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Fate of Herbicide-derived Aniline Residues During Ensilage*

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Microbial and plant metabolism of acylanilide, phenylurea, phenycarbamate and nitroaniline herbicides produce free and bound aniline residues (Herrett, 1969; Geissbühler, 1969; Bartha and Pramer, 1970; Cripps and Roberts, 1978). When formed in soil, aniline residues are efficiently absorbed by plant roots and are subsequently translocated into the shoot (Still, 1969). The preservation of forage crops by the ensilage process provides an additional opportunity for microbial transformations of plant-associated aniline residues. Katz et al. (1970) found that 3-(4-bromo-3-chloropheny)-1-methoxy-1-methylurea (chlorobromuron) disappeared within a week in laboratory ensilage studies. The authors concluded that the 4-bromo-3-chloroaniline moiety was either degraded or modified in a manner to prevent its recovery by the Bleidner procedure (Bleidner et al. 1954). The objective here was to study these as yet unexplored transformations that may alter the toxicity and bioavailability of the aniline residues in forage.

The selected test compounds were aniline, 3-chloroaniline (3-CA), 4-chloroaniline (4-CA), 3,4-dichloroaniline (3,4-DCA), 2,6-diethylaniline (2,6-DEA) and 2,6-dinitro-4-trifluoromethyl-aniline (2,6-DNTFA). Examples of parent herbicides for these anilines are isopropyl-carbanilate (propham), isopropyl-m-chlorocarbanilate (chlorpropham), 3-p-chlorophenyl)1,1-dimethylurea (monuron), 3',4'-dichloropropionanilide (propanil), 2-chloro-2',6'-diethyl-N-(methoxymethyl) acetanilide (alachlor) and α,α,α-trifluoro-2,6-dinitro-N,N-dipropyl-p-toluidine (trifluralin), respectively, (Weed Science Society of America, 1979). The aim of these investigations was to explore the type and range of the transformations that may take place in the reductive environment of the ensilage process and to relate these to aniline structure. It is not implied that all of the above herbicides are used on forage crops, nor that the aniline concentrations employed in our experiments would result from their use at recommended rates.

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MATERIALS AND METHODS

Anilines and substituted anilines were purchased from Aldrich Chemical Co. (Milwaukee, WI) and were purified to 98% or better by distillation and recrystallization from petroleum ether. The acylanilide standards formanilide, acetanilide, 3-chloroacetanilide, 4-chloroacetanilide, 3,4-dichloroformanilide, and 3,4-dichloroacetanilide were purchased from the same source. Uniformly ^{14}C-labeled 3,4-dichloroaniline (specific activity 61 µCi/mg) was purchased from Amersham-Searle (Des Plaines, IL). As determined by thin layer chromatography using toluene:ethylacetate (9:1) on 500 µm silica gel plates (Fisher Scientific Co., Pittsburg, PA), its radiochemical purity was 97%. The solvents used were pesticide quality.

To provide a continuous supply of material for experiments, freshly harvested young corn plants (Zea mays) chopped for silage were sealed in plastic bags and stored frozen. Microbial activity in the thawed corn samples was restored using a small inoculum of dilute soil suspension and fresh raw milk. The test anilines (3 mg per sample) were added dissolved in 0.5 mL absolute ethanol. The chopped corn was packed to capacity in 40 mL screw-cap glass jars (Wheaton, Millville, NJ). The caps were secured with tape and each jar was jacketed with a 150 mL beaker to contain any sample overflow due to fermentation gases. Unsupplemented blanks and steam-sterilized controls (121°C, 25 min) were included; for the latter the anilines were filter-sterilized using 0.2 µm pore size Teflon membranes (Gelman Instrument Co., Ann Arbor, MI). For each sampling point, jars were prepared in duplicates. The silage samples were incubated for 2 weeks at 28°C in an anaerobic chamber (Lab-line Instruments, Melrose Park, IL) using a gas mixture of 92% N_2, 3% H_2 and 5% CO_2.

At appropriate time intervals, the entire contents of the jars were homogenized with 200 mL ethyl acetate for 2 min in a Waring Blendor, transferred to the thimble of a Soxhlet apparatus and extracted for 3 h. The extract was made alkaline with 10 mL 1 N NaOH and steam-distilled for 10 min. The anilines were collected in a receiving flask containing 1 mL 1N HCl. The distillate was made alkaline again with 2 mL 1N NaOH, the water and solvent phases were separated, the solvent was dried with anhydrous Na_2SO_4 and concentrated on a steam bath to 1.0 mL for gas chromatography. Unknowns are represented by their retention times (RT) and their quantity was approximated by peak area.

To liberate reversibly bound anilines, the extracted silage solids were subjected to Bleidner distillation (Bleidner et al., 1954; You and Bartha, 1982b) for 3 h using 12.5 N NaOH and isooctane. The solvent phase was cleaned up with steam distillation and concentrated for gas chromatography as described.

