# Diazonium Cations as Intermediates in the Microbial Transformation of Chloroanilines to Chlorinated Biphenyls, Azo Compounds, and Triazenes

Bacteria containing a nitrate reductase enzyme system convert 3,4-dichloroaniline into 3,3′,4,4′-tetrachloroazobenzene, 1,3-bis(3,4-dichlorophenyl)triazene, and chlorinated biphenyls. Trapping experiments with 2-naphthol indicate the involvement of a diazonium salt in the formation of all these compounds. Preliminary experiments indicate that conversion to the diazonium salt is a general transformation pathway for aniline derivatives.

The persistence and fate of pesticides and their metabolites are matters of great environmental concern. One group of metabolites, the substituted anilines, is formed in soil by the microbial degradation of a variety of substituted urea, acylamide, and carbanilate pesticides. The transformations of two of these metabolites, 4-chloroaniline and 3,4-dichloroaniline (3,4-DCA), have been studied extensively. For example, in soil 3,4-DCA is transformed into 3,3′,4,4′-tetrachloroazobenzene (TCAB), 1,3-bis(3,4-dichlorophenyl)triazene, and 3,3′,4-trichloro-4′-(3,4-dichloroanilido)azobenzene (Figure 1). Engelhardt et al. (1977) isolated a soil bacterium *Bacillus firmus* which converted 4-chloroaniline to 4-chloroacetanilide, 4-chloropropionanilide, and 7-chloro-2-amino-3*H*-3-hydroxyphenoxazine.

Little is known about the toxicological significance of these compounds although TCAB has been implicated in severe outbreaks of chloracne among workers handling 3,4-DCA. This azobenzene is known to be a potent inducer of microsomal hydrocarbon hydrolase in mice and chick embryos (Poland and Glover, 1976) and thus falls under suspicion as a potential carcinogen.

Considerable evidence has been presented on the formation of various azo compounds in soils after amendment with either aniline-based herbicides or the anilines themselves (Bartha and Pramer, 1967; Bartha, 1968; Chisaka and Kearney, 1970; Sprott and Corke, 1971). The formation of azo compounds has been demonstrated to occur through the oxidation of the aniline by a peroxidase enzyme (Bartha et al., 1968; Bordeleau et al., 1972). Plimmer et al. (1970) proposed that the production of bis-substituted triazene in soil could result from the reaction of soil nitrite with aniline to form the diazonium cation; this cation then couples with the aniline to produce the triazene compound.

In our studies of the bacterial conversions of chloroanilines, we have shown that chlorinated azobenzenes, bis-substituted triazenes, and chlorinated biphenyls are synthesized under the following laboratory conditions. The bacterial species listed in Table I were cultured in a glucose-yeast extract liquid medium (pH 7.2) supplemented with 100 ppm 4-chloroaniline and 100 ppm nitrate nitrogen. Flasks were shaken for 6–12 h at the respective optimum temperature of each culture to obtain a high cell population; then the flasks were changed to a still culture incubation for an additional 5 days. The cells were harvested at 8000*g* and the pellet, which was dark-red in color, was extracted with acetone (3 × 20 mL). The pooled acetone extracts were flash-evaporated to dryness, and the residue was taken up in 2 mL of hexane and examined for the presence of 4,4′-dichloroazobenzene by gas–liquid chromatography against an authentic reference. All the bacterial species used were tested both for peroxidase activity (Pokallus and Pramer, 1964) and for nitrate-reductase activity (Skerman, 1969). The correlation between synthesis of 4,4′-dichloroazobenzene from 4-

