Microbial Oxidation of 4-Chloroaniline

Donald D. Kaufman,* Jack R. Plimmer, and Ute I. Klingebiel

4-Chloroaniline was degraded through at least two metabolic pathways by the soil fungus *Fusarium oxysporum* Schlecht. Oxidation of the aromatic amine group was the major mechanism of 4-chloroaniline metabolism, but acylation of the aromatic amine group also occurred. 4-Chlorophenylhydroxylamine, 4-chloronitrosobenzene, 4-chloronitrobenzene, 4,4′-dichloroazobenzene, 4,4′-dichloroazoxybenzene, and 4′-chloroacetanilide were isolated as metabolites of 4-chloroaniline. The identity of several of these metabolites was established by comparison of their chemical and physical properties with synthetic samples. It is probable that 4,4′-dichloroazoxybenzene was formed by oxidative condensation of two molecules of 4-chlorophenylhydroxylamine or by the condensation of 4-chlorophenylhydroxylamine with 4-chloronitrosobenzene. 4,4′-Dichloroazobenzene could be formed by either the condensation of 4-chloroaniline with 4-chloronitrosobenzene or by reduction of 4,4′-dichloroazoxybenzene. Further oxidation of 4-chloroaniline and its products to phenolic metabolites and the liberation of Cl^- were also observed.

The aniline moiety constitutes a basic part of numerous pesticides. These pesticides are nearly all degraded in the environment, with the ultimate liberation of the aniline as a free entity. Subsequent degradation of this moiety is not well understood. Mechanisms of chloroaniline transformation in soil and chemical systems have been examined by numerous investigators. Both degradative and synthetic reactions have been reported. Evolution of small amounts of $^{14}CO_2$ from soils receiving ^{14}C-phenyl-labeled 3′,4′-dichloropropionanilide (propanil) suggests that some degradation of the aniline moiety occurs (Chisaka and Kearney, 1970). Condensation reactions resulting in the formation of both symmetrical and asymmetrical azobenzenes have been reported (Bartha and Pramer, 1967; Bartha, 1969; Kearney *et al.*, 1969; Plimmer *et al.*, 1970). The condensation of three molecules of 3,4-dichloroaniline to form 4-(3,4-dichloroanilino)-3,3′,4′-tetrachloroazobenzene has also been reported (Rosen *et al.*, 1969). We reported the formation of 3,3′,4,4′-tetrachloroazoxybenzene during the metabolism of 3,4-dichloroaniline by the soil fungus *Fusarium oxysporum* Schlecht (Kaufman *et al.*, 1971, 1972a).

The formation of azobenzenes and anilinoazobenzenes *in vitro* has been examined with the aid of horseradish peroxidase (Bartha *et al.*, 1968; Bordeleau *et al.*, 1972; Knowles *et al.*, 1969; Lieb and Still, 1969). The involvement of several labile intermediates in the formation of these compounds has been indicated (Bordeleau *et al.*, 1970, 1972; Neuberg and Wilde, 1914; Neuberg and Reinfurth, 1923; Taylor and Baker, 1942). In our investigations we suggested that the isolation of 3,3′,4,4′-tetrachloroazoxybenzene provided indirect evidence for the presence of 3,4-dichlorophenylhydroxylamine and 3,4-dichloronitrosobenzene (Kaufman *et al.*, 1972a). The present paper describes the detection, isolation, and identification of labile intermediates produced during the initial stages of metabolism of 4-chloroaniline by the soil fungus *Fusarium oxysporum* Schlecht.

EXPERIMENTAL SECTION

Synthesis of 4-Chloroaniline Metabolites. 4-Chlorophenylhydroxylamine was prepared by a modification of the method for phenylhydroxylamine (Vogel, 1956). Ethanol-water (50:50) was used as solvent for the zinc dust reduction of 4-chloronitrobenzene; no cooling was necessary and the product crystallized from the filtrate which was poured onto crushed ice. The compound appeared pure on thin-layer chromatography or on silica gel HF-254 coated plates using benzene–ethyl acetate (4:1) as the solvent.

4-Chloronitrosobenzene was prepared by acidified sodium dichromate oxidation of 4-chlorophenylhydroxylamine (Vogel, 1956). The product was purified by steam distillation and recrystallized from ethanol. The purity of the compound was checked by thin-layer and gas–liquid chromatography. 4,4′-Dichloroazobenzene was synthesized by reacting the 4-chloronitrosobenzene with 4-chloroaniline. All compounds had the expected mass spectra. Melting points of 4-chlorophenylhydroxylamine and 4-chloronitrosobenzene were comparable to those reported in the literature (Bordeleau *et al.*, 1972; Farrow and Ingold, 1924; Ingold, 1925).

4-Chloroaniline, 4′-chloroacetanilide, and 4,4′-dichloroazoxybenzene were obtained from Eastman Organic Chemicals, and 4-chloronitrobenzene was obtained from Aldrich Chemical Co., Inc.

