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Structural Effects on the Microbial Diazotization of Anilines

Anna M. Lammerding, Nigel J. Bunce,* Robert L. Merrick, and Charles T. Corke

Thirty-six aniline derivatives were examined for microbial converstion to diazonium salts by using a 2-naphthol trapping technique. The reaction is general except for anilines which are either 2,6 disubstituted or which are substituted with strongly electron withdrawing groups. For five representative anilines, the rates of biological and chemical diazotization at pH 6.3 are identical and are not substituent dependent. This shows that the organisms act only to convert nitrate to nitrite and that the actual diazotization step occurs without biological assistance. At this pH, formation of the active nitrosating agent is rate limited. Yields of azonaphthols are lower for the biological system than for the chemical system, and it is proposed that cellular metabolites interfere by trapping some of the diazonium ions to give as yet unidentified byproducts.

Substituted anilines are formed during the microbial degradation of a variety of aniline-based herbicides applied to soils and are subject to many subsequent transformations. Chloro- and methyl-substituted anilines are converted to azobenzenes (Bartha and Pramer, 1967; Bartha, 1968; Chisaka and Kearney, 1970). The compound 3,4-dichloroaniline is transformed to such compounds as 1,3-bis(3,4-dichlorophenyl)triazene (Plimmer et al., 1970), and 3,3′,4′-trichloro-4-(3,4-dichloroanilido)azobenzene (Linke, 1970). The bacterium *Bacillus firmus* forms 4-chloroacetanilide, 4-chloropropionanilide, and 7-chloro-2-amino-3*H*-3-hydroxyphenoxazine from 4-chloroaniline (Engelhardt et al., 1977).

Plimmer et al. (1970) have proposed that 1,3-bis(3,4-dichlorophenyl)triazene formation in soil may involve the reaction of 3,4-dichloroaniline with soil nitrite to form the diazonium ion, which then couples with unreacted 3,4-dichloroaniline. The same mechanism was proposed by Minard et al. (1977) to occur in the transformation of anilines to triazenes by a *Paracoccus* sp. Corke et al. (1979) proposed that the diazonium ion was the key intermediate in the formation not only of the triazenes but also of azobenzenes and biphenyls from aniline derivatives. This was shown by trapping the diazonium ion (whose formation was initiated by the bacterial reduction of nitrate to nitrite) with 2-naphthol. In the presence of 2-naphthol, the production of biphenyl, azobenzene, and triazene was substantially reduced in favor of the coupling product between the diazonium ion and 2-naphthol. This pathway for forming the azo compound is different from the peroxidase mechanism advocated by Bartha et al. (1968), although it must be noted that these latter observations were made under very different experimental conditions.

This paper summarizes studies on the effects of ring substitution of anilines on the formation of coupled azonaphthols and comparisons of rates of formation of these compounds in chemical and microbial systems.

MATERIALS AND METHODS

Culture and Growth Medium. *Escherichia coli* B (No. 263), obtained from the culture collection of the Department of Microbiology, University of Guelph, was used in all experiments. The basal medium was as follows (grams per liter of distilled water): $MgSO_4 \cdot 7H_2O$, 0.2, $CaSO_4 \cdot 2H_2O$, 0.05, KH_2PO_4, 3.65, Na_2HPO_4, 5.7, and Difco yeast extract, 1.0, adjusted to a final pH of 6.9 after autoclaving. The complete growth medium was formulated by the addition of required volumes of filter-sterilized solutions of glucose and sodium nitrate to yield final concentrations of 1% (w/v) and 100 μg of NO_3–N mL^{-1}, respectively.

Chemicals. A total of 36 anilines was used in this study and these are listed in Table I. Liquid anilines were purified by vacuum distillation from zinc dust. Solid anilines were chromatographed on alumina columns and

Department of Environmental Biology (A.M.L., R.L.M., and C.T.C.) and Department of Chemistry (N.J.B.), University of Guelph, Guelph, Ontario, Canada N1G 2W1.

