fat gross energies even though they have a fairly high fat content. Lipids in shellfish meat contribute to the total caloric value of the shellfish meat energy, but at their low level in relation to other sources of energy, especially protein, the lipid would not be the dominant source of calories.

Two conclusions can be drawn from the results: (1) fat contributes toward the heat of combustion even in shellfish and (2) the kind of lipid as well as the quantity contributes to the combustibility of the lipid.

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Hydroxylation of Monochloroaniline Pesticide Residues by *Fusarium oxysporum* Schlecht

Metabolism of 2-, 3-, and 4-chloroanilines by isolated cultures of the soil fungus *Fusarium oxysporum* Schlecht, and chlorpropham in soil was investigated. Hydroxylated (phenolic) products were detected colorimetrically, extracted, and characterized by thin-layer and gas chromatography and mass spectrometry. The presence of ortho-hydroxylated chloroanilines in *F. oxysporum* culture solutions was established by a characteristic ortho-elimination process which resulted in the formation of chlorinated benzoxazolines during mass spectral analysis of acylated derivatives. 2-Amino-4-chlorophenol and 2-amino-5-chlorophenol were positively identified as metabolites of 3- and 4-chloroaniline, respectively, in *F. oxysporum* culture solutions by comparison of the mass fragmentation pattern with acylated reference standards. Chloroaminophenols were also detected as metabolites of 2-chloroaniline in *F. oxysporum* culture solutions and from chlorpropham-treated soil.

Chloroaniline-based pesticides may be degraded in soils by a variety of processes. Hydrolysis of several chloroaniline-based pesticides by soil microorganisms yields the chloroaniline moiety as the leaving aromatic group. The aniline moiety has been the subject of intensive investigation in soils and with isolated microbial cultures. Metabolism of 4-chloroaniline by cultures of *Fusarium oxysporum* results in oxidation of the amino group to a nitro group, and the formation of 4-chloronitrobenzene (Kaufman et al., 1973). Aromatic ring hydroxylation and acetylation or formylation of the amino and hydroxyl groups can also occur (Kaufman et al., 1972). A soil bacterium, *Bacillus firmus*, converted 4-chloroaniline to 4-chloroacetanilide, 4-chloropropionanilide, and 2-amino-7-chloro-3-hydroxy-3*H*-phenoxazine (Englehardt et al., 1977). Briggs and Walker (1973) tentatively identified a phenoxazinone from a soil bacterium which metabolized 4-chloroaniline and suggested a hydroxylation occurred ortho to the amino group yielding 2-amino-5-chlorophenol, although the phenol was not isolated. Ambrosi et al. (1977) also reported the detection of phenoxazinones in soil.

It has been suggested that microbial peroxidases in soils are reponsible for the transformation of chloroanilines to chloroazobenzenes (Bartha and Bordeleau, 1969; Kaufman et al., 1972). The subject of this paper is the identification and characterization of some of the hydroxylation products formed during metabolism of 2-, 3-, and 4-chloroanilines by cultures of *Fusarium oxysporum*. 2-Chloroaniline is a potential degradation product of the fungicide Dyrene [2,4-dichloro-6-(*o*-chloroanilino)-*s*-triazine], whereas 3- and

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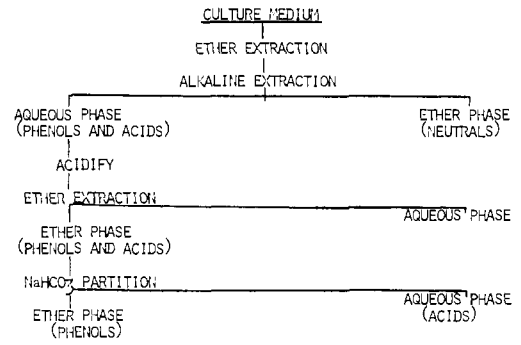

Figure 1. Fractionation scheme used on culture media.

4-chloroaniline are known degradation products of the herbicides chlorpropham (isopropyl *m*-chlorocarbanilate) and barban (4-chloro-2-butynyl *m*-chlorocarbanilate), and monuron [3-(*p*-chlorophenyl)-1,1-dimethylurea], respectively.