Detection of 4-Chloroaniline Metabolites. 4-Chloroaniline was fed to actively growing cells of *Fusarium oxysporum* Schlecht mass cultured in 1-l. quantities in 2-l. flasks on a gyrorotary shaker. The nutrient medium contained 0.2 g of K_2HPO_4, 0.3 g of NH_4NO_3, 0.2 g of $CaSO_4$, 0.2 g of $MgSO_4 \cdot 7H_2O$, 1 mg of $FeSO_4 \cdot 7H_2O$, 2.0 g of sucrose, 0.1 g of yeast extract, and 25 mg of streptomycin sulfate per 1000 ml of distilled water. 4-Chloroaniline (50 mg/l.) was added to the sterilized medium in 0.1 ml of acetone. One milliliter of a spore suspension of *F. oxysporum* was added and the culture was incubated for 8 days at 24°. Duplicate flasks containing the inoculum without 4-chloroaniline and 4-chloroaniline without inoculum were maintained as controls.

A 10-ml sample was removed daily from each flask and centrifuged to remove suspended fungal cells. Two-milliliter subsamples of the centrifugate were assayed for the presence of 4-chloroaniline according to the procedure of Pease (1962), chloride ion by the procedure of Iwasaki *et al.* (1952), and phenols by a modification of the procedure of Casida and Augustinsson (1959). 4-Chlorophenylhydroxylamine production was detected by the trisodium pentacyanoammine ferroate complex (Boyland and Nery, 1964). The development of a magenta color with the addition of 4 drops of a 0.2% (w/v) aqueous trisodium pentacyanoammine ferroate solution to 1 ml of the centrifugate was indicative of the presence of the 4-chlorophenylhydroxylamine–trisodium pentacyanoammine ferroate complex. The mixture was allowed to stand for 20 min to achieve full color development and then diluted to 6 ml with distilled water and analyzed spectrophotometrically at 540 nm with a Bausch & Lomb Spectronic 20 spectrophotometer.

Since nitrosobenzenes also form a similar complex with

Agricultural Environmental Quality Institute, Agricultural Research Center, Agricultural Research Service, U. S. Department of Agriculture, Beltsville, Maryland 20705.

trisodium pentacyanoammine ferroate (Bordeleau *et al.*, 1972; Boyland and Nery, 1964) it was necessary to correct the 4-chlorophenylhydroxylamine values obtained for the presence of 4-chloronitrosobenzene. 4-Chloronitrosobenzene was detected by reaction of 1 ml of the centrifugate with 0.5 ml of 5% (w/v) aqueous hydroxylamine hydrochloride followed by 0.5 ml of 5% (w/v) aqueous *N*-1-naphthylethylenediamine dihydrochloride and 4 ml of distilled water. A reddish-brown color developed immediately in the presence of 4-chloronitrosobenzene. This test appeared to be fairly specific for the nitrosobenzene, as it did not react with either 4-chloroaniline or 4-chloronitrobenzene. A color reaction with 4-chlorophenylhydroxylamine developed only after standing 15–20 min. All 4-chloronitrosobenzene determinations were made spectrophotometrically at 500 nm within 5–10 min after addition of the reagents. The color produced was stable for that period of time and followed Beer's law.

At the conclusion of the incubation period, the medium was extracted successively with 300 ml each of ethyl ether and ethyl acetate. The extracts were combined, dried over anhydrous magnesium sulfate, filtered, and evaporated to dryness in a rotary evaporator. The residues were examined by both tlc and gc analyses. Tlc solvent systems used were hexane–benzene–acetone (7:3:1) and benzene–dioxane–acetic acid (90:25:4). 4-Chloroaniline metabolites were visualized on the plates by their uv absorption, and by various spray reactions. 4-Chlorophenylhydroxylamine and 4-chloronitrosobenzene were visualized on the plates by spraying aqueous sodium pentacyanoammine ferroate solution. 4-Chloroaniline and 4′-chloroacetanilide were visualized by first exposing the tlc plates to nitrous oxide fumes in a closed container for 15–20 min, and then spraying with a 0.5% (w/v) *N*-1-naphthylethylenediamine dihydrochloride in ethanol. Phenolic materials were detected on tlc plates by spraying with the phenol reagents described above.

