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REVIEWS

Natural Product Interactions during Aniline Metabolism Including Their Incorporation in Biopolymers

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Anilines can be found in many agrochemicals. Their metabolism in biological systems often results in large portions of the aniline moiety being incorporated in the "bound" fraction of biological samples. The reaction of anilines with natural products and their binding to biopolymers, such as starch, lignin, and humus, are reviewed from the existing literature covering the period of the 1870s to the present. The structural characteristics of the aniline/biopolymer complex are discussed. Such binding will be noted in plants, soil, ecosystems, and ensilage. The analytical process for the complexed anilines is examined, including problems encountered. The interactions of anilines with natural products and biopolymers are complex. This makes the understanding of the metabolic consequences of anilines in biological systems difficult and not completely understood. In defining metabolites, insight is presented on the need for careful experimental design strategies, judiciously chosen biopolymer disruption procedures, well-planned isolation/purification techniques, and critical evaluation of results.

INTRODUCTION

In the metabolism of anilines in biological systems, a large portion of the aniline moiety is found in the "bound" fraction of biological samples (especially for soils and plants). The bound fraction is that portion not extracted by neutral polar and/or organic solvents. Acid or base hydrolysis can release additional aniline moiety, but a significant portion of the moiety remains and is considered "fixed". In a fairly slow process, soil microbes can mineralize the aniline moieties (free, bound, or fixed) to CO_2, NH_3, and H_2O, with the free or hydrolyzable moieties being mineralized more rapidly.

The incorporation of anilines, as well as phenols, into biopolymers has stimulated researchers to perform studies for elucidating the structures of the bound moieties. This has required developing and understanding the structures of the biopolymers themselves and, in turn, the structural attachment of the anilines.

This summary will address the metabolism and organic–analytical chemistry of chloroanilines (DCA or CA) (3,4-dichloroaniline will be referred to as DCA and chloroanilines as CA throughout this review), including the binding to biopolymers (particularly carbohydrates, lignins, and humus). While the papers reviewed covered the period from 1870 to the present, an extensive review covering all chloroaniline papers published in this period was not done. Key papers (often reviews) were selected to describe a general picture for the present understanding of these processes. By summarizing information from these various sources, the intent of this review is to provide a general overview of chloroaniline metabolism, mainly in plants and soil, and to guide and alert investigators to the potential problems in defining the metabolic end products. The discussion on aniline biopolymer binding is directed to the difficulties in isolating and identifying discrete chemical entities (or monomeric units) and in elucidating

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the aniline attachment to biopolymers. Several biological areas are addressed, including ecosystems and ensilage. The bioavailability of the aniline bound material to animals and plants is also discussed.

CHEMISTRY

Carbohydrates. The first reported condensation of aniline and toluidine was with anhydrous D-glucose (Schiff, 1870). While Schiff preferred the "Schiff base" amine–aldehyde condensation product type linkage, an *N*-glycoside was a formula later proposed (Marchlewski, 1894). In the first of several publications (Irvine and Moodie, 1908), it was demonstrated that the condensation product of glucose and aniline was an *N*-glycoside and not a Schiff base (Irvine and Gilmore, 1908, 1909; Irvine and McNicoll, 1910). Irvine and Hynd (1911) further showed that the amino group of anthranilic acid complexed to glucose in preference to the carboxylic acid group. Amadori (1925, 1929) found the conversion of *N*-glycosides of aldose sugars to amino derivatives of the corresponding ketoses, often referred to as isoglucosamines. The reaction is catalyzed by both acids and bases but proceeds best in acidic media. Weygand (1939) found sugar/aniline glycoside yields to be in the order of glucose > galactose > mannose > xylose. Berger and Lee (1946a,b) found xylidene plus glucose to yield both the pyranose and furanose ring forms but no Amadori reaction under their dilute acid conditions. The pyranose form could be converted to the furanose form in refluxing alcohol. Hanaoka (1940) found, in chloroaniline–glucose conjugates, the order of the chloro position as para > meta > ortho in rates of hydrolysis. Ellis and Honeyman (1952) also found ribose plus aniline to give both pyranose and furanose rings depending upon reaction conditions and that ketoses, like fructose, did *not* conjugate *directly* with aniline. Bayne and Holms (1952) studied the decomposition of glycosylamines in acid and found ortho-substituted aniline conjugates to rearrange less readily to isoglucosamines than meta or para. Capon and Connett (1965a,b) provided data to argue that acid-catalyzed hydrolysis of *N*-aryl-D-glycosylamines proceeds via intermediate Schiff base forms. They found hydrolysis of the glucosylamines to be preceded by a rapid anomerization to a mixture of 10% α and 90% β forms. With water present, hydrolysis with release of aniline occurred; anhydrous conditions yielded the Amadori isoglucosamines. Also, the isoglucosamines decomposed more slowly in acid conditions than the glycosylamines. Shafizadeh et al. (1974) pyrolyzed glycosides and glycosylamines. The glycosides quantitatively released the phenols, but glycosylamines showed chemical rearrangements with the nitrogen moiety remaining in the unknown nonvolatile residue. Some possible reaction products in pyrolysis could be similar to those proposed by Baltes and Franke (1978) for the Maillard reaction upon heating of D-glucose and *p*-chloroaniline. Yaylayan (1990) discusses the 1,2- and 2,3-enolization of the Amadori rearrangement product (the fructopyranose structure) after ring opening. He further proposes the formation of pyrylium betaines, by direct dehydration of the fructopyranoses, which he suggests leads to the formation of heterocyclics and polymers by the pyrilium ions. The results from the preceding papers are summarized in parts A (for Amadori reaction products) and B (for Maillard reaction products) of Figure 1 as a possible sequence of heat and acid/base catalytic action on glycosylamines. Thus, the interaction of anilines and carbohydrates can lead to a large number of nitrogen-containing products that are no longer structurally similar to the original aniline/carbohydrate metabolite and can no longer release the original aniline upon hydrolysis. Also,