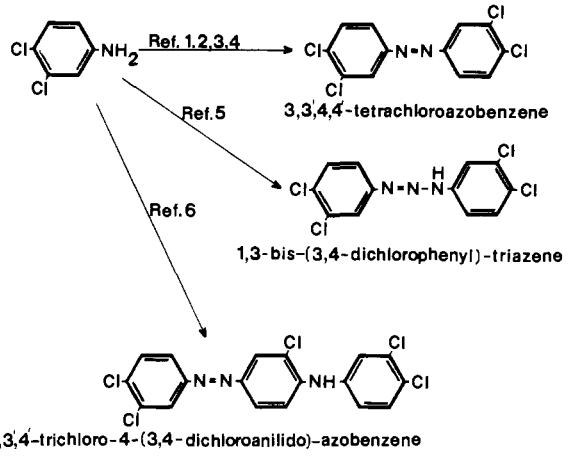


**Figure 1.** Compounds found in soil treated with 3,4-dichloroaniline and herbicides containing 3,4-dichloroaniline moiety: (1) Bartha and Pramer, 1967; (2) Bartha, 1968; (3) Chisaka and Kearney, 1970; (4) Sprott and Corke, 1971; (5) Plimmer et al., 1970; (6) Linke, 1970.

**Table I. Relationship between Azobenzene Formation from 4-Chloroaniline and Peroxidase and Nitrate Reductase Activity**

| culture | nitrate-reduction to nitrite | peroxidase activity | formation of 4,4′-DCAB |
|---|---|---|---|
| *Arthrobacter oxydans* | + | + | + |
| *Arthrobacter pascens* | + | − | + |
| *Bacillus cereus* | + | − | + |
| *Bacillus polymyxa* | + | − | + |
| *Bacillus circularis*[a] | + | − | + |
| *Bacillus subtilis* | + | + | + |
| *Corynebacterium insidiosum* | + | + | + |
| *Escherichia coli* | + | − | + |
| *Enterobacter aerogenes* | + | + | + |
| *Erwinia herbicola* | + | − | + |
| *Arthrobacter globiformis* | − | + | − |
| *Arthrobacter aurescens* | − | + | − |
| *Bacillus circularis*[a] | − | − | − |
| *Bacillus megaterium* | − | − | − |
| *Pseudomonas putida* | − | + | − |

[a] Different strains.

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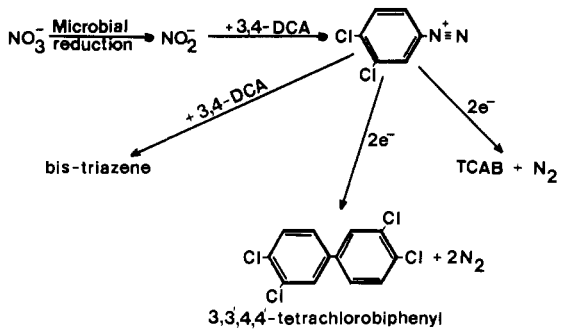


**Figure 2.** Proposed sequence of conversions of 3,4-dichloroaniline by *Escherichia coli* via the diazonium ion to azobenzene, bis-substituted triazene and chlorobiphenyl.

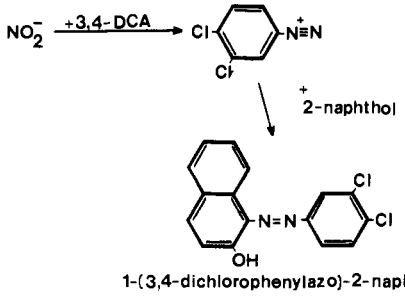


**Figure 3.** Azonaphthol formation by *Escherichia coli* from 3,4-dichloroaniline and nitrite nitrogen.

chloroaniline and the possession of a nitrate-reducing system was absolute (Table I).

The bacterium *Escherichia coli* and 3,4-dichloroaniline were selected for all subsequent experiments reported. Cells of *E. coli* were cultured in medium amended with 3,4-dichloroaniline and nitrate nitrogen as described previously. The hexane-soluble residues were chromatographed over alumina (neutral) to yield an eluted fraction which contained 3,3′,4,4′-tetrachloroazobenzene. A more polar solvent was used to elute the 1,3-bis(3,4-dichlorophenyl)triazene. The presence of TCAB was confirmed by thin-layer and gas–liquid chromatography, and the bis-substituted triazene by TLC. In the GLC scan of the fraction containing the azobenzene, three additional major peaks with shorter retention times were observed. These peaks were identified by combined GLC–mass spectrometry as tetra-, tri-, and dichlorobiphenyls. In addition, the retention time of the tetrachlorobiphenyl in GLC was identical with that of an authentic sample of 3,3′,4,4′-tetrachlorobiphenyl.

