identified as neoxanthin had spectral and partition coefficient properties corresponding to neochrome (Curl, 1964). Fraction VI contained a single pigment that was tentatively identified as neochrome, since it has spectral properties and a partition coefficient consistent with the values cited in the literature (Subbarayan *et al.*, 1965; Curl, 1964). Fraction IV contained three carotenoids tentatively identified by comparison with the spectral properties and partition coefficients cited in the literature (Jungalwala and Cama, 1962) as cryptoxanthin (3-hydroxy-β-carotene), chrysanthemaxanthin (3,3′-dihydroxy-5,8-epoxy-α-carotene), and antheraxanthin, (3,3′-dihydroxy-5,6-epoxy-β-carotene), none of which were found in the plant parts of cotton (Thompson *et al.*, 1968). Flavoxanthin (stereoisomer of chrysanthemaxanthin) and violaxanthin (3,3′-dihydroxy-5,6,5′,6′-diepoxy-β-carotene) were found in cotton plant tissue but were absent in the plant tissue of *H. syriacus*.

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3,3′,4,4′-Tetrachloroazoxybenzene from 3,4-Dichloroaniline in Microbial Culture

3,3′,4,4′-Tetrachloroazoxybenzene was isolated from cultures of *Fusarium oxysporum* Schlecht growing in the presence of ^{14}C-ring-labeled 3,4-dichloroaniline. Its identity was established by comparison of its chemical and physical properties with a synthetic sample. Mass spectra, infrared, and ultraviolet spectral properties were determined. It is probable that azoxybenzene is formed by oxidative condensation of two molecules of 3,4-dichlorophenylhydroxylamine or by the condensation of 3,4-dichlorophenylhydroxylamine with 3,4-dichloronitrosobenzene which are potential intermediates in the oxidation of 3,4-dichloroaniline to 3,4-dichloronitrobenzene.

Several condensation products have been isolated from soils treated with aromatic amine compounds. Bartha and Pramer (1967) demonstrated that 3,4-dichloroaniline (3,4-DCA) from 3,4-dichloropropionanilide (propanil) was converted to 3,3′,4,4′-tetrachloroazobenzene (TCAB) in Nixon sandy loam soil. Position of chlorine substituents as well as several other factors was found to affect azobenzene formation (Bartha *et al.*, 1968). Plimmer *et al.* (1970) identified 1,3-bis(3,4-dichlorophenyl)triazene from propanil-treated soils. They proposed that soil nitrite could react with 3,4-DCA to form an intermediate diazonium cation, which would react with another molecule of free aniline to produce the triazene. Condensations of 3-chloraniline and 3,4-DCA to form 3,3′,4′-trichloroazobenzene in addition to 3,3′-dichloroazobenzene and TCAB have also been observed in soil (Kearney *et al.*, 1969). Combinations of propanil and *N*-[3-chloro-4-methylphenyl]-2-methylpentanamide (solan) applied to soil were transformed to TCAB, 3,3′-dichloro-4,4′-dimethylazobenzene, and the asymmetrical 3,3′,4-trichloro-4′-methylazobenzene (Bartha, 1969). The condensation of three molecules of 3,4-DCA to form 4-(3,4-dichloroanilino)-3,3′,4′-trichloroazobenzene has also been reported (Rosen *et al.*, 1969; Linke and Bartha, 1970).

The formation of azobenzenes and anilinoazobenzene has been examined *in vitro* with the aid of horseradish peroxidase (Bartha *et al.*, 1968; Knowles *et al.*, 1969; Lieb and Still, 1969). Few attempts have been made to examine directly the microbial metabolism of halogenated aniline molecules. The formation of azo compounds and related metabolites by soil microorganisms should be examined in greater detail, since anilines are degradation products of many pesticides in soil. The present paper describes the isolation, identification, and probable *in vivo* synthesis of 3,3′,4,4′-tetrachloroazoxybenzene from 3,4-DCA by the soil fungus *Fusarium oxysporum* Schlecht.