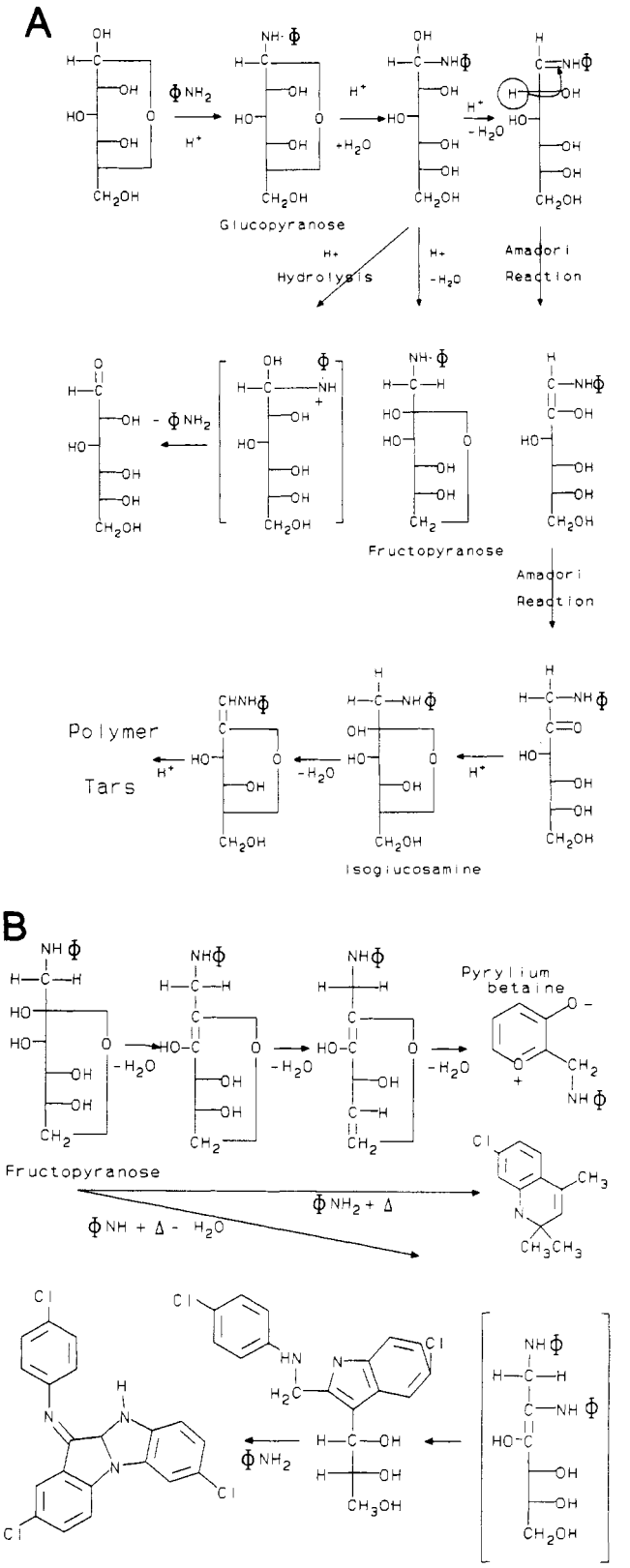

Figure 1. (A) Possible carbohydrate acidic interactions with anilines (possible Amadori reaction products). (B) Possible carbohydrate interactions with anilines (possible Maillard reaction products).

the sensitivity of aniline–carbohydrate conjugates to acids, bases, and heat makes isolation and identification of the true aniline conjugate difficult.

An additional consideration for the carbohydrates is

the encapsulation of anilines within the starch matrix. The capability of pesticides to encapsulate into starch has been studied by several investigators. Trimnell and Shasta (1988) encapsulated 15 pesticides, including two containing tertiary anilines (oryzalin and trifluralin), by mixing, at room temperature, a chloroform solution of either of two pesticides with pearl cornstarch suspended in NaOH/water, as one of their procedures. Wing et al. (1988) found the ability of starch to encapsule pesticides depended upon the amylopectin/amylose contents. They reasoned that the highly branched nature of the amylopectin provided a relatively larger volume than amylose in its matrix to accommodate the pesticide. It was indicated that commercially available starches have different amylose content: waxy (<1%) pearl (~25%), and Amylon type (50–70%). They found the higher the amylose content, the lower the efficiency of encapsulation of butylate. The analysis was done by using a hexane wash to remove surface compound and then dissolving the starch in 2 N HCl/100 °C and extracting the butylate with isooctane. These results suggest the possibility for the biosynthesized starch in corn or rice grain to also entrap organic compounds, such as the anilines, during their metabolism. Thus, not only could anilines form glycosidic linkages at the reducing ends of the starches but entrapment could also occur, increasing the nonextractable (bound) value in the metabolism of anilines. Therefore, the aniline–starch bound values could be higher than expected from glycosidic linkages alone, suggesting that some of the bound aniline can be released simply upon loosening the starch matrix, such as by enzymatic cleavage.

Lignin. Yih et al. (1968) were some of the first investigators to show DCA complexing to lignin in rice plants. To study the association of anilines in lignin, a disruption of the biopolymer into smaller units (preferably monomers) is desired. Bjorkman (1954, 1956) developed one of the milder processes used by many investigators. Dioxane/water and heat were used to disrupt the polymer; in a more rigorous version, HCl was added. Nooden (1970) found 2-aminoethanol an effective reductive reagent to degrade lignin. Cyr et al. (1988) studied the sulfite process, showing it to oxidatively cleave lignin at the ether linkages, releasing one phenylpropane monomeric unit at a time from the chain ends. Klason (1923) was the first to study the use of sulfuric acid to isolate lignin. Freudenberg and Ploetz (1940) found the method unsuitable for structural studies since no consistent procedure could be established in wood lignins and the conditions resulted in structural changes. The sulfuric acid procedure, with many modifications made over the years, is used mainly for quantitative determination of lignin. In all isolation/disruption procedures, oxygen is excluded from the reactions since the released products are sensitive to oxidation. This indicates that the presence of reductive reagents during hydrolysis would be useful.

Kirk et al. (1975) compared natural lignin with lignin synthesized from coniferyl alcohol and peroxidase, used to produce a free-radical mechanism. The synthetic lignin was a smaller polymer with more coniferyl alcohol end groups. In the synthetic lignin, there were more β–β linkages at the propyl side chains, more phenylcoumarin, and more 5–5 linkages on the phenyl rings (see Figure 2 for the structures discussed). Both natural and synthetic lignins were degraded by the white rot fungus. The synthetic process has more monomer–monomer couplings, while natural lignin has more monomer–polymer couplings produced during the course of synthesis. Thus, natural lignin has all of the linkages shown in Figure 2. The authors felt that synthetic lignin was acceptable as a model for lignin interaction studies. v.d. Trenk et al. (1981) presented the free-radical intermediate sequence of coniferyl alcohol polymerization with peroxidase (Figure 3A) The sequence shows only β-, 1-, 4-, and 5-positions available as reactive coupling sites by free-radical activation. By predominantly using NMR analysis, v.d. Trenk provided evidence that a quinone methide is the intermediate structure in a 1,6 addition of aniline (like DCA) to the side-chain α-carbon position. The quinone methide is generated by various oxidative processes in lignin but apparently not by catalase. Since the 1,6 addition is a nucleophilic one, there is reason to expect other functional groups with active hydrogen, like hydroxyl, sulfhydryl, and carboxyl, would add to the α-position. The locations of the reactive positions in lignin, with coniferyl alcohol as the example, are shown in Figure 3B.

Balba et al. (1979) attempted to use pyrolysis to analyze DCA from lignin complex with only partial success, getting 84–86% DCA released from DCA/lignin complexes that were *not* aged. Aging decreased the yield of DCA. Still et al. (1981) copolymerized aniline and coniferyl alcohol, using laccases and peroxidases, to study the release of aniline by pyrolysis. They obtained release of 85% of the aniline. The investigators used the Bjorkman extraction (not containing HCl) since it was believed carbohydrates and other biopolymers would not be extracted. They also felt that there was oxidation of aniline to form the ring hydroxyaniline, which would prefer carbohydrate conjugation, while the nonhydroxylated aniline would prefer lignin. Also, 3-chloroaniline (3-CA) was found to conjugate with carbohydrate faster than DCA. It was concluded that the 1,6 addition to the α–4-positions led to the aniline lignin complex with DCA and that its complex formation was faster than that of 3-CA. They also concluded that the ring-hydroxylated aniline could further couple to lignin through the phenol group reacting intramolecularly by either 1,6 or free-radical addition or both. These reactions would lead to heterocyclic structures similar to those proposed for humus (Figure 4B,C).

