Herbicide-Derived Chloroazobenzenes: Pathway of Formation

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In soil, the chloroaniline moieties of certain phenylamidine herbicides are liberated by microbial acylamidases, and are subsequently transformed by peroxidases to stable chloroazobenzenes residues. The intermediate steps of this transformation were studied by allowing 4-chloroaniline or 3,4-dichloroaniline to react under steady state conditions with peroxidase and H2O2. The results indicated that chloroazobenzenes and other chloroaniline moieties may involve the rapid autoxidation of the respective chlorohydroxylamines. Other workers (George, 1953a,b; Yamazaki and Piette, 1961) have observed the disappearance of 3,3',4,4'-TCAB residues in rice field soils that were treated with 3,4'-dichloropropionanilide (propanil) 2 and 3 yr prior to sampling. The weight of the evidence indicates that chloroazobenzenes and other chloroaniline transformation products (Rosen and Siewierski, 1971) are relatively persistent environmental pollutants. An understanding of their formation mechanism is basic to any attempt to prevent or reduce their production.

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To identify the formation of intermediates and azo compounds as a means of defining their formation pathway, with no attempt to measure either the rate or the extent of the transformation. For detection of short-lived labile intermediates of enzymatic chloroazobenzene production, a continuous flow system was constructed in which stoichiometric concentrations of peroxidase, H_2O_2, and substituted aniline were permitted to react at steady state. The system was modeled after those of Roughton (1953), Bray (1961), and Gibson and Milnes (1964). It is illustrated schematically in Figure 2. Channels A, B, C, and D carried to the reaction chambers solutions of mixture (1:1). The homogeneity of all compounds was established by gas-liquid and thin-layer chromatography. Melting points (uncorrected): 4-chloronitrosobenzene, 91°C; 4-chlorophenylhydroxylamine, 88°C; 3,4-dichloronitrosobenzene, 46°C; 3,4-dichlorophenylhydroxylamine, 38°C. The melting points of the 4-chloro compounds matched the ones reported in the literature (Ingold, 1925; Farrow and Ingold, 1924). The authenticity of the 3,4-dichloro analogs, which are new compounds, was established by the comparison of their ir spectra with those of the respective 4-chloro analogs. Further proof of authenticity was obtained by reacting the synthesized 3,4-dichloronitrosobenzene with 3-chloro-4-methylaniline in glacial acetic acid (Bray et al., 1957), and by the mass spectrometric characterization of the resulting 3,3′,4-trichloro-4′-methylazobenzene (Bartha, 1969). In the enzyme experiments, horseradish peroxidase Type II, 1.35 purpuragallin units/mg (Sigma Chemical Co., St. Louis, Mo.) was used.

Metabolites from the peroxidase reaction mixture were partitioned into benzene and were subjected to chromatographic and mass spectrometric analysis. Thin-layer chromatography was performed on 20 x 20 cm sheets coated with a 250-nm layer of silica gel with fluorescent indicator (Chromatogram 6060, Eastman, Rochester, N.Y.). Sheets were activated 1 hr at 100°C before use. Separation of substituted anilines was achieved using benzene, and azo compounds were separated from other reaction products by hexane:benzene:acetone (7:3:1, v/v). Individual azobenzenes were resolved by ligroin (60–70°C). R_t values are listed in Table I.

Preparatory separations were performed on 30 x 2 cm columns packed with neutral aluminum oxide (Woelm, Activity II). Nonpolar solvents (ligroin, 60–70°C, or benzene) were used for development.