Evidence for ring hydroxylation of chloroaniline as a metabolic pathway in *Fusarium* is provided by identification of phenolic compounds by thin-layer and gas chromatography and mass spectrometry. The presence of ortho-hydroxylated aniline derivatives as metabolic products has been demonstrated by the intramolecular rearrangement of *o*-aminophenol derivatives during electron impact fragmentation (Still, 1971; Kaufman et al., 1972). A 2-benzoxazoline intermediate ion was formed by elimination of a molecule of water from an 2-hydroxyacetanilide or by the loss of a molecule of acetic acid from an *o*-*O*-acetylacetanilide (Kaufman et al., 1972). The preferential formation of a fragment ion of this type would demonstrate the presence of an *o*-aminophenol group in the molecule. Still (1971) demonstrated formation of a 2-benzoxazalinone intermediate during electron impact fragmentation of isopropyl 2-hydroxy-5-chlorocarbanilate, a metabolite of chlorpropham.

MATERIALS AND METHODS

Culture Conditions. *Fusarium oxysporum* was mass cultured in 1-L quantities of a culture medium containing inorganic nutrients, sucrose, and yeast extract (Kaufman et al., 1973). Chloroaniline (50 mg/L) was added to the medium in ethanol (0.1 mL). Approximately 1 mCi of uniform ring-labeled [^{14}C]chloroaniline was also added. Samples (10 mL) were periodically removed during the study to determine the time of maximum phenol production. The samples were centrifuged and phenol content of the supernatant determined by color development with 4-aminoantipyrine/potassium ferricyanide reagent (Lacoste et al., 1959) and the optical density measured at 515 nm. The incubation periods were from 6 to 11 days, depending on rate of phenol production.

Product Isolation and Characterization. At the conclusion of the incubation period, the medium was extracted with ether and the phenolic products were isolated and purified as diagramed in Figure 1. The ether was evaporated, and the phenol fraction redissolved in ethyl acetate and applied to precoated silica gel HF_{254} thin-layer chromatography (TLC) plates to achieve further purification. Development was achieved in two solvent system: benzene/dioxane/acetic acid (90:25:4); and benzene/tetrahydrofuran/methanol (80:15:5). The TLC plates were partially covered and the exposed area sprayed with the same reagents used to colorimetrically measure phenol production in the culture medium. The phenolic areas were located by the development of a pink color. The phenolic areas also developed a blue color when sprayed with Folin's reagent (Stahl, 1965). The corresponding

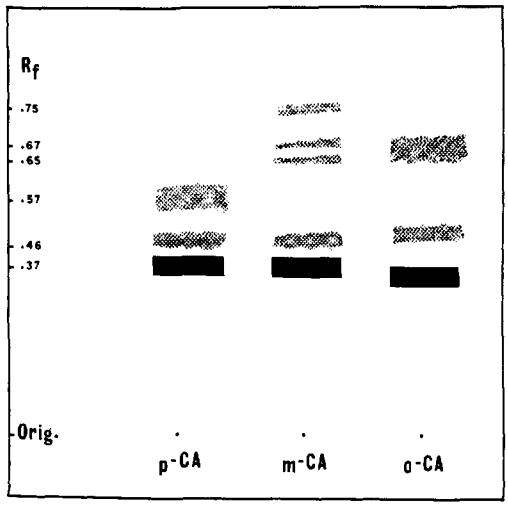

Figure 2. TLC of extracted phenols. Solid spots indicate the strongly reacting aminophenols characterized in this investigation. Shaded spots are indicative of trace amounts of additional phenolic-type materials present in this fraction, which were not further identified. o-, m-, and p-CA indicate 2-, 3-, and 4-chloroaniline metabolites, respectively.

covered phenolic areas were then scraped from the plate and eluted with ethyl acetate.

Acylation was achieved by allowing the metabolites to stand overnight in acetic anhydride/pyridine (1:2) under nitrogen at room temperature. After removal of the acetic anhydride/pyridine solvent, the acylated derivatives were rechromatographed by TLC with the solvents mentioned above. Autoradiographs were made to locate radioactivity. The acyl derivatives were again eluted from the silica gel with ethyl acetate.