Arjmand and Sanderman (1985) used the v.d. Trenk procedure to polymerize [^{14}C]DCA with coniferyl alcohol, showing 41% to be incorporated yielding a synthetic DCA/lignin complex with a molecular weight range of 1100–20 000. By the action of *Phanerochaete chrysosporium*, the complex yielded 62% $^{14}CO_2$ in 33 days. Hydrolysis was studied with the results shown in Table I. Thus, the thio alcohol was about as effective as acid in releasing DCA and provided a reductive atmosphere. The extract was mainly DCA. The authors followed this study by one with [^{14}C]DCA-treated wheat grown hydroponically. They prepared "control" synthetic lignin with [^{14}C]coniferyl alcohol. By use of a dioxane/water extraction, the wheat lignin provided fragments in the molecular weight range of 600–35 000 when analyzed on a Sephadex LH60 column with DMF as the eluting solvent. The same fungus previously used on the synthetic lignin showed, in a 33-day incubation, a $^{14}CO_2$ release of 71.3% for the wheat DCA/lignin complex and of 65.4% for the synthetically polymerized [^{14}C]coniferyl alcohol. The hydrolysis data for the wheat DCA/lignin complex were similar to those of the synthetic polymer, although overall the recoveries were somewhat lower (Table I). These data indicated to the authors that the white rot fungus would mineralize the DCA/lignin complexes in the soil as effectively as lignin not containing DCA. In another paper, Arjmand and Sanderman (1986) further supported this finding. These results suggested to the investigators that synthesized

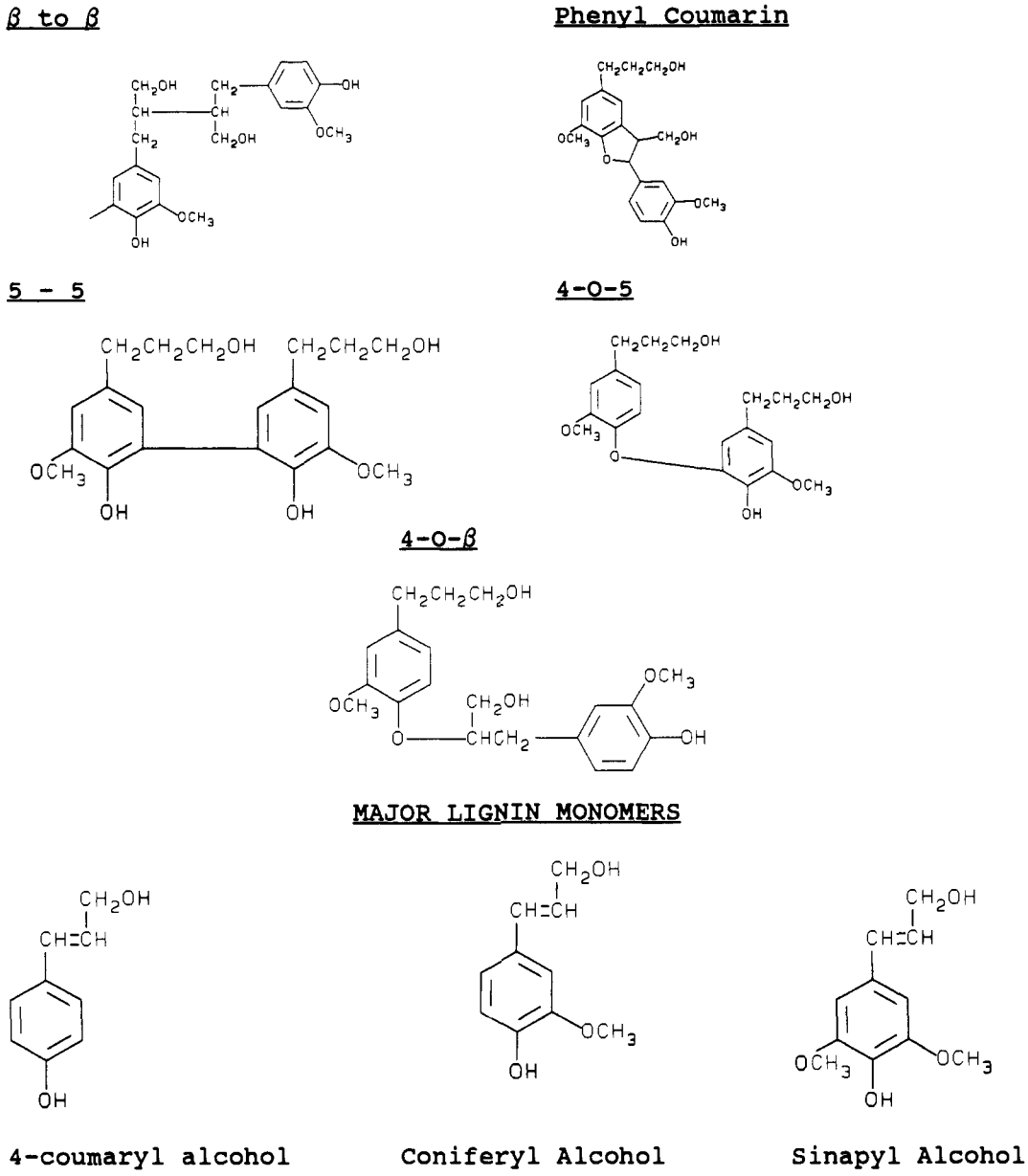

Figure 2. Linkages in lignin polymer (coniferyl alcohol as monomer).

aniline/lignin complexes, as well as the natural complexes, could be used to study aniline/lignin interactions and structures.

For investigators attempting to isolate aniline metabolites, the lignin interactions, as discussed above, can produce unique nitrogen compounds, either upon plant aging or during the isolation procedures. Such results could lead to confusion in the interpretation of the original, true aniline lignin metabolites in plants and to problems in the analysis for the original aniline.

Humus. Hsu and Bartha (1976) studied the binding of DCA to humic matter and concluded that quinone, catechol, and indamine structures in humus played a role in the binding as shown in Figure 4. They concluded that quinone adducts could be readily hydrolyzed to release DCA while the heterocyclic adducts could not. Saxena and Bartha (1983a–c) used a model adduct of DCA and tolylquinone (TQ–DCA) (Figure 4A) to compare its behavior to humic acid–DCA (HA–DCA) adducts. The TQ–DCA and HA–DCA adducts gave 64–65% release of DCA on acid hydrolysis, but with base, the model adduct released only 31% DCA vs 72% for HA–DCA. More basic amines, such as unsubstituted aniline, could displace DCA from both adducts. They indicated that these results supported the quinoid attachment site proposed by Parris (1980) and shown in Figure 4A. From the results of acid/base hydrolysis and CO_2 production, You et al. (1982) concluded that the model adduct of DCA and 4-methylcatechol (Figure 4B) did not represent a binding of DCA to humus. In a paper in *Soil Science* (Saxena and Bartha, 1983b), it was shown that anhydrous ammonia displaced DCA as readily as organic amines from freshly made HA–DCA adduct, but the displacement decreased to <1% after 105 days of incubation. Bartha (1980) reported his concept for the mechanism of pesticide attachment to humus (Figure 5). Therefore, as in the case of lignin, isolation of aniline metabolites from the bound fraction of soils can produce unexpected heterocyclics and less than expected amounts of the original aniline. Again, this can lead to confusion in the definition of soil metabolic end products and to problems in aniline analysis.