Gas-liquid chromatography was performed on an F&M Model 700 gas chromatograph equipped with dual flame ionization detector, temperature programmer, and an F&M Model 7127-A recorder with a disc integrator. The gas chromatographic columns were stainless steel, 1.8 m long, 3 mm o.d., packed with 10% UC-W 98 on Chromosorb W. The operational parameters for the gas chromatograph were as follows: injector temperature, 275°C; column temperature, 175°C isothermal for chloroanilines, and 250°C for chloroazobenzenes; detector temperature, 275°C; carrier gas (helium), 40 cm/ min; hydrogen, 40 cm/ min; oxygen, 250 cm/ min. Before use, the columns were aged at 270°C for 48 hr with a carrier gas flow of 30 cm/ min. Under the listed conditions the lower limits of detection for chloroanilines and chloroazobenzenes were 0.01 and 0.05 µg, respectively. Mass spectra were obtained using a Hitachi Perkin-Elmer Model RMU-7 combination gas chromatograph–mass spectrometer. High-resolution mass spectrometry was performed in the laboratories of the Morgan & Schaffer Corp., Montreal, Canada. The purpose of these experiments was to detect and identify the formation of intermediates and azo compounds as a means of defining their formation pathway, with no attempt to measure either the rate or the extent of the transformations.
RESULTS AND DISCUSSION

Evidence for Free Chloroanilino Radicals. Employing various flow rates in the described apparatus, the time required for formation of 4,4'-dichloroaizobenzene (4,4'-DCAB) from 4-chloroaniline (4-CA) was determined. Trichloroacetic acid solution (4%, w/v) was introduced into chamber 3 to stop the reaction. Formation of 4,4'-DCAB was detected only when the reaction time in chambers 2 and 3 was 0.6 sec or longer. No 4,4'-DCAB was detected when either peroxidase or H2O2 was omitted from the system, proving that enzymatic peroxidation was indeed an essential part of the transformation.

In a similar experiment the trichloroacetic acid solution used in chamber 3 to stop the reaction was supplemented with 3,5-dichloroaniline at the concentration of 10^{-4} M. This compound was found to resist transformation by horseradish peroxidase (Bartha et al., 1968). When in chambers 2 and 3 the reaction time was 0.6 sec, both the symmetric 4,4'-DCAB and the asymmetric 3,4',5-trichloroaizobenzene were detected. At longer reaction times production of 4,4'-DCAB increased, while that of 3,4',5-trichloroaizobenzene decreased. In the case of 3,3',5,5'-tetrachloroaizobenzene detected. These results indicated that the labile intermediate formed during peroxidatic oxidation of 4-CA in chamber 2 was present in chamber 3 at the time when the asymmetric azo compound was produced. It had to be present in a form which permitted its fast reaction with an unchanged aniline.

In the flow system used, the proper conditions for such a reaction sequence were met when the flow of each reactant was 0.66, 0.99, and 0.90 ml/sec, respectively. Other parameters of operation were as stated before. In this semiquantitative experiment, the flow apparatus was operated for a period of 33 sec, permitting the consumption of 1 μmol of each reactant. Gas chromatographic analysis of the products showed that about 25% of the 4-CA had not been oxidized. The amount of 4,4'-DCAB detected accounted for 10% of the amount of 4-CA that had been introduced into the system. Since all products of chloroaniline transformation
could not be detected, a balance was not obtained. However, a new compound that differed from any of the reactants or the known reaction products appeared. Its gas chromatographic retention time was 420-430 sec. This new compound, hereafter referred to as X, was chromatographed on silica gel thin-layer sheets. When developed with benzene it moved as a single spot with an Rf value of 0.66, and was separated completely from other reaction products.

Compound X was purified by repeated column chromatography using benzene as developing solvent. Fractions containing X were combined and concentrated on a rotary evaporator. Attempts to crystallize it had failed, and it was isolated as an oil. The purified compound exhibited a mass spectrum with a parent ion at m/e 326; chlorine was not present. The isotopic analysis by high-resolution mass spectrometry indicated the molecular formula to be C22H30O2. This suggested that two 2-(tert-butyl)-4-methylphenoxy radicals had coupled to form compound X. No further attempt was made to characterize this dimer. The antioxidant failed to trap the chloroanilino radical that was suspected of arising from the peroxidatic oxidation of 4-CA.