EXPERIMENTAL

Mass spectra were obtained with a Perkin-Elmer Model GC 270 combination gas chromatograph–mass spectrometer using direct or gc inlet systems. The gc column used here was a 50-ft surface-coated open tubular column (SCOT) of

0.02-in. internal diameter coated with SE 30 on Chromosorb W. Infrared spectra were recorded in KBr on a Perkin-Elmer Model 621 infrared spectrophotometer. Gas chromatographic analyses were performed with an F&M Model 700 gas chromatograph with a 6-ft stainless steel column ($^1/_8$-in. i.d.) containing UCC-W-982 methyl vinyl silicone gum rubber on diatoport S 80–100 mesh, and a flame ionization detector. The carrier gas (N_2) flow rate was 40 ml/min. Injection port and detector temperatures were 270 and 310°C, respectively. Column temperatures were 180, 180, and 250°C for 3,4-DCA, 3,4-dichloronitrobenzene (3,4-DCNB), and 3,4-DCA condensation products, respectively.

Synthesis of 3,3′,4,4′-Tetrachloroazoxybenzene. The compound was synthesized by the method described for azoxybenzene (Vogel, 1956). 3,4-Dichloronitrobenzene was used as starting material. A yellow solid was collected and purified by recrystallization twice from hexane. The chromatographic properties were identical with those of the metabolite. The synthetic material had a mass spectral fragmentation pattern with major fragments at *m/e* 334, 318, 243, and 145 and a melting point of 142.5–143°C.

Uniformly labeled phenyl-^{14}C-3,4-DCA was prepared from ^{14}C-phenyl-labeled 3-(3,4-dichlorophenyl)-1,1-dimethylurea (diuron) by basic hydrolysis in methoxyethanol. It was purified by thin-layer chromatography on silica gel GF 254 Merck by using a solvent system of hexane–benzene–acetone (7:3:1 v/v). Purity was verified by comparison with authentic 3,4-DCA on thin-layer chromatograms and autoradiography. The specific activity of 3,4-DCA-phenyl-^{14}C was 1.00 mCi/mmol.

Isolation of 3,3′,4,4′-Tetrachloroazoxybenzene. 3,4-DCA was fed to actively growing cells of *Fusarium oxysporum* Schlecht mass cultured in 10-l. quantities in a New Brunswick Model F-14 Fermentor. 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, 25 mg of chloromycetin, and 25 mg of streptomycin sulfate per 1000 ml of distilled water. 3,4-Dichloroaniline (50 mg/l.) was added to the sterilized medium in 1 ml of acetone. ^{14}C-3,4-DCA (2 μCi) was added in 2 ml of ethanol. One hundred milliliters of a spore suspension of *F. oxysporum* was added and the culture was incubated for 6 days.

At harvest the medium was extracted twice with ether (2 × 2.5 l.) and once with ethyl acetate. The extracts were combined and evaporated to dryness in a rotary evaporator.

Components of the residue were separated by column chromatography on silica gel which was eluted successively with mixtures of benzene–acetone (95:5, 95:10), benzene–ethyl acetate (50:50), ethyl acetate, ethyl acetate–methanol (80:20), and methanol. The column eluate was collected in approximately 12-ml vol of which 0.1 ml was used for ^{14}C determination by liquid scintillation. Eluates were combined according to major peaks of radioactivity.

RESULTS AND DISCUSSION

Eighty-five percent of the ^{14}C activity remaining in the culture solution after 6 days of incubation was recovered by the ether and ethyl acetate extractions. All of the ^{14}C material recovered was committed to column chromatography. A major peak of radioactivity containing 10.4% of the total ^{14}C material eluted was observed in the first 120 ml of column eluant. This material was concentrated, purified on silica gel GF 254 preparative thin-layer chromatographic plates, and then recrystallized from hexane or methanol. The unknown appeared as a yellow spot on tlc plates and had mobility characteristics distinctly different from TCAB and 1,3-bis-(3,4-dichlorophenyl)triazene. Mass spectral analyses revealed the compound to have molecular ion at *m/e* 334 (4 Cl), suggesting the empirical formula $C_{12}H_6N_2Cl_4O$. Fragment ions at *m/e* 318 (4 Cl) (M − 16), 243 (3 Cl) (M − 91), and 145 (2 Cl) were ascribed to probable structures $C_{12}H_6N_2Cl_4$, $C_{11}H_6Cl_3$ (formed by loss of Cl, CO, and N_2), and $C_6H_3Cl_2$, respectively. This evidence summarized in Figure 1 and Table I supported the probability of the compound having an oxygenated azobenzene structure (Budzikiewicz *et al.*, 1967).