ENVIRONMENT

Soil Metabolism. Since many agrochemicals containing anilines are deposited on the soil, the fate of anilines in soil has been studied by many investigators.

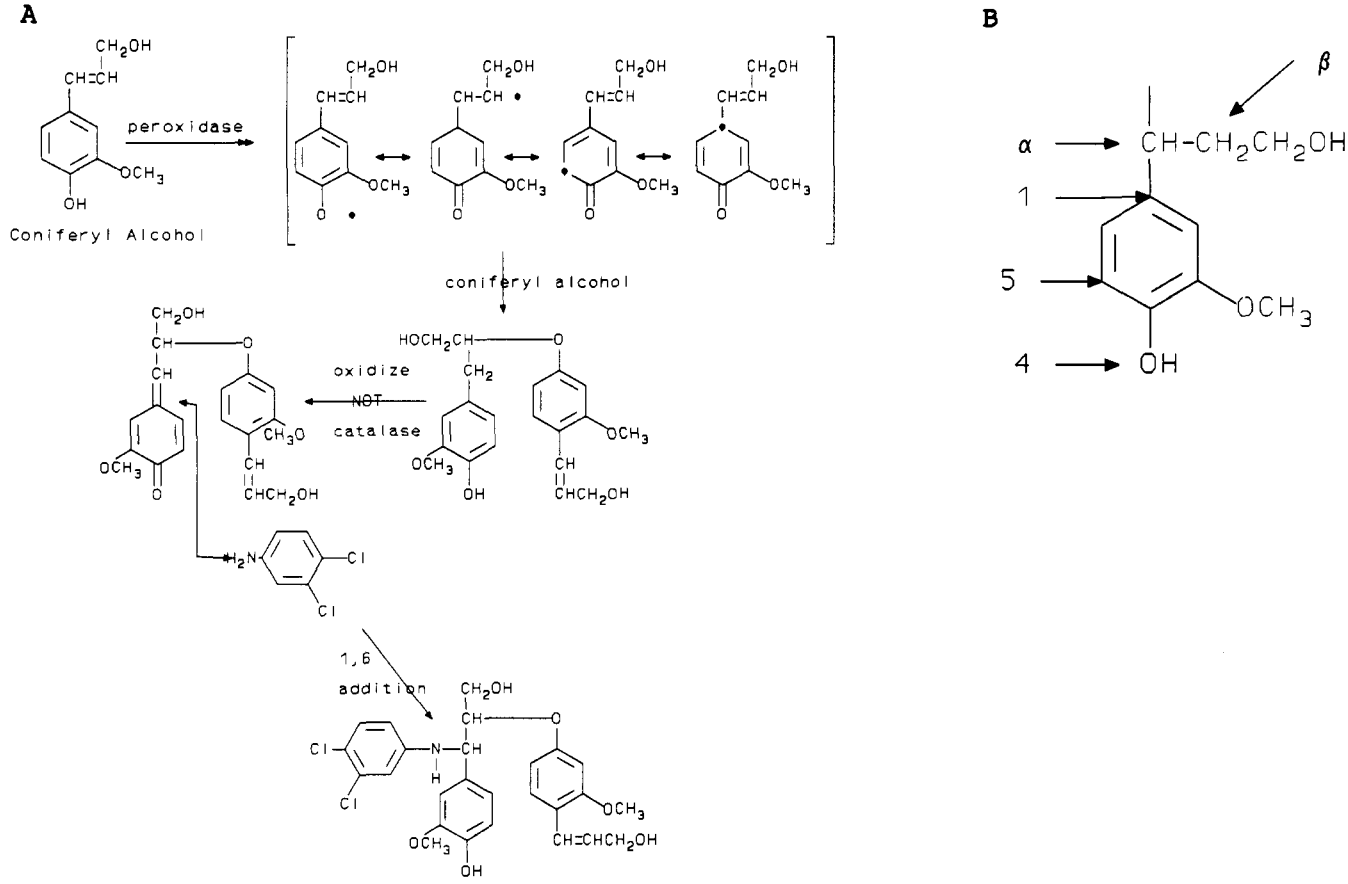

Figure 3. Free-radical sequence in lignin biosynthesis and nucleophilic aniline reaction. (A) Free radical; (B) position nomenclature.

One of the early concerns about herbicides was the release of anilines when exposed to soil and their subsequent conversion to other products, as well as translocation into plants. Lanzilotta and Pramer (1970) found a soil microbe, *Fusarium solani*, to have an acylamidase that hydrolyzed propanil to DCA. The earliest report of the transformation of DCA by soil was noted by Bartha and Pramer (1967), showing 3,3′,4,4′-tetrachloroazobenzene (TCAB) as a soil metabolite. The question of possible carcinogenicity of the azo product was raised in this paper, but no data were given or referenced. This concern led investigators to perform many studies on DCA and TCAB conversions in soils. Bartha (1968) found that the pesticide source of DCA, not just free DCA, produced the azobenzene, while *N*-alkyl substitution of the acylanilide (i.e., *N*-isopropylanilines) prevented azobenzene formation. Kearney et al. (1970) studied rice field soil repeatedly treated with propanil and found low concentrations, 0.1 ppm, of TCAB in the 0–4-in. layer with amounts decreasing with increasing depth. Linke (1970) had shown that peroxidase was involved in the generation of TCAB and a TCAB–DCA adduct (see Figure 6A for structures). Hughes and Corke (1974) found that soil at pH 4.5–5.5 gave the best yields of TCAB. Plimmer et al. (1970) found another azo metabolite, 1,3-bis(3,4-dichlorophenyl)triazene (Figure 6A), in soil and indicated the need for diazonium conversion of the amino group to the diazonium function for azo synthesis to occur (Figure 6B). Bunce et al. (1983) studied the microbial role in azobenzene formation using ^{15}N. They used *Escherichia coli*, which has nitrate reductase activity, an enzyme they felt was needed to produce nitrite from nitrate. The nitrite then diazotizes the aniline, which dimerizes to the azo compounds. Corke et al. (1979) studied 15 bacteria, and only those containing nitrate reductase produced TCAB from DCA; they found that peroxidase activity was not required. Diazonium ion did not survive above pH 6.5. Biphenyl derivatives and dechloronation were noted. Chiska and Kearney (1970) found DCA recoveries in Japanese silt loam to decrease over 105 days, and the higher the parts per million level in soil, the less was bound. TCAB plateaued in 15 days. Figure 6B shows the diazotization sequences. Other nonbound soil metabolites have been reported. Arjmand and Sanderman (1987) found that, in addition to CO_2 as an end product of DCA/lignin transformation by *P. chrysosporium*, a single major new DCA metabolite, *N*-anilinosuccinimide, was formed. Bollag and Russel (1976) found the soil bacteria, *Paracoccus* sp., to metabolize *p*-chloroaniline to an unknown volatile component with some acetanilide metabolite in anaerobic conditions, while the major metabolite under aerobic conditions was the acetanilide. Thus, in the soil, anilines can be converted to a variety of phase I, nonconjugated metabolites with DCA conversion to TCAB still of major interest.