Another attempt, using 3,4-dichloroaniline (3,4-DCA) as hydrogen donor, was made under the same experimental conditions. Again, part of the 3,4-DCA was oxidized to 3,3',4,4'-tetrachloroazobenzene (3,3',4,4'-TCAB) with the simultaneous oxidative dimerization of the antioxidant.

Oxidative dimerization of 2-(tert-butyl)-4-methylphenol is known to occur when the compound is attacked by a free radical (Fieser and Fieser, 1961). Therefore, the presence of free radicals in the flow system can be inferred from the ability of the mixture to initiate the polymerization of the antioxidant. Furthermore, since dimer X was produced only in the complete reaction system, chloroanilino radicals appear to be the initiators of the dimerization. No dimer was produced when any of the reactants (peroxidase, hydrogen peroxide, or chloroaniline) were omitted from the system. This also proved that hydrogen peroxide, subject to homolysis of the weak O-O bond, did not initiate the dimerization reaction. Since it has been reported that amino radicals of the type R-NH2 act as polymerization initiators (Walling, 1957), we believe that the chloroanilino radical was the initiator in the present case. Whether or not chloroanilino radical was generated directly by an initial abstraction of one amino hydrogen atom as a result of peroxidatic oxidation of chloroaniline, or indirectly by homolysis of 4,4'-dichlorohydroxybenzene, is unknown. The latter compound was probably present in the reaction mixture, since it was suggested (Holland and Saunders, 1968) as an intermediate in the formation of chloroazobenzene.

**Evidence for Chlorophenylhydroxylamine Intermediates.** By indirect evidence, earlier experiments (Bordeleau and Bartha, 1970) suggested the possible participation of phenylhydroxylamine analogs in the chloroazobenzene formation pathway. Direct proof was sought using the flow system under the previously described conditions, but with the substitution of trisodium pentacyanoaminoferrate solution in chamber 3 for trichloroacetic acid. Separate tests established that this agent not only formed complexes with arylhydroxylamines, as reported by Boyland and Nery (1964), but also served as reaction terminator by inhibiting peroxidase. Figure 3 shows the results of the experiment in which 3,4-DCA was used as the hydrogen donor. Nitrosobenzene may interfere with the determination of phenyldihydroxyamine, but aniline and other aniline derivatives do not. The absorption spectra of reaction product complexes were compared with those of 3,4-DCA, 3,4-dichlorophenylhydroxylamine, and 3,4-dichloronitrosobenzene. The results indicate the presence in the reaction mixture of either 3,4-dichlorophenylhydroxyl-
amine or 3,4-dichloronitrobenzene, or both. Similar results were obtained when 4-CA served as hydrogen donor for peroxidase. As previous experiments demonstrated that in soil or in aqueous reaction mixtures chloronitrosobenzenes were not the prime precursors of chloroazobenzene formation (Bordeleau and Bartha, 1970), the intermediates in question could be narrowed down to the respective chlorophenylhydroxylamines.

More than one mechanism can be visualized for the peroxidase-mediated production of chlorophenylhydroxylamines from chloroanilines, and the available evidence does not allow a clear choice between these possibilities. Peroxidases have been reported to act also as mixed function oxidases (Dure and Cormier, 1964; Saunders et al., 1964; Evans, 1970; Siegel and Siegel, 1970; Thomas et al., 1970a,b). Consequently, chlorophenylhydroxylamines may arise from direct enzymatic oxidation by abstraction of a hydrogen atom and the attachment of a hydroxy radical to the chloroaniline. The hydroxy radical is supplied by the peroxidase-hydrogen peroxide complex (Chance, 1952); thus the process is completely enzymatic. Another possible route of chlorophenylhydroxylamine formation is the nonenzymatic coupling of hydroxy and chloroanilino radicals. In this case, the enzyme action is required only for the formation of the chloroanilino radical, while hydroxy radical may be generated by the homolysis of hydrogen peroxide. It is less likely that the hydroxy radical is generated by the chloroanilino radical's attack on water, since the H-OH bond (119 kcal/mol) of the latter is much stronger than the HO-OH bond (51 kcal/mol) of the hydrogen peroxide (Kerr and Trotman-Dickenson, 1968-69).