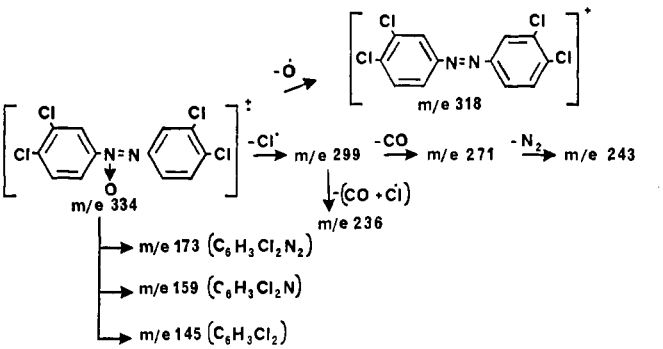

Figure 1. Fragmentation processes of 3,3′,4,4′-tetrachloroazoxybenzene

Comparisons were made with the authentic material. The infrared spectrum of the metabolite was identical with that of the synthetic sample of 3,3′,4,4′-tetrachloroazoxybenzene. The melting point of the metabolite was 135–139°C, as compared to 142.5–143°C for the synthetic sample. Sufficient quantities of the metabolite were not available to permit further purification by recrystallization. The uv absorption maxima for the metabolite and the synthetic compound were 334 and 335 nm, respectively. Silica gel GF 254 plates developed in hexane were used for tlc comparisons with known standards. 1,3-Bis(3,4-dichlorophenyl)triazene, TCAB, the metabolite, and the 3,3′,4,4′-tetrachloroazoxybenzene sample had R_f values of 0, 0.33, 0.17, and 0.17, respectively. A gc retention time of 3.8 min was observed for TCAB under the conditions discussed above, whereas both the metabolite and the 3,3′,4,4′-tetrachloroazoxybenzene sample had an identical retention time of 5.8 min.

Several pathways exist for the formation of azoxybenzenes. Oxidation of an azobenzene may yield the corresponding azoxybenzene (Taylor and Baker, 1949). A second pathway involves the condensation of nitrosobenzene and phenylhydroxylamine. Sequentially, the oxidation of an amino group proceeds *via* the hydroxylamino and the nitroso stages to the nitro group (Figure 2). The hydroxylamino and nitrosoaryl compounds are reactive and can give an azoxyaryl compound by mutual reaction. Condensation of the nitrosoaryl compound with unreacted amine would yield the azoaryl compound. Alternatively, a phenylhydroxylamine itself in neutral or basic solution could yield an azoxy compound (Williams, 1959). We cannot exclude the possibility that the azoxybenzene is formed from the labile phenylhydroxylamine during the workup procedure. The reactivity of this compound, however, is such that it would be rapidly lost from the culture medium by chemical reaction during incubation if it were not rapidly metabolized. The reduction of a nitro group to an amine function requires the reverse of the above sequence with the same potential for formation of intermediates and condensation products. The pathway illustrated here appears to be the more probable explanation for the