However, as in plant lignins, anilines have the propensity to be bound to the soil biopolymer, humus. The soil fungus *P. chrysosporium* was discussed in the lignin section to compare synthesized and natural wheat lignins containing aniline. A broader view of microbial metabolism of soil-bound anilines follows. Hsu and Bartha (1973) noted that aromatic moieties (like anilines) persisted in soils and were bound to the organic rather than clay fraction. Also, microbial mineralization of the bound aromatics proceeded at a slow rate. In a later paper, Hsu and Bartha (1974) showed soil-bound DCA to yield about 1% CO_2 per week. Acid or base released only about half of the DCA added to the soil, while the nonhydrolyzable fraction increased with time. They also found different fungi to transform humus differently; that is, *Penicillium frequentans* produced humus oligomer and then monomer units, while *Aspergillus versicolor* could mineralize aniline while still attached to the humus. Reber et al. (1979) studied the

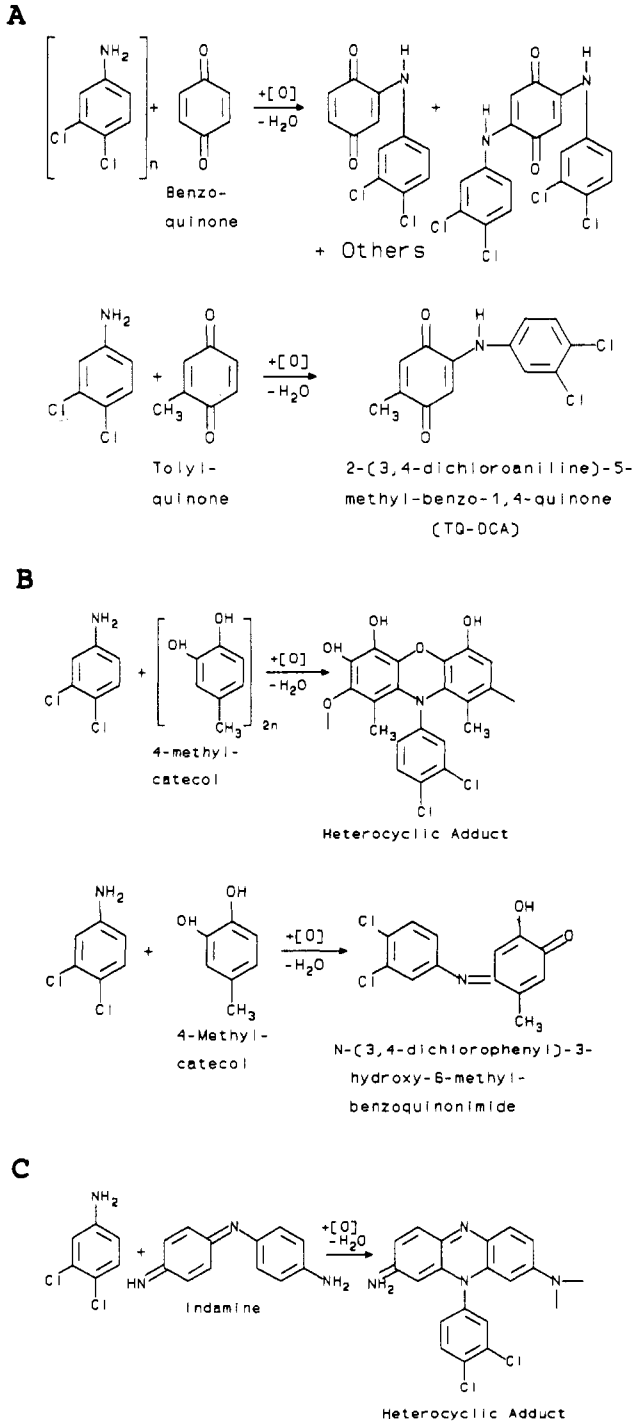

Figure 4. DCA binding reactions to humus components. (A) Quinone; (B) catechol; (C) indamine.

Table I. Hydrolysis of Synthetic and Wheat DCA/Lignin Complex

hydrolysis condition	% DCA extracted	
	synthetic	wheat
70% H_2SO_4 (sealed, 100 °C, 1 h)	92	74
50% NaOH (sealed, 100 °C, 1 h)	32	20
$HSCH_2CH_2OH$ (sealed, 190 °C, 2 h)	84	68

soil bacterium *Pseudomonas multivarans* strain A1 and found anilines to induce synthesis of aniline oxygenating enzyme to produce chlorocatechols as intermediates to CO_2 generation. The 2-chloroaniline was oxygenated fastest, and DCA was the slowest. They also indicated that chlorocatechols could undergo nonbiological oxidations. Bartha et al. (1983) showed *Pseudomonas putida*

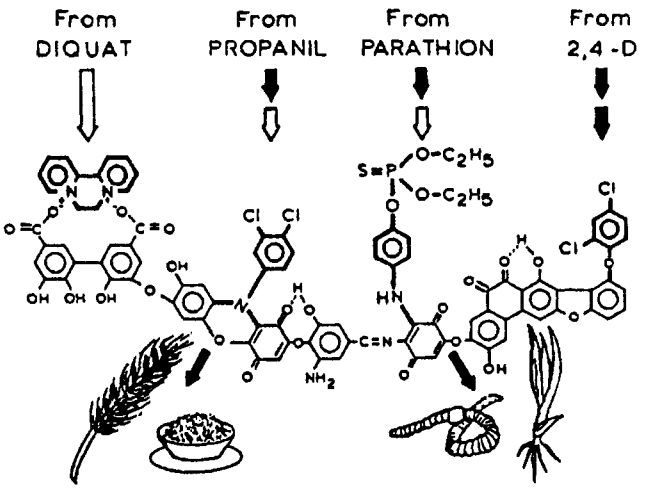

Figure 5. Proposed pesticide residue attachment to humus. Subsequent microbial mobilization of the residues may result in contamination of organisms and agricultural products. Chemical processes are represented by white arrows; enzymatic processes are represented by black arrows. The xenobiotic residues are drawn with a heavy line; the type structure of natural humus is drawn with a thin line. Chemical names of the parent compounds generating the humus-bound residues are 6,7-dihydrodipyrido-(1,2-*a*;2′,1′-*c*)pyrazidinium ion (diquat, herbicide), 3′,4′-dichloropropionanilide (propanil, herbicide), *O*,*O*-diethyl *O*-*p*-nitrophenyl phosphorothioate (parathion, insecticide), and 2,4-dichlorophenoxyacetic acid (2,4-D, herbicide). Reprinted with permission from Bartha (1980). Copyright 1980 ASM International.

to degrade DCA to 2-chlorosuccinimide and acetate with production of 4,5-dichlorocatechol as the first step (Figure 7). Zeyer and Kearney (1982) showed the soil microbe *Pseudomonas* sp. to have no growth on DCA as the sole carbon/nitrogen source, but soils supplemented with the organism and [*ring*-^{14}C]propanil produced 25–50% $^{14}CO_2$ within 5 days. Without microbe supplementation, the CO_2 generated was <1%. They gave a reference that showed DCA-treated soils to produce <11% CO_2 in 4 months. The authors suggest the low activity of catabolic enzymes on DCA, the high microbial toxicity of DCA, and the low potential of DCA to induce catabolic enzymes as reasons for the variable results of DCA catabolism in soils reported by different investigators. However, they indicated that there was a slow degradation of DCA by soil microbes. It appears that soil microbes can readily produce oxidative products of anilines but further metabolism to disrupt the ring and produce CO_2 is slow.