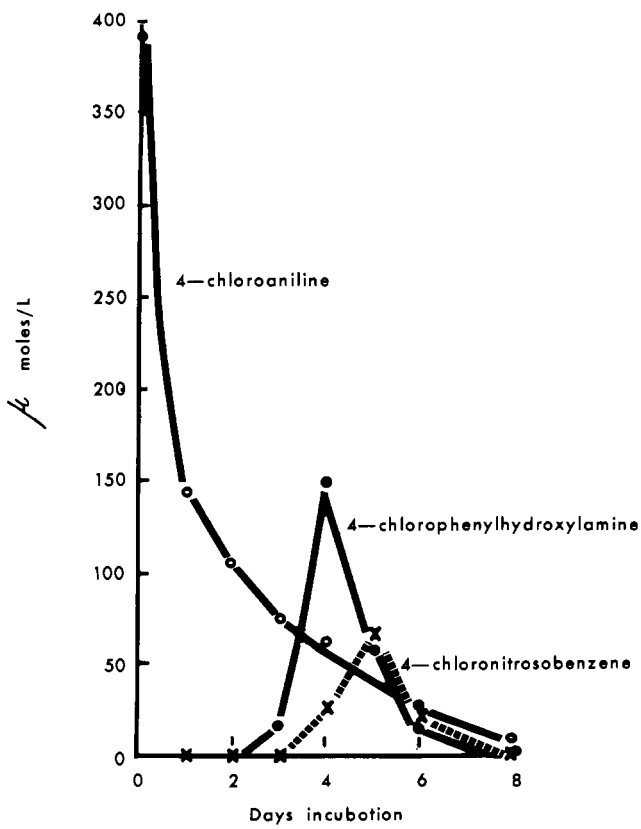

Figure 1. Metabolism of 4-chloroaniline, and formation and subsequent metabolism of 4-chlorophenylhydroxylamine and 4-chloronitrosobenzene.

Gas chromatographic analyses were performed with an F&M Model 700 gas chromatograph with a 6-ft stainless steel column (⅛ in. i.d.) containing UCC-W-982 methylvinyl silicone gum rubber on diatoport S 80–100 mesh and equipped with a flame ionization detector. The carrier gas (N_2) flow rate was 40 ml/min. Injection port and detector temperatures were 270 and 310°, respectively. Column temperatures were 130, 180, and 250°, isothermal.

Isolation of 4-Chlorophenylhydroxylamine. 4-Chlorophenylhydroxylamine was isolated and characterized as a complex with trisodium pentacyanoammine ferroate. Fifty milligrams of sodium pentacyanoammine ferroate were added to 1 l. of filtrate from a 3-day-old culture of *F. oxysporum* growing in nutrient medium containing 4-chloroaniline. A magenta water-soluble complex was formed. The filtrate was then evaporated to dryness in a rotary evaporator and the residue was washed twice with ether to remove other less polar compounds. The trisodium pentacyanoammine ferroate–4-chlorophenylhydroxylamine complex was then dissolved in methanol and concentrated for tlc comparison with the complex prepared with authentic 4-chlorophenylhydroxylamine. Two solvent systems were used: isopropyl alcohol–H_2O–ammonia (30:10:2), and methanol–H_2O–ammonia (30:10:2) on Brinkman silica gel HF_{254} plates. The absorption spectra of the two complexes in methanol were compared using a Cary 15 recording spectrophotometer.

Metabolism of 4-Chlorophenylhydroxylamine and 4-Chloronitrosobenzene. Three-day-old cultures of *F. oxysporum* were used to examine the short term metabolism of 4-chlorophenylhydroxylamine and 4-chloronitrosobenzene. Prior to use in these studies the cultures were initiated and incubated in the presence of 4-chloroaniline as described previously. After 3 days' incubation, the cells were harvested by filtration on Whatman No. 1 filter paper and washed twice with 250 ml of mineral salts solution (medium minus yeast extract, sucrose, and 4-chloroaniline). The cells originating from each liter of culture medium were then resuspended in 100 ml of fresh mineral salts medium containing 200 μmol/l. of 4-chlorophenylhydroxylamine or 4-chloronitrosobenzene and incubated on a gyrorotary shaker at 24°. Samples were removed at hourly intervals, centrifuged, and assayed for residual 4-chlorophenylhydroxylamine and 4-chloronitrosobenzene. At the conclusion of an 8-hr incubation period, the medium was also assayed for chloride ion, 4-chloroaniline, and phenol content. The remaining medium was then extracted as described previously and the residues were submitted to gc and tlc examination.

RESULTS AND DISCUSSION

4-Chloroaniline Metabolism. 4-Chloroaniline was rapidly degraded by growing cells of *F. oxysporum* (Figure 1). 4-Chlorophenylhydroxylamine reached detectable levels within 3 days of incubation and accumulated to a concentration of 149 μmol/l. before beginning to disappear. 4-Chloronitrosobenzene formation lagged behind 4-chlorophenylhydroxylamine formation and reached a peak 1 day later than 4-chlorophenylhydroxylamine. At its point of maximum accumulation the 4-chlorophenylhydroxylamine concentration was approximately 38% of that theoretically possible. In other experiments the 4-chlorophenylhydroxylamine concentration occasionally reached as high as 76% of that theoretically possible. This observation would indicate that oxidation of the aromatic amine moiety is a major pathway for the degradation of 4-chloroaniline by *F. oxysporum*.