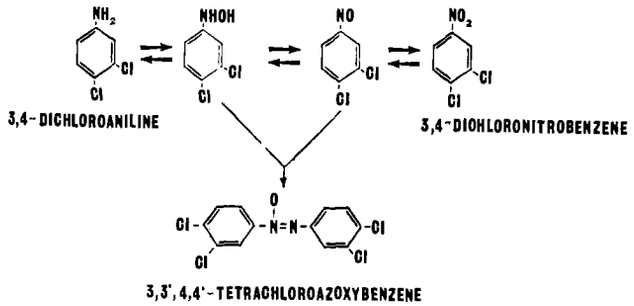

Figure 2. Probable pathway for 3,3′,4,4′-tetrachloroazoxybenzene formation

Table I. Mass Spectral Data for 3,3′,4,4′-Tetrachloroazoxybenzene

	Relative abundance	
m/e	Authentic	Metabolite
73	12	10
74	24	17
75	26	19
84	8	5
85	6	7
88	9	7
97	12	10
98	6	3
99	6	5
109	48	34
110	14	12
111	17	14
112	7	5
124	48	51
125	6	7
126	19	19
133	14	14
135	10	10
145	100	100
146	12	10
147	72	77
148	8	7
149	27	15
159	28	29
161	30	24
163	12	9
172	6	7
173	35	26
174	6	7
175	26	20
177	6	5
236	10	10
238	7	7
271	6	7
272	7	5
273	6	6
274	6	3
318	12	7
320	14	9
322	7	5
334	19	15
336	22	19
338	12	10

formation of the 3,3′,4,4′-tetrachloroazoxybenzene observed in our investigations. Gas chromatographic, tlc, and mass spectral analysis of 3,4-DCA culture extracts also revealed the presence of trace amounts of 3,4-dichloronitrobenzene. Similarly, when 3,4-dichloronitrobenzene is supplied to the *Fusarium* culture, 3,4-DCA can be detected in culture extracts. These observations are supported by data of Neuberg and Wilde (1914) and Newberg and Reinfurth (1923), who considered the formation of an azoxybenzene from a nitrobenzene as evidence for the intermediate formation of the corresponding nitroso- and hydroxylamino compounds. The reduction of aromatic nitro compounds to arylamino compounds by tissue slices and homogenates and by extracts of *Escherichia coli* was demonstrated by Bueding and Jolliffe (1946) and Saz and Slie (1954). A similar amine oxidation and nitro group reduction phenomenon was observed in the metabolic degradation of 2,4-dinitrophenol by *Fusarium oxysporum* Schlecht (Madhosingh, 1961). In subsequent experiments we removed a few milliliters of the culture medium at daily intervals and treated them with a few drops of sodium pentacyanoammine ferroate solution (Boyland and Nery, 1964). The formation of a bluish purple complex was noted at a specific point during the incubation period and was considered indicative of the presence of 3,4-dichlorophenylhydroxylamine or 3,4-dichlorophenylnitrosobenzene.

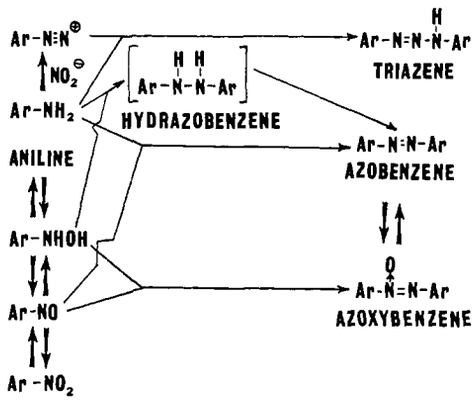

Figure 3. Reactions of substituted anilines

The significance of our observation in its relationship to the further isolation of products of aniline metabolism is open to question. Academically, it provides further insight into possible condensation products and degradation pathways of aniline molecules derived from pesticides. TCAB appears to be the major condensation product occurring in soils treated with 3,4-DCA or 3,4-DCA-containing pesticides. Bartha and Pramer (1967) proposed that the formation of TCAB occurred *via* oxidation of 3,3′,4,4′-tetrachlorohydrazobenzene, which was formed by condensation of a peroxidatically generated 3,4-dichloroanilino free radical with 3,4-dichlorophenylhydroxylamine (Figure 3). Bordeleau *et al.* (1972) reported the peroxidatic formation of 3,4-dichlorophenylhydroxylamine from 3,4-dichloroaniline. The presence of the hydrazobenzene, however, was not demonstrated in biological systems. TCAB formation could also occur by either a reduction of the 3,3′,4,4′-tetrachloroazoxybenzene or by condensation of 3,4-dichloronitrosobenzene with 3,4-DCA (Taylor and Baker, 1949). The mechanism by which TCAB formation actually occurs may therefore be dependent upon the oxidative capability of the biological system involved and the relative concentration of the various potential reactants. The microbial oxidation of amine groups to nitro groups is well documented, as is the reduction of nitro groups to amines. Therefore, the oxidation of 3,4-DCA to 3,4-dichloronitrobenzene is not surprising. The isolation of 3,4-dichloronitrobenzene and the azoxybenzene would support the probability that TCAB formation in biological systems could also arise from condensation of the 3,4-dichloronitrosobenzene with 3,4-dichloroaniline. In this investigation the formation of the azoxybenzene was observed under *in vitro* conditions in a well aerated culture and the compound is believed to be an artifact