By using the Bleidner procedure for DCA analysis, You and Bartha (1982) studied the aging of DCA in soils. Soil treated freshly with DCA gave a recovery over 90%; by 99 days, the recovery of DCA had decreased asymptotically to about 50% of dose, while CO_2 generation increased linearly to 6.9% of the DCA dose. The poisoning of the soil with $HgCl_2$ essentially stopped CO_2 production, and the nonreleasable DCA value was similar (15%) to that in viable soil, indicating that microbial action is not required for DCA to bind irreversibly to soil. On the basis of these results and other data in their paper, the conclusion was reached that about 25% of the original DCA is converted to CO_2 per year and that half of the original DCA is releasable. Thus, the picture of aniline conversion products in soils becomes rather complex. Heterocyclic products produced in bound anilines, as well as some azo compounds, would no longer release the original anilines upon hydrolysis. The aniline recovery values, as measured by standard analytical procedures, could produce values lower than expected, again posing problems for the analyst.

Another concern of soil residues is the possibility of

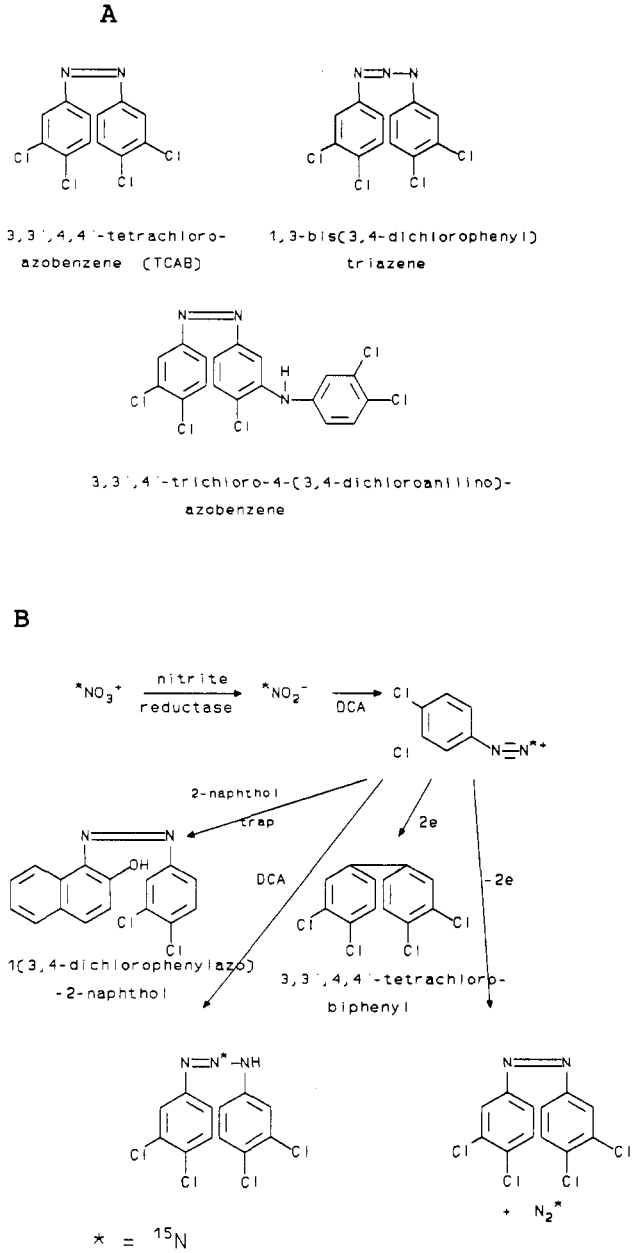

Figure 6. (A) Soil azo metabolites. (B) Diazotization sequence in soil.

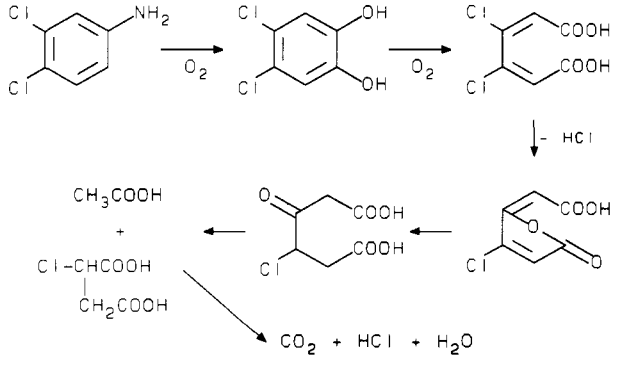

Figure 7. Catechol intermediate in DCA mineralization.

their transfer to crops grown in those soils. Still (1969) found that [^{14}C]TCAB was poorly transferred to rice plants from nutrient solution with 5.2% absorbed in roots and only 3.2% translocated to the shoots. The translocated ^{14}C was shown to be TCAB, but neither propanil nor DCA, when absorbed into rice plants, was converted to TCAB by plants. Still and Mansager (1969), upon analysis of commercial rice grain, found that the rice DCA was contaminated with DCA and concluded that the source was from DCA bound in the soil. Additional support for this concept came from work by Still et al. (1980) in which rice grain from foliage or soil treated with [^{14}C]propanil showed only the soil treatment to give grain residues. The structures of these grain residues were not determined. In a paper in *Soil Science*, Saxena and Bartha (1983b) showed that anhydrous ammonia displaced DCA from a HA–DCA adduct (a humus model) and suggested that fertilization with anhydrous ammonia can mobilize soil-bound aniline which crops could translocate to produce contamination. They also showed that anaerobic conditions would reduce the binding of DCA to humus. These studies show the ability of chloroanilines but little, if any, TCAB to be translocated into plants grown in soils containing either the free or bound anilines or TCAB.

ECOSYSTEMS

Only a few of the published studies are given here to show how ecological biological systems can handle the anilines.

Isensee et al. (1982) studied the fate of DCA in a rice paddy environment using a laboratory glass microecosystem containing rice, soil, water, and aquatic organisms (daphnid, fish, snails, and algae). The soil was treated with 10 ppm of DCA, rice planted, and the system flooded at the 2-leaf rice stage. After flooding, the aquatic organisms were added. A maximum of 2.8% of the DCA from soil desorbed into the water. By 30 days, the water in the tank soil contained 5% of the original ^{14}C with 22% of the water ^{14}C as DCA and the remaining activity as polar metabolites (acetanilide, formanilide, and unknowns). A second experiment, not designed to study leaching, lasted 60 days, but by the 15th day, only 69% of the ^{14}C dosed was recovered with 83% nonextractable by chloroform/methanol/water. This compared to a field trial at a DCA level of 1.2 ppm showing 69% recovered after 1 year. In both experiments, rice plants (above the roots) showed that, of the <0.5% uptake of the [^{14}C]DCA in the soil, 35–50% was MeOH/H_2O extracted, indicating more polar metabolites in rice plants than in soil. For aquatic organisms, ^{14}C accumulated in the order of algae > fish > snails > daphnids, with 25 cpm/mg in algae representing about 340× the water level. The metabolism of DCA by the biological systems was not discussed. Wyndham (1986) studied river water microbes such as *Acinetobacter* and found that aniline was metabolized preferably to catechol and then cleaved similarly to the pathway shown in Figure 7. The author also proposed that the microbes could adapt upon aniline exposure.