Analysis of the acyl derivatives was initially performed on a Glowall Model 310 gas chromatograph (GC) with a 3% SE 30 on 60/80 Chromosorb W column and flame ionization detector. Low-resolution mass spectral analysis was determined using both GC and solid probe inlets on a Dupont Model 21-491-B mass spectrometer. Analysis of the hydroxylated 3- and 4-chloroaniline metabolites was compared with 2-amino-4-chlorophenol and 2-amino-5-chlorophenol standards. A high-resolution mass spectrum of the acylated soil metabolite of chlorpropham was determined on an AEI MS-902 at a resolving power of 10000 and a block temperature of 200 °C by the Florida State University High Resolution Mass Spectrometry Laboratory, Tallahasee, FL.

RESULTS AND DISCUSSION

In one test, it appeared the *Fusarium oxysporum* had metabolized 4.1% of the original 4-chloroaniline; 0.7% of the 3-chloroaniline and 1.2% of the 2-chloroaniline to a phenolic product. These percentages varied with additional experiments, probably due to variation in inoculum age and size, and different incubation periods. Also, Kaufman et al. (1972, 1973) reported acetylation and formylation of hydroxy and amino groups occurred during 4-chloroaniline metabolism. Thus, it is conceivable that additional phenolic products were present in the acylated form. The TLC pattern of extracted phenols is shown in Figure 2. There appeared to be only one major phenolic metabolite produced during the metabolism of each of the chloroanilines, although additional spots provided much weaker color reactions. These latter products constituted less than 2–5% of the total radioactivity appearing on the TLC plates. GC analysis of the major product eluted from TLC plates indicated the presence of only a single com-

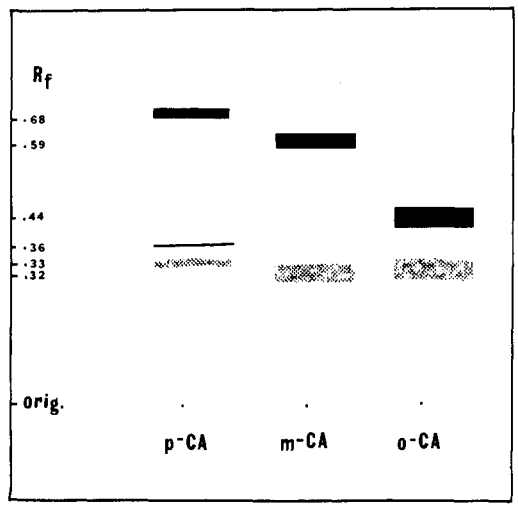

Figure 3. TLC of acylated phenols. Solid spots indicate acylated products. Shaded spots indicate presence of trace amounts of unacylated aminophenols. o-, m-, and p-CA indicate 2-, 3-, and 4-chloroaniline metabolites, respectively.

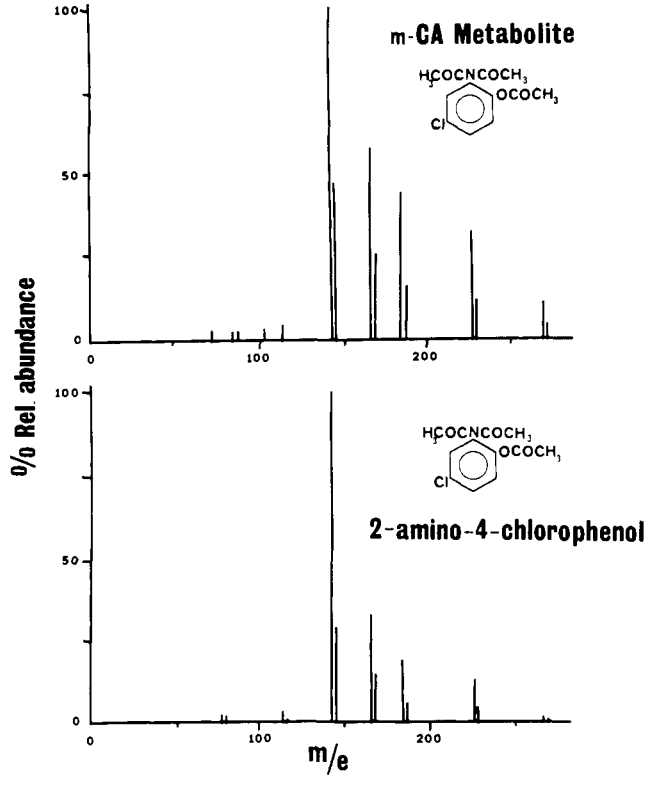

Figure 4. Mass spectral fragmentation pattern of the acylated 3-chloroaniline metabolite and the acylated 2-amino-4-chlorophenol standard.

pound. The R_f values for the extracted phenols were 0.35, 0.37, and 0.37 for the 2-, 3-, and 4-chloroanilines, respectively (Figure 2). The R_f values for the principle acylated derivatives were 0.43, 0.58, and 0.63, respectively (Figure 3).