It has been suggested that bis-substituted triazene may be formed in soil when a diazonium ion, formed by the reaction of nitrite with an aniline, reacts with residual aniline (Plimmer et al., 1970). On this basis we hypothesized that a possible route to the various aniline-based compounds we have detected involves the formation of this reactive ion (Figure 2). To confirm this hypothesis, 2-naphthol, a known trapping agent for the diazonium ion, was added to cells of *E. coli* growing in a medium supplemented with 3,4-DCA and nitrate nitrogen. In this case, formation of TCAB, bis-substituted triazene, and chlorobiphenyls was suppressed, and instead the 2-naphthol coupling product 1-(3,4-dichlorophenylazo)-2-naphthol was formed (Figure 3). This compound was identified by thin-layer chromatography, light absorption scan (max 475 nm), and its melting point (160 °C).

This experiment provided substantial evidence as follows: (i) 3,4-Dichloroaniline was converted to its corresponding diazonium cation. (ii) Nitrate nitrogen reduction to nitrite nitrogen was required before the aniline was converted to the diazonium ion. This was confirmed with other experiments which showed that when nitrate in the medium was replaced by ammonium, conversion of the anilines did not take place; and bacterial cultures without a nitrate-reductase did not convert aniline and nitrate to diazonium ions. (iii) The diazonium ion appeared to be a key intermediate since, when the ion was trapped by 2-naphthol, there was a marked reduction (greater than 90%) in the production of the metabolites discussed.

These data indicate a pathway of metabolism of aniline residues which is quite different from the peroxidase system described by Bartha et al. (1968). The key step in our pathway involves the conversion of the aniline to a reactive diazonium ion and is consistent with the correlation of azobenzene production with nitrate reducing activity of the bacteria examined (Table I). The proposed pathway through the diazonium ion accounts for all the products from 3,4-dichloroaniline we have identified so far.

Using the 2-naphthol trapping technique, a variety of anilines including the parent and derivatives substituted with methyl, methoxy, or chloro groups were screened for diazonium ion formation. Of 21 different anilines tested, all were transformed to their azonaphthol coupling products except for three anilines which were substituted in both the 2 and 6 position with either chlorine or methyl groups.

The bacterial participation in these reactions appears to be as follows: (i) The generation of nitrite nitrogen from nitrate. (ii) Since the majority of metabolites are extracted from the cells, the bacteria appear to concentrate the reactants and may thus speed up the reaction leading to the diazonium cation. (iii) The diazonium cation is not formed chemically from anilines above pH 6.5; therefore the bacteria may function in either maintaining a favorable internal cell pH in which the chemical reaction can take place, or the bacteria may be actively involved in the reaction. (iv) It seems feasible that the bacteria could be involved in the reduction of the diazonium ion with the liberation of nitrogen, thus permitting the formation of the biphenyls and azobenzene. (v) The tri- and dichlorobiphenyls may be formed either through dechlorination of 3,4-DCA prior to formation of the diazonium cation, or else the tetrachlorobiphenyl, which is formed intercellularly, may be dechlorinated. This aspect is under study because of its implication as a possible new route for the metabolic degradation of substituted biphenyls. (vi) Biphenyls, azobenzenes, and bis-substituted triazenes were not found in extracts of chemical controls (no bacteria present). The pH of the medium does not favor the chemical formation of the diazonium cation, and in addition it is known that azo compounds are formed from diazonium salts only in a strongly reducing medium (Bunce et al., 1976).

In summary, we have proposed a pathway for the conversion of 3,4-dichloroaniline, and perhaps anilines in general, which proceeds to biphenyls, azo compounds, and bistriazenes through the intermediate diazonium salt. Our model system uses pure cultures of bacteria, many of which can be readily isolated from soils, sediments, and sewage treatment plants. The requirements for this pathway are as follows: availability of nitrate nitrogen or nitrite nitrogen, aniline, and an ecosystem with sufficient reducing conditions to allow the microbial reduction of nitrate to nitrite. The ecosystems mentioned above are locations where these conditions pertain.