To obtain recovery estimates and a complete materials balance, 4 mg ^{14}C-labeled 3,4-DCA (9.5×10^4 dpm) was added to ensilage samples. Incubation and extraction was as described, but the recovered radioactivity was measured by liquid scintillation counting in Econofluor (New England Nuclear, Boston, MA) using a Beckman Model LS-230 instrument (Irvine, CA) after each extraction and cleanup step. Radioactivity remaining in the silage solids after Bleidner distillation (irreversibly bound residues) was determined after wet combustion (Allison et al., 1965) and trapping of the evolved CO_2 in Oxifluor (N.E.N.). Gas chromatographic analysis of residual anilines and their metabolites was performed as described elsewhere (Lyons et al., 1984). At each sampling time, a small amount of the free liquid was removed from the spiked silage and filtered through 0.2 μm pore size membranes; 0.1 mL of this liquid was added to each agar plate in the Ames test to determine the mutagenic potential (Maron and Ames, 1983). Details of the Ames test procedure as applied in this study were described by Lyons et al. (1985).

RESULTS AND DISCUSSION

Total recovery of radiolabeled 3,4-DCA during a 2-week sampling period declined from 100% on day 0 to 87% on day 14 (average of duplicate samples). The lowest single recovery value was 80%. The above values included recovery of $^{14}CO_2$ by wet combustion. Consequently, a maximum analytical loss of 20% was assumed also for the other aniline compounds, although the average loss was probably around 5%. This assumption was necessary because conventional recovery measurements were affected by the relatively rapid and, in part, irreversible binding of the anilines to plant constituents, primarily lignins (Yih et al., 1968; Sanderman et al., 1983). The 0 day (recovery) samples were extracted within 2 h of spiking. At this time, only 88% of the added 3,4-DCA radioactivity was recoverable by exhaustive solvent extraction. Of the missing radioactivity, 7% was recovered by Bleidner distillation (reversible binding) but 5% could be recovered only as $^{14}CO_2$ after wet combustion (irreversible binding). The amount of irreversibly bound 3,4-DCA gradually increased with the time of incubation from 5% on day 0 to 38% on day 14. Since the actual amounts of irreversibly bound residues in case of the non-radiolabeled anilines could not be determined, the total amounts not recovered were listed. By projection from the measurements on radiolabeled 3,4-DCA, the anilines "not recovered" include an average of 5% and a maximum of 20% analytical loss. Recoveries lower than the aforementioned values are interpreted as evidence for irreversible binding.

The fate of the six anilines during 14 days of simulated silage fermentation is summarized in Table 1. All six compounds were metabolized during two weeks of silage fermentation, but to different extents. As judged by the amount of unchanged parent compound in biologically active samples after 14 days, the

Table 1. Fate of aniline residues during two weeks of ensilage of corn (Zea mays)[1]

Compound	Days of incubation					
	0	3	7	10	14	14[2]
Aniline	28	4	6	6	5	0
Formanilide	0	37	67	59	43	0
Propionanilide	0	6	14	12	7	0
Bleidner recovery	58	39	9	11	19	79
Not recovered	14	14	4	4	26	21
3-CA	52	-	15	16	16	30
3-Chloroacetanilide	0	-	14	14	8	0
3-Chloropropionanilide	0	-	30	25	7	0
Bleidner recovery	25	-	18	21	23	39
Not recovered	23	-	34	34	46	31
4-CA	40	-	28	34	36	29
RT 13.2 min	0	-	21	26	22	0
4-Chloroacetanilide	0	-	40	36	28	0
Bleidner recovery	21	-	7	3	11	37
Not recovered	39	-	4	1	3	34
3,4-DCA	88	76	20	8	9	58
3,4-Dichloroacetanilide	0	0	11	26	16	0
3,4-Dichloropropionanilide	0	0	10	12	5	0
Bleidner recovery	7	18	19	21	19	16
Not recovered	5	6	40	33	51	26
2,6-DEA	77	8	6	10	7	40
RT 6.5 min	0	7	2	9	4	0
RT 17.9 min	0	4	5	5	6	0
RT 27.1 min	0	21	27	27	30	0
Bleidner recovery	3	31	39	27	36	38
Not recovered	20	29	21	22	17	22
2,6-DNTFA	39	-	38	25	49	49
RT 7.9 min	0	-	8	16	8	0
RT 22.0 min	10	-	42	44	5	0
RT 25.0 min	19	-	12	15	15	0
Bleidner recovery	18	-	7	4	16	22
Not recovered	14	-	0	0	7	29

[1]All numbers are in percentage of the originally added aniline and represent the average of duplicate determinations. "Bleidner recovery" designates bound parent anilines that resisted solvent extraction but were released intact by Bleidner distillation. "Not recovered" included analytical losses to a maximum of 20%.
[2]Sterile controls.

compounds were metabolized in decreasing order as follows: aniline > 2,6-DEA > 3,4-DCA > 3-CA > 4-CA > 2,6-DNTFA. Persistence of the parent compounds was generally higher in sterilized corn silage, but extensive binding, both reversible and irreversible, occurred also in these samples. Binding was often higher in the sterile than in the biologically active samples, probably because of a lack of competing metabolic transformation processes. Remobilization of reversibly bound anilines by microorganisms offers another plausible explanation.