Numerous metabolites were extracted from the culture medium at the conclusion of the 8-day incubation period. Identifiable metabolites included 4,4′-dichloroazoxybenzene, 4,4′-dichloroazobenzene, 4-chlorophenylhydroxylam-

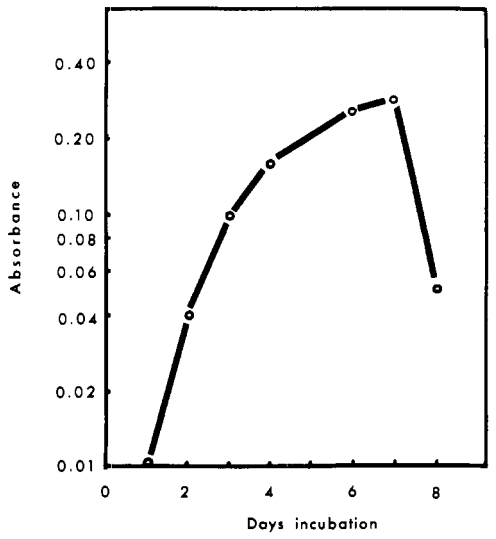

Figure 2. Production and disappearance of phenolic metabolites during 4-chloroaniline metabolism.

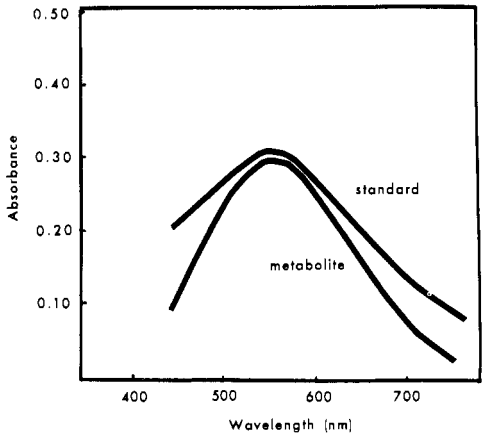

Figure 3. Absorption spectra of trisodium pentacyanoammine ferroate complex of 4-chlorophenylhydroxylamine standard and metabolite.

ine, 4-chloronitrosobenzene, 4-chloronitrobenzene, and 4-chloroacetanilide, in addition to the parent 4-chloroaniline (Table I). 4-Chlorophenylhydroxylamine and 4-chloronitrosobenzene could both be visualized as magenta spots on developed tlc plates sprayed with aqueous trisodium pentacyanoammine ferroate solution. 4′-Chloroacetanilide and residual 4-chloroaniline spots were visualized on tlc plates by diazotization and spraying with a coupling reagent. All of these products had tlc R_f values or gc retention times corresponding to those of known standards (Table I; tlc R_f data comparable to Figure 5).

Chloride ion was detected during 4-chloroaniline metabolism, with as much as 98% of the organically bound Cl^- being liberated after incubation periods of 10–12 days. Phenolic materials (Figure 2) were also detected during 4-chloroaniline metabolism. Several types of phenolic metabolites could be produced from 4-chloroaniline; *e.g.*, aminophenols, nitrophenols, or chlorophenols, and at least three chemically distinct phenolic metabolites were detected on thin-layer chromatograms of 4-chloroaniline culture extracts. One of these has been tentatively identified as 2-chloro-4-nitrophenol. The presence of this compound would imply migration of the chlorine substituent. Further identification of this product and other phenolic metabolites will be the subject of subsequent reports.

Identification of 4-Chlorophenylhydroxylamine. Trace amounts of 4-chlorophenylhydroxylamine were readily detectable in culture extracts by tlc and gc methods. 4-Chlorophenylhydroxylamine is an unstable compound and readily decomposes in solution or in the solid state (indeed, 4,4′-dichloroazoxybenzene, its major chemical decomposition product, is often obtained as the sole product during the preparation of 4-chlorophenylhydroxylamine). Due to the lability of the 4-chlorophenylhydroxylamine, we were unable to extract sufficient amounts of unchanged material for more complete chemical characterization. Therefore, it was isolated as a complex of trisodium pentacyanoammine ferroate. This permitted identification by comparison with a complex prepared with authentic material. Uv absorption spectra of the two were identical (Figure 3). R_f values of the standard and metabolite 4-chlorophenylhydroxylamine complexes were 0.38 and 0.38 in isopropyl alcohol–water–ammonia (30:12:1), and 0.91 and 0.90 in methanol–water–ammonia (30:12:1), respectively.

Metabolism of 4-Chlorophenylhydroxylamine and 4-Chloronitrosobenzene. Both 4-chlorophenylhydroxylamine and 4-chloronitrosobenzene were readily metabolized by cells of *F. oxysporum* when provided as a sole substrate (Figure 4). 4-Chlorophenylhydroxylamine was degraded almost completely within the 8-hr incubation period. 4-Chloronitrosobenzene was also readily degraded. Measure-