We still must determine whether these transformations take place in the environment. In particular, the proposed mechanisms of production of substituted biphenyls via the liberation of aniline residues from certain widely used

pesticides presents a completely new potential source of these compounds. It is significant indeed that other metabolites, e.g., TCAB and bis-substituted triazene have already been detected in soils that have been treated with anilines or aniline-containing compounds.

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Received for review September 1, 1978. Accepted December 27, 1978. This investigation was supported by research funds from The Pesticides Advisory Committee of the Ontario Ministry of the Environment and the Ontario Ministry of Agriculture and Food.

# Identification of Offensive Odor Compounds from Potato Processing Plant Waste Effluent Irrigation Fields

The volatile components from an Idaho potato processing waste effluent irrigation field were isolated and analyzed using chemical and adsorption chromatography separation techniques together with combined capillary gas chromatography–mass spectrometry. The components with the most offensive odors were identified as skatole (ca. 0.5 ppm of the soil) and geosmin (ca. 24 ppb of the soil).

The aqueous waste from major potato processing plants in Idaho generally is first neutralized and treated to remove solids. The resulting effluent, which may amount to more than 1000 gal/min, contains some small amount of solids and dissolved matter. This is run out onto fields which are used frequently for growing crops, taking advantage of the soluble nitrogen content of the effluent. Unfortunately, at certain times of the year, particularly during warm weather, the fields may develop odors which are offensive. The present study was undertaken to determine the nature of the compounds responsible for the offensive odors. Such basic information could aid in finding a solution to this problem.

## EXPERIMENTAL SECTION

**Materials.** Authentic skatole was Eastman No. 1357. Other authentic samples were obtained from reliable commercial sources and purified by gas chromatography (GLC).

Several samples of soil were obtained from the waste effluent irrigation fields of a major potato processor in Idaho. They were shipped to Berkeley in polyethylene bags contained in wooden boxes.

**Isolation of Volatile Oil.** A 4-L volume of soil (4.7 kg) was placed in a 12-L flask together with sufficient water (ca. 4 L) to cover it. The mixture was treated for 3 h using vacuum steam distillation continuous extraction (Likens:Nickerson head) at 100 mmHg pressure with the aqueous soil at about 50 °C. Hexane was used as the extracting solvent. A refrigerated water/ethanol mixture at 0 °C was used to cool the condensor. The hexane extract was dried over sodium sulfate and filtered, and the solvent was removed at atmospheric pressure using low hold-up Vigreux distillation columns.

**Separation into Fractions.** The volatile oil was taken up in hexane (100 mL) and extracted in a separatory funnel with hydrochloric acid (4 N, 2 × 20 mL) to give (after neutralization with excess $NaHCO_3$) a basic fraction (1). The hexane solution was then extracted with sodium hydroxide solution (10%, 2 × 20 mL) to give (after acidification with dilute HCl) an acidic fraction (2). The remaining hexane solution (the neutral fraction, 3) was dried over sodium sulfate and placed on a column of activated alumina (Woelm neutral activity grade 1, 14 × 1.5 cm). The column was washed through with more hexane (200 mL) to give a hydrocarbon fraction (3a), then with diethyl ether (200 mL) to give an intermediate polarity fraction (3b), and finally with ether/methanol (10:1, 100 mL) to give an "alcohol" fraction (3c).

**Capillary GLC–MS.** A Pyrex glass capillary column, 150 m long by 0.64 mm i.d. coated with Tween 20 containing 5% Igepal CO-880 was used. Several different temperature programming conditions were used with combined GLC, mass spectrometry (GLC–MS). For the identification of skatole, the column was kept at a constant temperature of 170 °C with a helium carrier gas flow velocity of 50 cm/s. The column was coupled to a modified Consolidated 21-620 mass spectrometer through a silicone rubber membrane molecular separator.

**Odor Evaluation.** The odor threshold of skatole was obtained using procedures described previously (Guadagni et al., 1963) with Teflon bottles and tubing as containers for the odor solutions. Odor quality evaluation of fractions throughout was carried out by three-four experienced odor judges.