2.128×10^7 cpm/g to 7.724×10^6 cpm/g or by a factor of 2.75. When the absolute $^{14}CO_2$ production from the residual material was corrected by this factor, it became apparent that *A. versicolor* made little, if any, distinction between intact and residual DCA–humic acid complexes, and oxidized both at comparable rates. No significant radioactivity was found in the acid traps. The results show that the residual, i.e. nonhydrolyzable, DCA residues are not inherently recalcitrant to biodegradation, and an alternative explanation has to be found for their apparent stability in soil. It is possible that, in natural soils, *A. versicolor* and similar humus-degrading microorganisms are either infrequent, inactive, or utilize alternate substrates preferentially. Compared to this entirely speculative explanation we find more attractive the proposition that some of the nonhydrolyzable chloroaniline residues are in fact degraded, but a gradual shift of physically absorbed DCA and of hydrolyzable DCA to the nonhydrolyzable form compensates for this loss. Our data (Table I) lend some support to the latter theory.

Suggested Mechanisms of Covalent Binding of Some Chloroanilines and the Significance of the Bound Residues. The fact that the humic component is a random polymer prevents the precise chemical definition of the DCA–humic complex in terms of total molecular structure and molecular weight. Yet it is of importance to know whether the radiolabel attached to the humic material represents intact DCA that may be remobilized under appropriate circumstances, or whether the complex is essentially indistinguishable from normal humic substances. In an attempt to offer at least a partial answer to the above questions, we wish to evaluate the available direct and indirect evidence and to offer some theoretical considerations as a basis for future experimental work.

There is no sufficient reason to doubt that "hydrolyzable" DCA and 4-chloroaniline are attached with the chlorophenyl ring intact, since they were determined

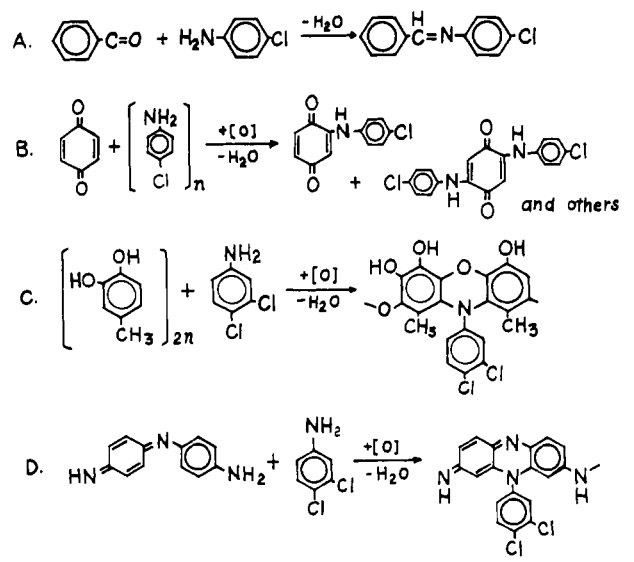

Figure 4. Model reactions suggesting some hydrolyzable and nonhydrolyzable binding mechanisms for chloroanilines to humic substances. Radiolabeled chloroanilines reacted under conditions resembling the soil environment with monomeric constituents of humic substances. Hydrolyzable binding of 4-chloroaniline occurred to benzaldehyde (A) and to *p*-benzoquinone (B) (Hsu and Bartha, 1974a). Nonhydrolyzable binding of DCA occurred to 4-methylcatechol (C) and to indamine (D) (see Table II).

directly after alkaline or acidic hydrolysis (Bartha, 1971; Hsu and Bartha, 1974a). The resistance of the residues to solvent extraction and ion exchange combined with a sensitivity to acid as well as alkaline hydrolysis strongly suggest a covalent binding of the nitrogen atom of these chloroanilines to the carbon of a carbonyl group, or to a quinoidal ring of the humic compounds. While other reactions resulting in hydrolyzable attachment are conceivable, those of the described type were demonstrated to occur with monomeric constituents of humic acid (Figure 4, A and B) under conditions that prevail in the experimental soil (Hsu and Bartha, 1974a). Biodegradation of a DCA–humic acid complex by *Penicillium frequentans* in mineral culture solution resulted in the release of free DCA and other radioactive compounds that are believed to be humic oligomers with attached DCA (Hsu and Bartha, 1974b). $^{14}CO_2$ was also produced, indicating that some of the released material was mineralized. Biodegradation by another soil fungus, *A. versicolor*, resulted mainly in $^{14}CO_2$ and low amounts of soluble radioactivity (DCA and oligomeric complexes).