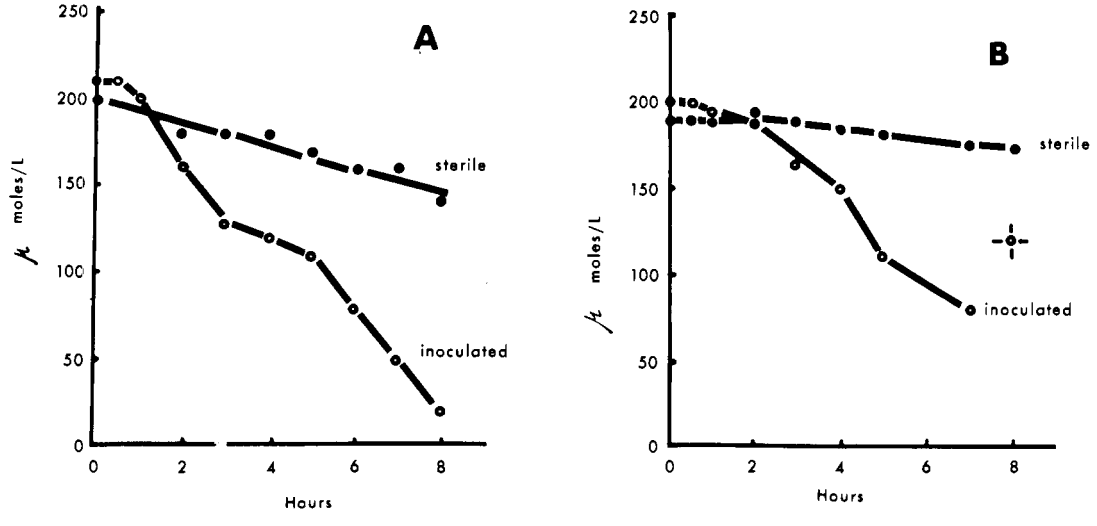

Figure 4. Metabolism of 4-chlorophenylhydroxylamine (A) and 4-chloronitrosobenzene (B).

Table I. Relative Gas Chromatographic Retention Times (min) of Standard Chemicals and 4-Chloroaniline Metabolites

	Column temperatures, °C					
	130°		180°		250°	
Chemical identity	Standard	Unknown	Standard	Unknown	Standard	Unknown
4-Chloronitrobenzene	2.1	2.0	0.5	0.5		
4-Chloroaniline	1.8	1.8	0.4	0.4		
4-Chlorophenylhydroxylamine	1.0	1.0				
4-Chloronitrosobenzene	0.9	0.9				
4′-Chloroacetanilide			2.1	2.2		
4,4′-Dichloroazoxybenzene					1.5	1.5
4,4′-Dichloroazobenzene					1.0	1.0

ment of the rapid degradation of this compound, however, was somewhat obscured by the increased presence of a metabolite which also formed a trisodium pentacyanoammine ferroate complex. Although the complex produced by this metabolite was distinctly different in color (blue, as opposed to magenta), its presence interfered with measurement of the rate of 4-chloronitrosobenzene metabolism. This was particularly noticeable in the last sample assay, which produced a higher reading than the preceding one. A similar color was also present but barely perceptible in 4-chlorophenylhydroxylamine cultures at the end of the incubation period. Although the chemical nature of this complex is not presently known, Boyland and Nery (1964) observed various shades of green, red, magenta, and bluish-purple among ferroate complexes with arylhydroxylamines and arylamines.

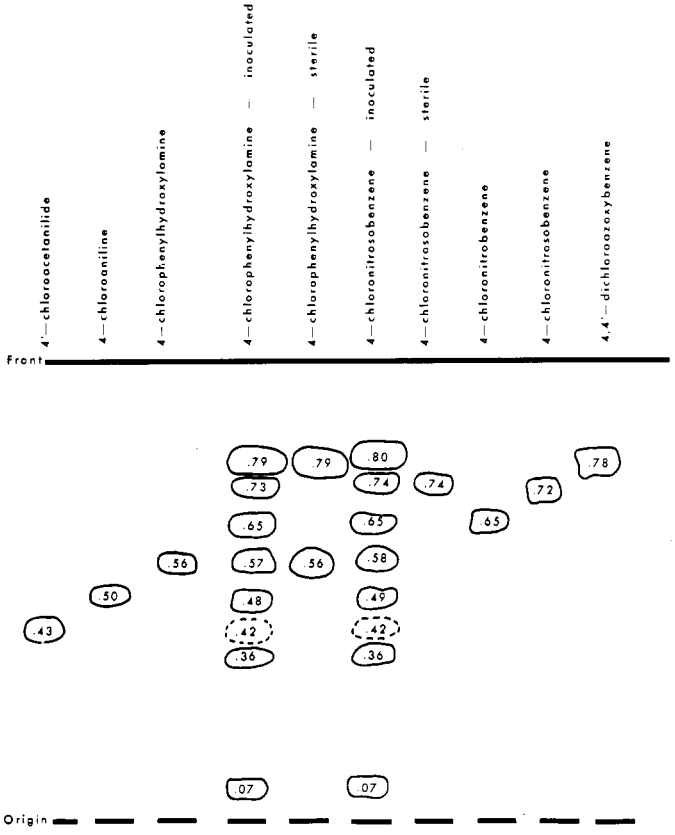

Figure 5. Composite of tlc plates illustrating separation and identification of standards and metabolites of 4-chlorophenylhydroxylamine and 4-chloronitrosobenzene. Solid circles indicate uv detected spots; dotted circles indicate additional spots detected after spraying. Numbers indicate R_f values. Solvent system: benzene–dioxane–acetic acid, 90:25:4.