Fusarium hydroxylated 3- and 4-chloroanilines in the ortho position. Ortho hydroxylation of aromatic compounds has been detected with liver microsomal mixed function oxygenases (Anders, 1969) and some fungi (Auret et al., 1971; Ferris et al., 1973). Low-resolution mass spectral fragmentation patterns of the acylated 3-chloroaniline metabolite and the acylated 2-amino-4-chlorophenol standard are illustrated in Figure 4. A similar mass spectral fragmentation pattern was also

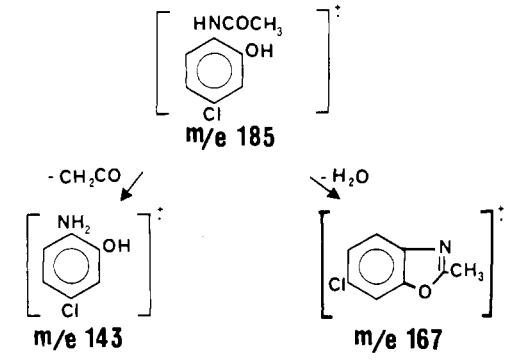

Figure 5. Mass spectral fragmentation of acylated *o*-aminophenol.

obtained with the acylated 2-amino-5-chlorophenol standard. It is interesting to note that diacylation of the amine group occurred during derivatization. TLC and GC analyses of the underivatized phenol indicated that the amino or hydroxy groups had not been acylated by *F. oxysporum*. Replacement of the N-H of 2-acylamino compounds by a second acyl group yielding diacyl amines is readily accomplished chemically (Inch and Fletcher, 1966).

Evidence for ortho hydroxylation is indicated by the *m/e* 167 molecular ion peak. A large fragment ion at *m/e* 167 which corresponds to a chlorobenzoxazoline is observed in the mass spectral analysis of the acylated derivative of the 2-amino-4-chlorophenol standard (Figure 5). The same molecular ion peak at *m/e* 167 occurs in the mass spectral analysis of the diacyl derivatives of the *o*-aminophenol metabolites of 3- and 4-chloroaniline. Further confirmation that hydroxylation occurred at the 6 position on the 3-chloroaniline molecule and not at the 2 position would require NMR analysis. However, sterically, hydroxylation would be expected to preferentially occur at the less hindered 6 position. Still and Mansager (1972) observed that in soybeans arylhydroxylation of the 3-chloroaniline moiety of chlorpropham occurred at the 6 position rather than at the 2 position. Also, the relative abundance of the molecular ions obtained with our acylated 3-chloroaniline metabolite and standard are more closely comparable to those obtained by Still (1971) with isopropyl 5-chloro-2-hydroxycarbanilate than those obtained with isopropyl 3-chloro-2-hydroxycarbanilate.

The low-resolution mass spectrum of the hydroxylated metabolite of 4-chloroaniline follows the pattern of that of the 3-chloroaniline hydroxylated metabolite (Figure 6), and the 2-amino-5-chlorophenol standard. The molecular ion peak at *m/e* 167 is again prominent. The hydroxylated metabolite of 2-chloroaniline, however, undergoes a somewhat different fragmentation. There is no *m/e* 167 peak, which indicates that hydroxylation did not occur ortho to the amine.