As to the residual DCA–humic complex, no direct proof is available that the chlorophenyl ring is still intact. Nevertheless we consider this to be a reasonable assumption on the basis of the following considerations: (1) the attachment of uniformly ring-labeled DCA is not accompanied by substantial CO_2 release (Bartha, 1971); (2) attachment occurs in sterile soil where no previous biodegradation can take place (Hsu and Bartha, 1974a); (3) the 3,4-dichloro substitution conveys substantial resistance against biodegradation of the phenyl ring (Alexander, 1965) yet lowers the reactivity of the amino group only moderately (Bordeleau and Bartha, 1972).

The resistance of the attached radioactivity against release by hydrolysis suggests the incorporation of the nitrogen atom into heterocyclic ring systems of the phenoxazine and phenazine type. Such mechanisms were suggested for the nonhydrolyzable attachment of ammonia to humic compounds (Nommik, 1970; Lindbeck and Young, 1965; Murphy and Moore, 1960). These suggestions were based on model experiments with various monomeric constituents of humic acid including phenolic and quinoidal compounds. Believing that the attachment mechanism of ammonia and DCA may be similar, we tested the behavior of radiolabeled DCA in terms of reactivity and nonhydrolyzable bond formation in two such model systems. While various reaction conditions were employed by the cited authors, we did not use any other oxidant than atmospheric air and we maintained a pH and temperature resembling our experimental soil.

Table II. Nonhydrolyzable Binding of Radiolabeled DCA to Some Model Compounds under Ambient Conditions[a]

Reactants[b]	Product weight, g	Radioactivity: Product, cpm	Hydrolyzable, %	Nonhydrolyzable, %	Recovery, %
4-Methylcatechol + DCA	0.51	2.88×10^7	16.4	59.0	75.4
Indamine + DCA	1.21	3.14×10^7	8.8	83.1	92.0

[a] In pH 6.0 phosphate buffer at 28° C, with shaking under air atmosphere for 4 days. [b] 4×10^{-4} mol of each.

Under the described mild reaction conditions both 4-methylcatechol and indamine yielded products with DCA that precipitated. No precipitate or any visible change was observed in individual solutions of 4-methylcatechol, indamine, or DCA during the same time period. The results of the experiment are summarized in Table II. In analogy to ammonia, the bulk of the radiolabeled DCA was bound in a nonhydrolyzable manner. No attempt was made at this time to chemically characterize the heterogeneous and polymeric products, but based on the cited model experiments with ammonia (Murphy and Moore, 1960; Lindbeck and Young, 1965) and on known reactions of aniline with similar chemical compounds (Joule and Smith, 1972), we suggest the reaction mechanisms illustrated in Figure 4 (C and D). The type of receptor sites suggested in the figure for 4-chloroaniline and DCA binding is common in humic substances (Stevenson, 1972).

Assuming the basic validity of our theory, i.e. that the residual DCA–humic complex represents an intact 3,4-dichlorophenyl ring attached through the nitrogen atom in a manner suggested by our model reactions, we have to face the practically significant question whether or not residues bound in this manner will be re-mobilized in an intact form. Obviously, only further experimentation will yield a satisfactory answer, but we wish to submit that this is at least a reasonable possibility, since microorganisms are known to break heterocyclic bonds that resist acid as well as alkaline hydrolysis (Dagley, 1972).