Degradation of 4-chlorophenylhydroxylamine occurred in both sterile and inoculated culture solutions (Figure 4). 4,4′-Dichloroazoxybenzene was the only product in sterile culture solutions. The lability of phenylhydroxylamines and their condensation to azoxybenzenes is well known (Taylor and Baker, 1942). 4-Chloronitrosobenzene appeared to be stable under the experimental conditions employed. Analyses of inoculated culture extracts revealed that 4-chlorophenylhydroxylamine and 4-chloronitrosobenzene were metabolized to identical products (Figure 5). The relative distribution of these products was only partially determined. Approximately 10% (20 μmol) of the 4-chlorophenylhydroxylamine remained in solution at the conclusion of the incubation period. It was not possible to accurately quantitate the residual 4-chloronitrosobenzene for the reasons discussed above. Chloride ion and 4-chloroaniline were among the metabolites detected from both compounds. Sixty-three percent of the organically bound chloride was liberated as chloride ion from 4-chloronitrosobenzene, whereas 48% was liberated from 4-chlorophenylhydroxylamine. 4-Chloroaniline accounted for 1.8% of the 4-chloronitrosobenzene and 2.4% of the 4-chlorophenylhydroxylamine products. A positive phenolic reaction was also obtained with samples of the inoculated culture medium at the conclusion of these experiments. Again the exact nature of these phenols has not been determined. However, they appear identical to those obtained from 4-chloroaniline culture extracts. These products are represented by at least three unknowns, including the two most polar compounds illustrated in Figure 5. Both of these compounds react positively to the phenol-detecting reagents used for tlc spraying, but not to the aromatic amine or sodium pentacyanoammine ferroate sprays.

The ability of heterotrophic soil microorganisms to oxidize ammonium N or amino N to nitrite is well known. The detection of hydroxylamine-type intermediates in such conversions has been observed in microorganisms (Gunner, 1963) and animals (Booth and Boyland, 1964) and chemical reactions (Bordeleau *et al.*, 1972). We recently reported the isolation and characterization of 3,3′,4,4′-tetrachloroazoxybenzene from culture solutions of *F. oxysporum*, which was metabolizing 3,4-dichloroaniline (Kaufman *et al.*, 1972a). We suggested that isolation of this compound provided indirect evidence for the presence of 3,4-dichlorophenylhydroxylamine alone or in combination with 3,4-dichloronitrosobenzene. Similar conclusions were reported earlier by Neuberg and Wilde (1914) and Neuberg and Reinfurth (1923). Arylhydroxylamines may condense with aromatic amines and form azobenzene compounds (Taylor and Baker, 1942). Azoxybenzenes are formed through the condensation of two molecules of the arylhydroxylamine, by condensation of the arylhydroxylamine with a nitrosoaryl compound, or by oxidation of the azo compound. Powerful chemical oxidants are usually

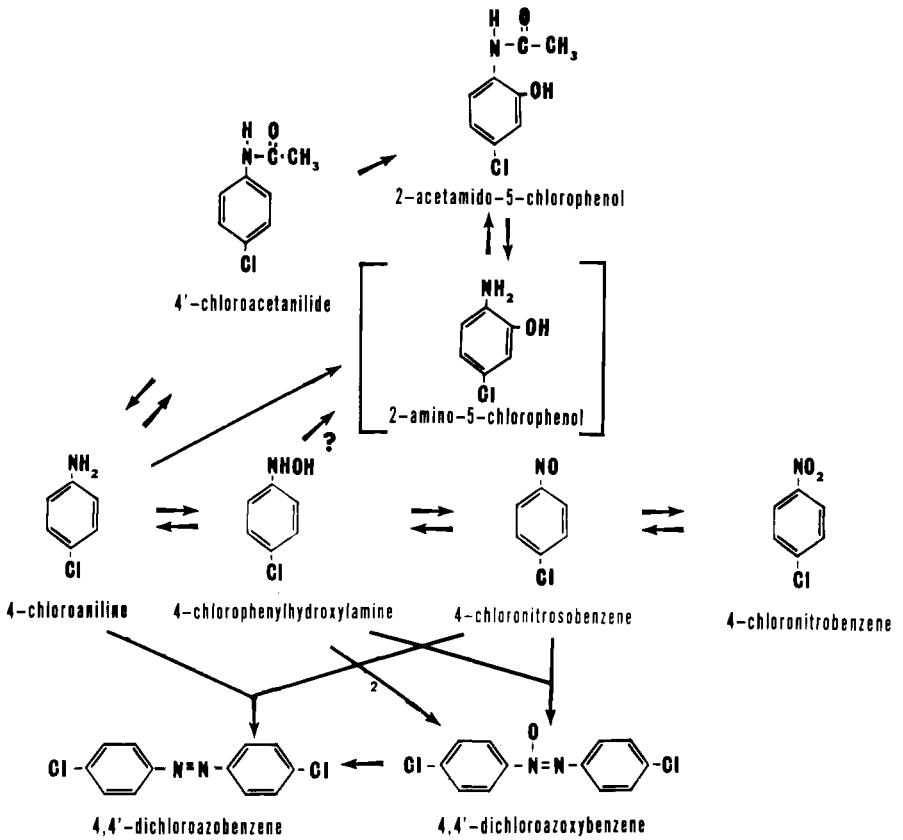

Figure 6. Metabolic scheme for degradation of 4-chloroaniline.

needed to oxidize azo compounds to the azoxy compounds. Thus, the presence of an azoxy compound may be interpreted as indirect evidence for the presence of at least the arylhydroxylamine and quite possibly a nitrosoaryl compound.