Although *o*-aminophenols can be formed by *Fusarium* in pure culture, their presence in the soil environment has not been previously established. By using the fractionation procedure outlined in Figure 1, 2-amino-4-chlorophenol was isolated and similarly identified in extracts of soil originally treated with chlorpropham. The high-resolution electron-impact mass spectrum of this product (acylated) was consistent with the empirical formula $C_{10}H_{10}NO_3Cl$ (calculated *m/e*, 227.0371; observed *m/e*, 227.0334). In assessing the environmental fate of the chloroaniline moiety of aniline-based pesticides, it must be considered that this hydroxylation reaction is linked in a complex manner with other reactions of the dynamic soil environment cycle and investigating one isolated reaction does not necessarily provide any information about the envi-

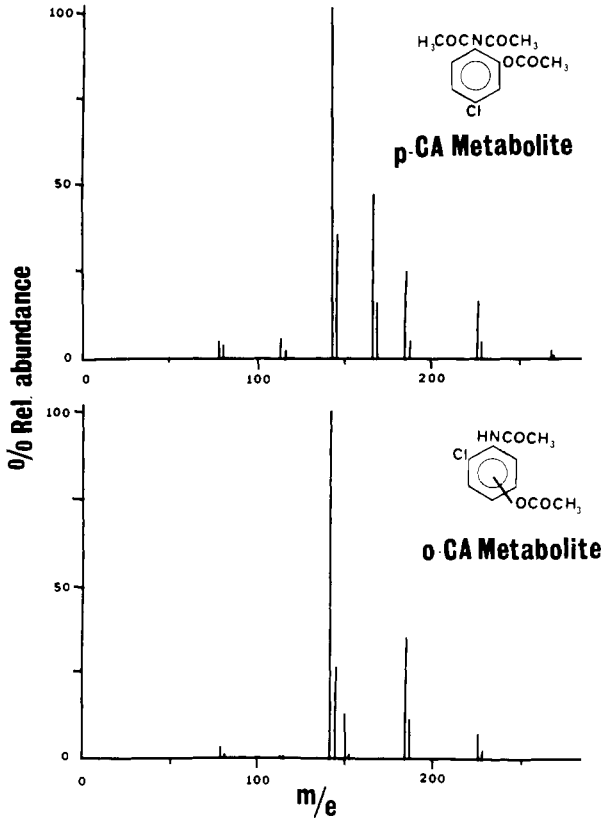

Figure 6. Mass spectral fragmentation pattern of acylated 4-chloro- and 2-chloroaniline metabolites.

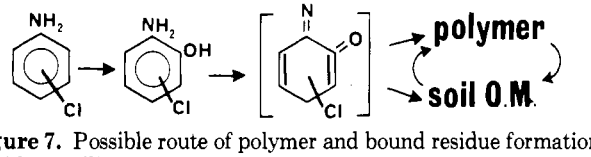

Figure 7. Possible route of polymer and bound residue formation of chloroanilines.

ronmental significance of that reaction (Figure 7). *o*-Aminophenols are relatively unstable molecules in that they can form quinonide structures which are very reactive in condensation and polymerization reactions and with the organic materials in soils. This is evidenced by the fact that there is a steady loss of parent *o*-aminophenol with time after extraction, and formation of more polar, most likely polymerized products, when analyzed by TLC. Interactions of aminophenols with soil organic matter would account for some of the soil-bound residues observed from aniline-based pesticides.

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Growth Response of Mice and *Tetrahymena Pyriformis* to Lysinoalanine-Supplemented Wheat Gluten

Tetrahymena pyriformis, a microorganism suggested for determinations of protein nutritional value, is capable of utilizing lysinoalanine instead of L-lysine. Similarly, L-lysine-dependent mutants of *Escherichia coli*, *Bacillus subtilis*, and *Aspergillus niger* can grow on media where L-lysine was replaced with lysinoalanine. In contrast, the poor growth response of mice indicated their inability to significantly utilize lysinoalanine when challenged with a wheat gluten–lysinoalanine diet.

Recent interest in lysinoalanine was spurred by the controversial detection of kidney lesions, called nephrocytomegalia, in rats fed a diet containing this amino acid (O'Donovan, 1976; Struthers et al., 1978). Lysinoalanine [N^{ϵ}-(DL-2-amino-2-carboxyethyl)-L-lysine] appears in proteins subjected to specific conditions of heating (Sternberg et al., 1975) or alkali treatment (Bohak, 1964; Patchornick and Sokolowski, 1964), associated with a decrease of cystinyl, lysyl, and/or seryl residues by β elimination and condensation reactions (Bohak, 1964; Nashef et al., 1977).

Alkali treatment of casein, peanut meal, and soya protein caused a decrease of protein nutritional quality as determined by net protein utilization (NPU) in rats (deGroot and Slump, 1969); however, no direct correlation was made between the NPU decrease and appearance of

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