BIOLOGY

Plant Metabolism. Some of the earliest studies on the metabolism of anilines in plants were by Still (1968) and Yih et al. (1968) concerning the fate in rice plants of propanil (3,4-dichloropropionanilide) labeled with [^{14}C]-phenyl. In the plants, both investigators found propanil to readily hydrolyze to the free aniline (DCA), which then conjugated to carbohydrates as the earliest metabolites. One identified metabolite was *N*-(3,4-dichlorophenyl)glucosamine. However, by 21 days after treatment, the free aniline and the methanol-soluble metabolites decreased to <10% of the plant radioactivity. In a more recent study (Winkler and Sanderman, 1989), chlorinated anilines produced *N*-glucosyl and *N*-malonyl conjugates in plants. However, the pesticide, its released aniline, and simple aniline conjugates are short-lived in plants with nonex-

Table II. Biopolymer Distribution in Plants Treated with [^{14}C]DCA

	% of ^{14}C in tissue		
	soil treated		cell culture
	tomato	corn	wheat
bound	5.6	32.4	31.0
starch	37.2	11.4	3.4
protein	20.1	15.6	3.4
pectin	14.6	27.4	20.9
lignin	9.2	32.9	52.1
hemicellulose	7.8	4.8	11.5
cellulose	3.4	3.9	0.2
unknown	3.8	2.6	0.2

tractable metabolites increasing with age to become the major constituent.

Therefore, the metabolism of anilines into plant biopolymers has been of major interest, because most of the metabolized aniline resides there, because of the challenge in the definition of the structures produced, and because of the difficulty in analysis for the bound anilines. To present the typical aniline distribution observed in plant cell fractions, two recent papers will be reviewed that define the distribution for DCA, especially in biopolymers. Pawlizki (1988) studied the distribution in tomatoes and corn grown in soil treated with DCA, while Harms and Langebartles (1988) studied wheat cell cultures exposed to DCA. Pawlizki used the same natural product fractionation procedures as Harms. The distributions found are shown in Table II. The highly rigid plants like corn and wheat have high levels of lignin, and the majority of the bound ^{14}C was in the lignin. Tomato had the majority of its ^{14}C in the starch fraction. A contributing factor to the observed high starch values in tomatoes could be aniline encapsulation into starch, as was discussed under Carbohydrates.

Ensilage. Since pesticide-treated crops can be ensilaged for animal feeding, several investigators have studied the fate of anilines and pesticides in this system.

Lyons et al. (1985) studied a variety of anilines, including DCA, in the reductive environment of the ensilage process. Chopped corn plants were inoculated with dilute soil suspension and raw milk. The activated corn along with six anilines and their six respective pesticides were placed in glass jars and incubated for 2 weeks at 28 °C in an anaerobic chamber using an atmosphere of 92% N_2/3% H_2/5% CO_2. The samples were analyzed according to the You and Bartha (1982) improvement of the Bleidner et al. (1954) procedure for anilines. Within 2 h of DCA treatment, 88% of the DCA was recovered by exhaustive ethyl acetate extraction. An additional 7% was recovered by use of the Bleidner procedure and was considered reversibly bound. By 14 days, the nonrecoverable, irreversibly bound DCA was 38% of dose. Of the extractable ^{14}C at 14 days, the major components were the DCA acetanilide and the DCA propionalide. Thus, ensilage fermentations lead to acylation and binding as the main transformation processes with some of the bound aniline not releasable upon hydrolysis.

Animal Toxicology. This section is not meant to provide an in-depth review of aniline toxicology. The intent is to indicate why "free" vs "bound" anilines can be of toxicological concern. In the past, it was though that bound anilines were not digestible and had no toxicological significance. However, as noted above, some DCA is releasable from the bound form, at least by base hydrolysis. Therefore, the concern of DCA's reaction with natural products is the possible DCA release by animal systems. As noted below, DCA itself can have toxicological consequences.

Singleton and Murphy (1973) showed that a sublethal dose of DCA administered interperitoneally to male mice produced substantial methemoglobinemia. Chow and Murphy (1975) showed rats, mice, and guinea pigs to have acylamidase activity in liver (as plants and microbes had) to hydrolyze propanil to DCA. Whole liver homogenates, a NaDPH generating system, and hemoglobin from heparinized rat blood served as the system incubated with DCA. Activity was measured by the quantity of methemoglobin formed. The liver formation of active methemoglobin metabolites was in the order mice > guine pig ≫ male rat > female rat and was related to the amount of *N*-hydroxy-DCA produced. Rashid and Arjmand (1987) studied 16 anilines and succinic acid conjugates of anilines (including DCA) for mutagenic activity in *Salmonella typhimurium* strains TA98 and TA100. None of the compounds were mutagens or promutagens in this assay. McMillan et al. (1988) tested propanil, DCA, and their *N*-hydroxy derivatives. None of these compounds were mutagenic on the basis of the *S. typhimurium* reversion assay (TA97, TA98, TA100, and TA104) with or without metabolic activation. The compounds were not mutagenic in the Chinese hamster ovary/hypoxanthine guanine phosphoribosyl transferase assay in the absence and presence of S9. Both TCAB and its azoxy form, TCAOB, were inactive in these tests. None of the compounds induced DNA damage. However, hepatocyte toxicity, as measured by the release of lactate dehydrogenase, was induced by all of the compounds. Incubation of *N*-hydroxy-DCA with DNA showed a low level of binding.

Few bioavailability studies have been done to observe possible absorption of plant nonextractable aniline residues by animals. In one study, rice plants 12 weeks after [^{14}C]propanil soil treatment were extracted with chloroform/methanol/water, and the resulting nonextractable radioactive plant material was dosed to rats (Sutherland, 1976). In two trials 76.1% and 78.3% of the dose was found in feces with 2.4% and 6.5% found in urine. In an additional test with bile cannulation, <0.5% of nonextractable rice plant material appeared in the bile of rats or dogs. Sanderman et al. (1990) fed rats enzymatically prepared ring-U-^{14}C-labeled chloroaniline/lignin complex containing either 4-CA or DCA. It was found that 62% and 68% were bioavailable for 4-CA and DCA, respectively. In a later study (Musick et al., 1991), a biosynthetically produced [^{14}C]DCA/lignin complex was fed to rats and lambs. On the basis of urine, bile, and fecal soluble radioactivity, only 11–14% of the fed radioactivity was bioavailable in rats and almost 16% in lambs. In contrast to the earlier study, low bioavailability was found. These results indicate that use of biopolymer models can produce controversial data which need careful evaluation and interpretation.

Thus, while free aniline, when ingested, can be of toxicological concern, the bound aniline does not seem to be readily released and appears to be minimally bioavailable to animals.