The results of our present investigation confirm that the metabolism of 4-chloroaniline indeed involves the oxidation of the amine group and the formation of 4-chlorophenylhydroxylamine, and that this compound is further metabolized to 4-chloronitrosobenzene and 4-chloronitrobenzene (Figure 6). The presence of 4,4'-dichloroazoxybenzene in culture extracts adds additional but indirect evidence for the presence of 4-chlorophenylhydroxylamine and 4-chloronitrosobenzene or both. N hydroxylation thus appears to be a major pathway for the oxidation of chloroanilines by this soil microorganism. Similar results have been obtained with 3-chloroaniline, 3,4-dichloroaniline, and 3-chloro-4-toluidine.

The process of acylation provides an alternative pathway for the degradation of chloroanilines. Direct gas chromatographic–mass spectrographic analysis of culture extracts in previous investigations revealed the presence of both acetylated and formylated chloroanilines (Kaufman *et al.*, 1971, 1972b). In the extracts examined in this investigation only 4'-chloroacetanilide was detected. Comparison of 4'-chloroacetanilide concentrations detected in culture extracts by gc analyses with those of known standards indicated that approximately 2.9% of the parent aniline was present as 4'-chloroacetanilide. Acylanilides are readily hydrolyzed under alkaline conditions. Total 4-chloroaniline determinations on hydrolyzed and unhydrolyzed samples of the culture solution at the conclusion of the 8-day incubation period confirmed the presence of 3.8% of the parent aniline as hydrolyzable product(s).

Subsequent metabolism of the acylanilide may proceed by either hydrolysis or hydroxylation. Hydrolysis of 4'-chloroacetanilide would result in the formation of acetate and 4-chloroaniline, thus returning the aniline to the metabolic pool. In earlier investigations we observed the formation of *o*-hydroxylated aniline derivatives (Kaufman *et al.*, 1972b). The presence of *o*-hydroxylated derivatives in the mixture of metabolic products can be demonstrated by gas chromatography and mass spectrometry. The mass spectral fragmentation pattern of the *N*-acyl derivatives of *o*-hydroxychloroanilines can be interpreted by the existence of an ortho elimination process characteristic of this structural type (Still, 1971). Whether hydroxylation of the ring occurs directly or by rearrangement of an *N*-hydroxyacylanilide compound is not known at this time. Detection of 4'-chloroacetanilide suggests that hydroxylation may follow acylation.

Some stages of the chloroaniline metabolic pathways

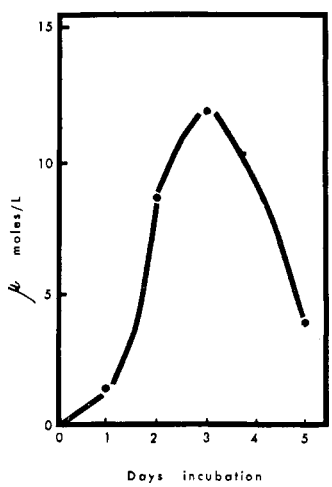

Figure 7. Formation and degradation of 4-chloroaniline from 4-chloronitrobenzene.

appear to be reversible. Chloroanilines are readily produced during the metabolism of halogenated formyl- and acetanilides by *F. oxysporum* (Kaufman *et al.*, 1972c). In identical experiments with 4-chloronitrobenzene as a substrate, we have observed the production of small amounts (3–4%) of 4-chloroaniline (Figure 7). 3,4-Dichloroaniline was also detected as a metabolite of 3,4-dichloronitrobenzene (Kaufman *et al.*, 1972a). The reduction of nitro aryl compounds to the corresponding arylamine by *F. oxysporum* (Madhosingh, 1961) and other microorganisms (Chacko *et al.*, 1936) has also been observed by others. The apparent reversibility of these reactions has made stoichiometric measurement of individual metabolic reactions difficult. Although such a dynamic relationship exists among the various metabolites, in experiments with ^{14}C-phenyl-labeled anilines the percentage of labeled carbon that can no longer be extracted from the aqueous medium increases with the length of incubation (Kaufman *et al.*, 1972b). Studies are continuing to characterize the microbial production and degradation of water-soluble metabolites of chloroanilines.