In biologically active samples, all compounds were transformed in part to solvent-extractable metabolites. No such products were detected in the sterile controls. In case of aniline, 3-CA, 4-CA and 3,4-DCA, all the identified metabolites were acylanilides. They were not detected on day 0, achieved a maximum concentration between 3 and 10 days, and declined by day 14. In the case of aniline, formanilide and propionanilide were positively identified but acetanilide was not detected. 3-CA was acylated to 3-chloroacetanilide and 3-chloropropionanilide. 4-CA was acylated to 3-chloroacetanilide and 3-chloropropionanilide. 4-CA was transformed to 4-chloroacetanilide and RT 13.2 min; the latter probably represents 4-chloroformanilide. 3,4-DCA was transformed to 3,4-dichloroacetanilide and 3,4-dichloropropionanilide.

Of the unidentified metabolites formed from 2,6-DEA, RT 6.5 min and RT 7.19 min were formed in rather low amounts. RT 27.1 min was a major metabolite but behaved differently from the acyl products inasmuch as it showed no decline at day 14. Of the unidentified 2,6-DNTFA products, RT 7.9 min exhibited synthesis-disappearance patterns similar to the acyl products, but RT 22.0 min and RT 25.0 min behaved atypically in the respect that they were detected already in the 0-day sample. They may represent conjugates or products of nitro group reduction, but are probably not acyl products. Both reversible and irreversible binding was low for 4-CA and 2,6-DNTFA, higher for aniline and 2,6-DEA, and very high for 3-CA and 3,4-DCA.

The identity of the three extractable metabolites produced from each of 2,6-DEA and 2,6-DNTFA is not known at this time. Although 2,6-DEA showed considerable mutagenic potential in the Ames test (Lyons et al., this issue) the extractable metabolite mixtures of 2,6-DEA and 2,6-DNTFA showed no significant mutagenic potential with or without activation by S-9 microsomal liver enzymes (data not presented). With the exception of 2,6-DEA, only marginal mutagenic potential was exhibited by the anilines studied (Lyons et al., 1985). Assays for mutagenic activity on the metabolites formed during ensilage fermentation were negative (data not presented).

Acylation, the major transformation pathway for aniline, 3-CA, 4-CA and 3,4-DCA during ensilage fermentation, was previously reported to occur in other microbially active environments.

Specifically, Tweedy et al. (1970) and Kearney and Plimmer (1972) reported the acetylation of 4-bromoaniline and 3,4-DCA in soil environments. Lyons et al. (1984) reported acetylation and, to a lesser degree, formylation of aniline in pond water. Acylation was also observed repeatedly in pure culture studies. 4-CA was acetylated by Fusarium oxysporum (Kaufman et al., 1973), 3,4-DCA by Pseudomonas putida (You and Bartha, 1982a). Russel and Bollag (1977) reported acetylation and formylation of 4-CA in culture of a Streptomyces sp. Bollag and Russel (1976) found acetylation to be the major transformation pathway for aniline, 2-chloroaniline, 3-CA and 4-CA in cultures of Paracoccus. All the above reports pertained to essentially aerobic systems. Bollag and Russel (1976) found acylation to be low in cultures of Paracoccus incubated under anaerobic conditions. The cited papers interpret the acylations of anilines as reversible reactions that have the effect of reducing the toxicity of excess substrate. As aniline concentrations drop due to mineralization and binding, the acyl metabolites are cleaved and utilized. The maximum amount of total acylated residues appears to decline with a decreasing electron density on the amino group in the order to aniline > 4-CA > 3-CA > 3,4-DCA. On the other hand, total binding (reversible plus irreversible) is highest for the least reactive 3-CA and 3,4-DCA, a phenomenon probably explainable by competition between the acylation and binding processes. At this time, there is no convincing explanation as to why certain anilines seemed to select for acyl moieties of certain carbon numbers. It is possible that the anilines influenced the relative amounts of the fatty acids formed in the silage fermentation, but this parameter was not investigated in the present study.

The overall pattern of aniline metabolism during ensilage fermentation is towards detoxification via acylation and binding. At least some of the binding appears to be irreversible, leading to a reduction of aniline bioavailability. The identity of the metabolites formed from 2,6-DEA and 2,6-DNTFA has not been determined, but the extractable metabolite mixtures were not mutagenic in the Ames test, while intact 2,6-DEA exhibited considerable mutagenic potential in the same test. Therefore, the unknown transformation products of 2,6-DEA and 2,6-DNTFA produced during ensilage fermentation also may be regarded as products of detoxification reactions.

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