dependent upon the experimental culture conditions. Variations in experimental culture conditions resulted in the formation of TCAB or the triazene instead of or in addition to the azoxybenzene.

At present the environmental significance of the azoxybenzene is not known. The chemical and physical characteristics of the azoxybenzene are such that if it were present in aniline-based pesticide-treated soils, it would be detected by the same technique used for monitoring TCAB formation. A cursory examination of all of our experimental data previously published (Kaufman *et al.*, 1971; Kearney *et al.*, 1969, 1970; Plimmer and Kearney, 1969; Plimmer *et al.*, 1970; Chisaka and Kearney, 1970) indicates that azoxybenzene type compounds were not present even in soils treated with exceptionally high rates of 3,4-DCA- or 3,4-DCA-containing pesticides.

Plimmer and Kearney (1969) detected the formation of 3,3′,4,4′-tetrachloroazoxybenzene during the photolysis of 3,4-DCA. Their system, however, involved irradiation by light wavelength >280 nm in benzene solution with benzophenone as a sensitizer in the presence of oxygen. They were unable to detect the azoxybenzene in soils under more natural conditions. Nevertheless, the chemical and physical behavior of azoxybenzenes in the environment will be experimentally assessed. Note: While this manuscript was in its final preparation the authors learned of a recent report that 3,4-dichlorophenylhydroxylamine was formed from 3,4-dichloroaniline by the action of peroxidase (Bordeleau *et al.*, 1972).

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Effect of Gallic (3,4,5-Trihydroxybenzoic) Acid on Iron Availability

Gallic acid added at 2% to experimental rat diets increased hemoglobin and hematocrit repletion of anemic rats fed ferrous sulfate or ferric citrate. This measure of biological availability of iron was more pronounced in the poorer utilized ferric salt than the better absorbed and utilized ferrous sulfate. Concord grape juice, known to contain free gallic acid and compounds containing galloyl groups, did not have a similar effect on the availability of iron from ferric citrate.

Gallic acid is present in a large variety of edible plants either in free form or as part of naturally occurring phenolic compounds, *i.e.*, anthocyanins, catechins, leucoanthocyanidins, gallotannins. (For occurrence of these compounds, see Hegnauer, 1962–1969). Metabolism studies in rats and rabbits ingesting gallic acid have shown that 4-*O*-methylgallic acid is the major metabolite in the urine. Decarboxylation accounted for a second metabolite identified as pyrogallol (Booth *et al.*, 1959). Gallic acid appears to increase the requirement for dietary methyl donors such as choline and methionine (Booth *et al.*, 1961).

Propyl gallate, a synthetic antioxidant widely used in fats, oils, and other food products (National Academy of Sciences, 1965), was shown to lower slightly hemoglobin levels in rats when fed at high dietary levels (Orten *et al.*, 1948). This suggests a chelation effect that interferes with the absorption of iron (Kuhn *et al.*, 1968). In contrast, however, Concord grape juice, which contains both free gallic acid and galloyl groups in the B-ring of its major anthocyanin, delphinidin 3-monoglucoside (Mattick *et al.*, 1967), enhanced iron availability and increased hemoglobin concentration in children (Fishbein *et al.*, 1938).

The objective of this study was to investigate the availability of iron from two iron salts by hemoglobin and hematocrit repletion in young anemic rats when gallic acid is incorporated into experimental diets.