LITERATURE CITED

Bartha, R., *Science* **166**, 1299 (1969).
Bartha, R., Pramer, D., *Science* **156**, 1617 (1967).
Bartha, R., Linke, H. A. B., Pramer, D., *Science* **161**, 582 (1968).
Booth, J., Boyland, E., *Biochem. J.* **91**, 362 (1964).
Bordeleau, L. M., Bartha, R., *Bull. Environ. Contam. Toxicol.* **5**, 34 (1970).
Bordeleau, L. M., Rosen, J. D., Bartha, R., *J. Agr. Food Chem.* **20**, 573 (1972).
Boyland, E., Nery, R., *Analyst* **89**, 95 (1964).
Casida, J. E., Augustinsson; I., *Biochim. Biophys. Acta* **36**, 411 (1959).
Chacko, C. I., Lockwood, J. L., Zabik, M. L., *Science* **154**, 893 (1966).
Chisaka, H., Kearney, P. C., *J. Agr. Food Chem.* **18**, 854 (1970).
Farrow, M. D., Ingold, C. K., *J. Chem. Soc. London* **125**, 2543 (1924).
Gunner, H. B., *Nature (London)* **197**, 1127 (1963).
Ingold, C. K., *J. Chem. Soc. London* **127**, 513 (1925).
Iwasaki, I., Utsumi, S., Ozawa, T., *Bull. Chem. Soc. Jap.* **25**, 226 (1952).
Kaufman, D. D., Plimmer, J. R., Iwan, J., 162nd National Meeting of the American Chemical Society, Washington, D. C., September 1971.
Kaufman, D. D., Plimmer, J. R., Iwan, J., Klingebiel, U. I., *J. Agr. Food Chem.* **20**, 916 (1972a).
Kaufman, D. D., Plimmer, J. R., Iwan, J., Klingebiel, U. I., 163rd National Meeting of the American Chemical Society, Boston, Mass., April 1972b.
Kaufman, D. D., Plimmer, J. R., Iwan, J., Klingebiel, U. I., unpublished data, 1972c.
Kearney, P. C., Plimmer, J. R., Guardia, F. B., *J. Agr. Food Chem.* **17**, 1418 (1969).
Knowles, C. O., Gupta, A. K. S., Hassan, T. K., *J. Econ. Entomol.* **62**, 411 (1969).
Lieb, H. B., Still, C. C., *Plant Physiol.* **44**, 1672 (1969).
Madhosingh, C., *Can. J. Microbiol.* **7**, 553 (1961).
Neuberg, C., Reinfurth, E., *Biochem. Z.* **139**, 561 (1923).
Neuberg, C., Wilde, E., *Biochem. Z.* **67**, 18 (1914).
Pease, H. L., *J. Agr. Food Chem.* **10**, 279 (1962).
Plimmer, J. R., Kearney, P. C., Chisaka, H., Yount, J. B., Klingebiel, U. I., *J. Agr. Food Chem.* **18**, 859 (1970).
Rosen, J. D., Siewerski, M., Winnett, G., Abstr 24, Division of Pesticide Chemistry, 158th National Meeting of the American Chemical Society, New York, N. Y., September 1969.
Still, G. G., *Org. Mass Spectrom.* **5**, 977 (1971).
Taylor, T. W. J., Baker, M. A., "Sidgwick's Organic Chemistry of Nitrogen," Oxford, 1942.
Vogel, A. I., "A Textbook of Practical Organic Chemistry," Longmans, Green and Co., New York, N. Y., 1956.

Received for review June 16, 1972. Accepted October 12, 1972.

COMMUNICATIONS

Insecticidal and Anticholinesterase Activity of Benzotriazolyl Methyl and Dimethylcarbamates

A new series of heterocyclic carbamates based on 1*H*-1-benzotriazol have been synthesized and evaluated as insecticides and as inhibitors of fly and bovine cholinesterases. Only in the dimethylcarbamates do regression analyses show high correlation between cholinesterase inhibition and chemical reactivity of the inhibitors as expressed by Hammett's σ constant. Anticholinesterase activity of the monomethylcarbamates increases and that of the dimethylcarbamates decreases with an increase in the σ value of the substituents.

Several groups of heterocyclic carbamate esters have been shown to possess insecticidal properties. Weismann *et al.* (1951), followed by Gysin (1954), were the first to study the pyrazolyl dimethylcarbamates. Their extensive work led to the development of insecticides such as isolan and dimetilan. In a search for safe and hydrolytically stable carbamates, the same group developed, later on, the hydroxyquinoline derivatives such as the 2-methylquinolyl-8 methylcarbamate (Gubler *et al.*, 1968). Kilsheimer *et al.* (1969) describe yet another class of heterocyclic methylcarbamates based on benzothiophene; preeminent among these is benzo[*b*]thien-4-yl methylcarbamate (Mobam). This communication presents data on a new group of heterocyclic carbamates with interesting anticholinesterase properties.

EXPERIMENTAL SECTION

Chemicals. The benzotriazolyl dimethylcarbamates were prepared by reaction of the corresponding 1-hydroxybenzotriazoles (0.05 mol) in dry pyridine (40 ml) with dimethylcarbamoyl chloride (0.06 mol) at room temperature for 24 hr. Addition of water precipitated the crude prod-