**Possible Role of Chlorohydrazobenzenes.** The involvement of chlorohydrazobenzenes as intermediates of chloroazobenzene formation has been suggested (Saunders et al., 1964; Holland and Saunders, 1968). When 3',3',4,4'-tetrachlorohydrazobenzene was incubated in soil, this compound was rapidly transformed to its corresponding chloroazobenzene (Bordeleau et al., 1969). Furthermore, in phosphate buffer solutions, 3',3',4,4'-tetrachlorohydrazobenzene was quantitatively transformed within minutes to 3',3',4,4'-TCAB, indicating the great susceptibility of the former compound to undergo autoxidation. Because of the required presence of H2O2, autoxidation of 3',3',4,4'-tetrachlorohydrazobenzene could not be prevented in the flow system. Although theoretical considerations and indirect evidence strongly suggest the existence of chlorohydrazobenzene intermediates, attempts to obtain direct proof of their involvement were unsuccessful.

**Proposed Pathway of Chloroazobenzene Formation.** Current evidence for the biochemically mediated transformation of chloroanilines to chloroazobenzenes is consistent with the sequence of events illustrated by Figure 4. The pathways of 4-CA and 3,4-DCA transformation are analogous, and only the latter is illustrated. Intermediates that are postulated on the basis of indirect evidence are enclosed in brackets, and all compounds have been assigned Roman numerals to expedite the discussion. The main pathway is indicated by solid arrows. The peroxidatic oxidation of 3,4-DCA (I) produces compounds II and III, via reactions A and A', respectively. Reaction A' is also a possible route for the formation of II. Compounds I and II react together to generate 3',3',4,4'-tetrachlorohydrazobenzene (reaction B, compound IV). Compound IV may also arise from dimerization of two radicals (III) via reaction B'. Whichever way it is formed, compound IV undergoes autoxidation (reaction C) to 3',3',4,4'-TCAB (V). Since the concentration of radicals in solution is much less than the concentration to other reactants (Gould, 1959), it might therefore be expected that the yields of product IV resulting from reaction B' would be negligible in comparison to the yields of IV via reactions A' and B. Similarly, reaction A is considered to predominate over reaction A'. Following the same line of reasoning, and considering the results of the asymmetric azobenzene formation experiment in that the unchanged 3,5-DCA failed to give the corresponding azobenzene, it is logical to propose that formation of azobenzene results from interaction of compound II with excess aniline (I). Thus the main pathway proceeds by the reactions A, B, and C. This route also accounts for the formation of an asymmetric azobenzene when two differently substituted anilines are incubated simultaneously in biological media (Bartha, 1969; Kearney et al., 1969; Bordeleau and Bartha, 1970).

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**LITERATURE CITED**

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Fate of Formothion on Bean Plants in the Greenhouse

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The fate of formothion is studied on bean plants following foliar application. In general, the distribution pattern between the vapor phase, surface, and subsurface area is similar to that of dimethoate. The half-life of formothion breakdown amounts to 1.2 days. Hydrolytic attack causes rapid formothion degradation to approximately equal amounts of dimethoate and O,O-dimethyl dithiophosphoryl-acetic acid. Further breakdown products are dimethoxon, O,O-dimethyldithiophosphoric acid, and bis(O,O-dimethylthiophosphoryl) disulfide. Only dimethoate and dimethoxon are insecticidally active metabolites. Although their residual amounts are considerably lower after application of Anthio than of dimethoate, and although formothion itself dissipates very fast, the insecticidal efficacy of Anthio is equal to dimethoate. It is concluded that the initial biological action after formothion treatment is caused by formothion itself. The long-term efficacy, however, is generated by potentiation of the insecticidal activity of the Anthio metabolites dimethoate and possibly dimethoxon by synergistic action of O,O-dimethyldithiophosphorylacetate acid and bis-(O,O-dimethylthiophosphoryl) disulfide.