Humus-bound DCA residues, even at excessive concentrations, do not inhibit microbial respiration (Hsu and Bartha, 1974b) and can be regarded as temporarily detoxified as well as immobilized. They may attain practical significance, however, if released by microorganisms as intact DCA. Rice plants readily take up DCA through their root system and translocate it (Still, 1968). DCA residues were demonstrated in samples of experimentally grown as well as in commerical rice grain samples, though some of the plants were not themselves treated with 3,-4-dichloropropionanilide (propanil), but grew in soil with a previous treatment history (Still and Mansager, 1969). Even in case of direct treatment, it is difficult to explain how propanil, that is applied at an early growth stage of

the rice plant, is stored in the form of metabolites throughout the growth cycle, and is eventually translocated into the ripening grain. In our opinion, it is a more likely possibility that the DCA in the rice grains originates from soil-bound residues, and enters the plant during the formation and ripening of the grain.

Whether or not aniline residues derived from other phenylamide herbicides behave similarly to DCA is at this time largely a matter of conjecture. 4-Chloroaniline was mineralized at a slightly faster rate than DCA, but otherwise behaved in soil (Bartha, 1971) and in model reactions (Hsu and Bartha, 1974a) very similarly to DCA. Rice grains that were harvested in 1958 (before the introduction of propanil), and had a *N*-(3-chlorophenyl)-carbamate (chloropropham) treatment history, contained 3-chloroaniline (Still and Mansager, 1969).

Whether or not soil-bound DCA and perhaps other similarly bound aniline residues constitute a source of crop contamination of practical importance remains to be established by future experimental work. We do not wish to imply that such a hazard was conclusively demonstrated here. Nevertheless, it is hard to conclude from the presented evidence that soil-bound DCA residues can be ignored safely. Our present inability to reliably monitor the humic complexes of DCA and of perhaps other chloroanilines is a highly unsatisfactory state of affairs. A more general awareness of this analytical challenge will hopefully hasten the development of a suitable procedure.

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Detection of Triazine Herbicides and Their Degradation Products in Tile-Drain Water from Fields under Intensive Corn (Maize) Production

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The herbicides cyanazine, cyprazine, atrazine, and metribuzin were applied (post-emergence) to plots which were drained by separate tile drains (depth 1.2–1.6 m). Water samples were collected from the tile outlets and extracted with ethyl acetate. In the first season of monitoring, herbicide concentrations ranged from 0.30 to 1.49 μg/l. for atrazine, from 0.00 to 0.68 μg/l. for cyanazine, and from 0.00 to 0.57 μg/l. for cyprazine. Similar levels of the chloro-*s*-triazine herbicides were found in the tile-drain water during the second year of the study. Metribuzin, which was applied during the second year, was found in the tile-drain water in concentrations ranging from 0.00 to 1.65 μg/l. The metabolites deethylated atrazine (2-chloro-4-isopropylamino-6-amino-*s*-triazine) and cyanazine amide [2-chloro-4-(1-carbamoly-1-methylethylamino)-6-ethylamino-*s*-triazine] and deisopropylated atrazine (2-chloro-4-amino-6-ethylamino-*s*-triazine) were detected at concentrations which were similar to those of the parent compounds. The discharge (grams/hectare) of herbicide residues in the tile-drain water was determined over a 9-month period.

There are relatively few published reports on field studies of herbicide residues in ground water despite the widespread use of these chemicals. Wheeler and Mansell (1974) found that Terbacil and 2,4-D were detected (10 to 110 μg/l.) in subsurface drainage water collected from a citrus grove under irrigation. Mackenzie and Viets (1974) have discussed the question of insecticide residues in tile-drain water and have pointed out there is a scarcity of information on the fate of herbicides in drainage water.

Sirons et al. (1973) reported on the persistence in soil of the major phytotoxic degradation products of atrazine and cyanazine. Their findings indicate that relatively large quantities of deethylated atrazine and deisopropylated atrazine are present in the upper 6 cm of the soil profile. Beynon et al. (1972) reported that the levels of cyanazine amide (0–10 cm depth) were double those of the parent compound 4 weeks after application of the herbicide.

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