ANALYSIS

This section is intended to show the status in the analysis of releasable DCA that has interacted with natural products, especially biopolymers. This should be considered a guide to analyzing DCA complexed with natural products and not a methods manual. One of the major problems in the analysis of anilines in biological samples is the possible re-reaction of the hydrolytically released aniline with natural product components, such as sugars, simultaneously released from the matrix itself. Bleidner

(1954) introduced a process to minimize this interaction by using a simultaneous hydrolysis/steam distillation/solvent extraction procedure. This procedure is applicable to all biological matrices, but that specific for humic acid in soils is presented next.

You and Bartha (1982) studied the effectiveness of the Bleidner distillation process for recovering DCA from humic complexes. The yield of DCA was improved by increasing the alkali concentration to 12.5 N NaOH with a distillation run time increased to 23 h. Before alkali hydrolysis, the soils were cold extracted with acetone and glacial acetic acid added to the extract which was concentrated in a rotary evaporator to 5 mL (*not* to dryness). The concentrate was made alkaline and extracted with isooctane, which was analyzed for parent pesticide and free DCA. Any azo compound in the soil should also be in this fraction. The extracted soil was further processed according to the Bleidner procedure using isooctane as the organic extractant. Both isooctane extracts were analyzed by gas chromatography. The gas chromatographic procedure quoted by You and Bartha (1982), and later updated (Bartha, 1989, private communication), used a Model MT 200 Tracor instrument equipped with a ^{63}Ni electron capture detector and a nonpolar fused silica open tubular (FSOT) siloxane capillary column using either RSL-150 [poly(dimethylsiloxane)] or RSL-300 [poly(phenylmethylsiloxane)]. Operating conditions were as follows: gas (CH_4–Ar, 95:5); carrier flow, 90 mL/min; oven temperature, 200 °C; detector temperature, 250 °C. Under these conditions, DCA had a retention time of 1.46 min.

The Bleidner type procedure for the analysis of anilines in biological samples remains a widely used method for residue analysis. The procedure is satisfactory for most matrices containing natural products and biopolymers.

Should there be an interest in TCAB content of a sample such as soil, an organic solvent extraction, such as acetone, followed by evaporation to dryness and re-extraction with hexane prepares the sample for gas chromatography. Chiska and Kearney (1970) and Hughes and Corke (1974) give the details for the extraction and chromatography of TCAB.

Except for radioactive analyses when [*ring*-^{14}C]aniline is used, no residue analytical procedure, to date, accounts for all of the aniline applied to plants or soil.

REVIEWS

In addition to the papers mentioned thus far, several pertinent review papers are noted for obtaining a broader discussion of some of the above topics. Bartha and Pramer (1970) give a good review for soils, microbes, and plants as known to that date. Bartha (1980) and Bartha et al. (1983) update the understanding of DCA binding in humus. The schematic noted in Figure 5 comes from these references. While not new, the book by Pearl (1967) is still a good source of methodology for use in lignin investigations.

CONCLUSIONS

While the biochemical release of anilines by acylamidase from parent pesticides is relatively straightforward, the chemico-physico-biological interactions of the freed anilines with natural products make the understanding of the metabolic consequences of anilines in biological systems extremely complex. For chloroanilines like DCA and CA, oxygen, light, heat, enzymes, reactive functional groups, and acid/base environments can produce artifacts of the true metabolites during the extraction and processing procedures.

In the case of carbohydrate conjugation in plants, which involves *N*-glycosyl linkage at the aldehyde carbon, different spatial isomers (α or β), ring isomers (pyranose or furanose), and rearrangements (aldose or ketose) are all possible reactions that can occur even during the fractionation of the biological sample and metabolite isolation/purification procedures. The *true* unaltered carbohydrate metabolite, based on the investigations to date, is considered the β-D-glycosylamine, that is, conjugation at the C-1 aldehydic carbon of glucose. Any other structures can be considered artifacts and will serve to confuse metabolite identification and metabolite pathway interpretation. The possibility of aniline encapsulation into starch should be considered. Mild procedures should be used to loosen the starch matrix and release anilines, yet prevent their reattachment to carbohydrate entities.

In the case of lignin conjugation in plants, the main linkage of anilines is considered to be at the α-carbon next to the phenyl ring of the propylphenyl monomeric structure of lignin, that is, a quinone methide conjugate. Steps leading to isolation of lignin, before its fragmentation, can generate artifacts by oxidation or acid/base activation. Disruption of the lignin leads to readily oxidizable fragments that can alter the aniline/lignin binding site, possibly releasing moieties that are not the original aniline. In the case of DCA, the "aged" DCA/lignin complexes from plants have possible heterocyclic structures caused by a second reaction at the amino or ring hydroxyl groups. The original chloroaniline moiety can be altered and not released as chloroaniline. The releasable chloroaniline is considered to exist as the quinone methide conjugate. Thus, procedures that exclude oxygen should be used for isolating aniline monomeric units in lignins to minimize the generation of artifacts.

In the case of aniline conjugation with soil humus, the main linkage is considered to be at the ortho position of the quinone ring. Similar concerns are noted for fragmentation of soil humus complexes as for lignin complexes since disruption of the humus complex can also lead to readily oxidizable fragments. Aging effects are similar in lignin and in humus, leading to heterocyclic structures that do not release the original chloroaniline upon hydrolysis. Soil microbes appear to catabolize DCA to CO_2 through a catechol intermediate. The major noncomplexed DCA metabolites, i.e., phase 1 metabolites, were several azo compounds, with 3,3′,4,4′-tetrachloroazobenzene (TCAB) the most prevalent one.

For ecosystems containing soil, water, rice, and aquatic organisms, soil binding was noted as seen in terrestrial soils. Water microbes generate anilides as the major metabolites. For the aquatic organisms, DCA accumulated in the order algae > fish > snails > daphnids. Their metabolites were not investigated.

In DCA distribution studies for plants, tomato, corn, and wheat culture cells were studied using the same tissue fractionation techniques. The decreasing order of nonextracted DCA was corn > wheat > tomato. The amount of bound material found in starch was in the decreasing order tomato > corn ≫ wheat. For the lignin fraction, the decreasing order was wheat > corn ≫ tomato.

In ensilage studies, the major DCA metabolites were acetanilide and propionalide. By the 14th day of incubation, 38% of the DCA was irreversibly bound, that is, not extracted by cold organic solvent or Bleidner hydrolysis.

In animals, the major toxicological effect of DCA is methemoglobinemia. All DCA metabolites, including *N*-hydroxy derivatives and TCAB, were nonmutagenic.

A few studies show that bound aniline residues are minimally bioavailable to animals.

In the analysis of biological samples, the Bleidner procedure, which combines base hydrolysis, steam distillation, and simultaneous organic solvent extraction, is still the preferred general method for DCA determinations. The advantage of the procedure is the removal of DCA as it is released to prevent reattachment of the freed aniline with reactive fragments of natural products produced during hydrolysis. The frequently used analytical equipment is gas chromatography using capillary columns and NPD or MS detectors. However, except when [*ring*-^{14}C]-anilines are used, no analytical methods, to date, appear to account for all of the aniline applied to plants and soils.

For the investigator studying the plant and soil metabolism of products containing chloroanilines, the definition of metabolic end products requires careful experimental design strategies and constant critical evaluation of results. The propensity of chloroanilines to accumulate in the bound fractions imposes use of judiciously chosen biopolymer disruption procedures and well-planned sophisticated isolation and purification techniques.

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