Anthio is one of the least toxic systemic organophosphorous insecticides with an LD_{50} of 370–400 mg/kg (Klotzsche, 1966). By systemic and contact action it controls a wide range of sucking, mining, and some biting pests on various crops. Its active ingredient is formothion [O,O-dimethyl S-(N-methyl N-formylcarbamoylmethyl) phosphorodithioate], which is closely related in its molecular structure to dimethoate. Comparative biological trials in the field under conditions of good agricultural practice as well as in the greenhouse revealed almost identical data of performance of both formothion and dimethoate (Staub, 1964; Wood and Tyson, 1965; Almeida and Cavalcante, 1966; Damiano, 1967; Thompson, 1967; Bassand and Klotzsche, 1970; Jalloul, 1968). Residue investigations, however, analyzing for formothion, dimethoate, and dimethoxon, yielded consistently lower residues in plants treated with Damiano, 1967; Thompson, 1967; Bassand and Klotzsche, 1967.

**Materials and Methods**

**Syntheses of Radio-Labeled Compounds.** FORMOTHION-carbonyl-^{14}C. To prepare formothion-carbonyl-^{14}C, 14C-BaCO_{3} was treated with concentrated H_{2}SO_{4}. The evolving 14C-CO_{2} was converted to 14C-carbonyl acetic acid by a Grignard reaction with CH_{2}MgI in ether. After distillation with unlabeled acetic acid, bromine was added to yield 14C-carboxylbromocarbonate acid, followed by conversion into the corresponding acid chloride by adding phthaloylchloride. The acid chloride was dissolved in trichloroethylene and refluxed with N-methylformamidine for 2 hr. The solvent was removed at 50°C in vacuum. The crude 14C-bromoacetic acid was dissolved in trichloroethylene and refluxed with N-methylformamide in dioxane for 3 hr at 35°C. Water-soluble by-products were partition into 2 N KHC\textsubscript{2}O. Purification of the material was stored in benzene at 5°C.

Purity of the described labeled compounds was determined by tlc chromatography on silica gel G with ethyl acetate. Visualization of the compounds was done by spraying with potassium iodoplatinate or by treatment with I\textsubscript{2} vapor. Radiochemical purity was determined by tlc-radioscanning on a Berthold Scanner No. 2 (Berthold Frieseke GmbH, 75 Karlsruhe-Durlach, Germany) and by scratching off the silica gel layer in 0.5-cm zones which were transferred into counting vials, extracted with the scintillator solution, and counted as described later.

FORMOTHION-methoxy-^{14}C. 14C-Methanol obtained from 14C-BaCO_{3} (specific activity 54 mcCi/mmol) by reduction of liberated 14C-CO_{2} with LiAlH\textsubscript{4} in tetrahydrofururyloxytetrahydropropyrene was reacted with P_{2}S_{5} in shellsol R for 2 hr at 60°C and an additional 2 hr at 80°C. Gaseous reaction products (H_{2}S) were removed in vacuum. The remaining solution was diluted with benzene and extracted with 1 N NH_{4}OH to yield the ammonium salt 14C-dimethylphosphorodithioic acid. The synthesis of formothion-methoxy-^{14}C was completed by reacting an aqueous solution of the ammonium salt with chloroacetic acid N-methylformamidine in dioxane for 3 hr at 35°C. Water-soluble by-products were removed from the reaction mixture dissolved in benzene by partition into 2 N KHCO_{3}. Purification of the material diluted with unlabeled carrier was done as described for