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Table I. Conversion of Anilines to the Corresponding (Phenylazo)naphthols by *E. coli* in the Presence of Nitrate[a]

ring substitution	reaction	ring substitution	reaction
none	++	2,3-dimethyl	++
2-chloro	+	2,4-dimethyl	++
3-chloro	++	2,5-dimethyl	++
4-chloro	++	2,6-dimethyl	−
2-methyl	+	3,4-dimethyl	++
3-methyl	++	3.5-dimethyl	++
4-methyl	++	2,5-dimethoxy	++
2-methoxy	++	3,5-bis(trifluoromethyl)	−
4-methoxy	++	2,6-diisopropyl	−
2-bromo	+	3-chloro-2-methyl	+
4-bromo	++	2-chloro-6-methyl	−
2-isopropyl	+	2,3,4-trichloro	−
2-phenyl	+	2,4,5-trichloro	−
2-fluoro	+	2,4,6-trichloro	−
2-trifluoromethyl	−	2,4,6-trimethyl	−
4-nitro	−		
2,4-dichloro	+		
2,5-dichloro	+		
2,6-dichloro	−		
3,4 dichloro	++		
3,5-dichloro	++		

[a] (−) No color development during 7 days of incubation. (+) Color development during 7 days of incubation. (++) Color development within 24 h.

Table II. Absorbance Data for Substituted and Unsubstituted 1-(Phenylazo)-2-naphthols

compound	λ_{max}, nm	extinct. coeff (ϵ)[a]
1-(phenylazo)-2-naphthol	478	15 900
1-(4-chlorophenylazo)-2-naphthol	480	15 600
1-(3,4-dichlorophenylazo)-2-naphthol	474	16 700
1-(4-methylphenylazo)-2-naphthol	484	13 800
1-(4-methoxyphenylazo)-2-naphthol	460	17 800

[a] The extinction coefficient (ϵ) was calculated from the absorbance (A) of a 6.67×10^{-5} mol/L (c) solution at λ_{max} in a 1.0-cm (l) cuvette and the equation $A = \epsilon lc$. Solvent: acetone-water (1:1).

recrystallized from benzene and hexane. The 2-naphthol was recrystallized from benzene with a small amount of cyclohexane added at the boiling point. The crystals were collected and washed with cold cyclohexane.

The reference 1-(phenylazo)-2-naphthols were synthesized by diazotizing the anilines under acidic conditions and then adding an alkaline solution of 2-naphthol. The red-colored products were recrystallized from ethanol, and their absorption maxima and molar extinction coefficients were determined in acetone–water (1:1) (Table II).

Azonaphthol Production with *E. coli*. Fourteen-milliliter volumes of the complete medium amended with (1) each aniline (50 μg mL^{-1}) and (2) each aniline plus 2-naphthol (50 μg mL^{-1}) were transferred aseptically to sterile culture tubes (16 × 125 mm) stopped with open-top screw caps fitted with 7-mm Tuf-bond silicone disc septa. All experimental tubes of medium were inoculated with a 1-mL suspension of mid-log phase cells (1×10^9 cells) by means of sterile Plastipak disposable syringes fitted with 22 gauge needles. The controls for this experiment consisted of medium amended with anilines or anilines plus 2-naphthol without a bacterial inoculum. Triplicate control tubes and five replicates of all experimental treatments were incubated at 37 °C in total darkness for 1 week.

Rate Studies. Rates of disappearance of anilines and the formation of the (phenylazo)naphthols were determined for aniline, 4-chloroaniline (4-CA), 3,4-dichloroaniline (3,4-DCA), 4-methylaniline (4-MeA) and 4-methoxyaniline (4-MeOA). The bacterial test system described above was used, but the concentrations of anilines and 2-naphthol were each 4.5×10^{-4} M. The chemical transformation of anilines was studied in a similar system, except that the pH of the medium was adjusted to 6.3 and nitrite (100 μg of NO_2–N mL^{-1}) was used in place of the nitrate employed in the bacterial study. Controls for these transformations included uninoculated medium with NO_3–N containing the aniline or aniline plus 2-naphthol.

All tubes were incubated at 37 °C in total darkness. At intervals, triplicates of all experimental and control reaction tubes were analyzed for residual aniline and for the amounts of the phenylazonaphthol produced. Aniline was determined after diazotization by using the procedure described by Pease (1962) for 3,4-dichloroaniline and 4-chloroaniline. Under these conditions, any triazene formed was estimated along with the aniline. The absorbances of the diazotized products were determined with a Zeiss PM2A spectrophotometer at 520 nm for aniline, 560 nm for 4-CA, 3,4-DCA, and 4-MeA, and 580 nm for 4-MeOA. The amounts of coupled products were calculated from absorbance vs. concentration standard curves.

Amounts of the phenylazo compounds formed in biological and chemical systems were determined by mixing equal volumes of acetone and medium, followed by sonication for 5 min. The mixture was filtered through acetone-resistant fluoropore filters (Millipore, 0.4 μm), and absorbances of filtrates were determined at their absorption maxima (Table II). Concentrations of azonaphthols were calculated from their respective extinction coefficients.

RESULTS AND DISCUSSION

The results for the qualitative experiments in which the appearance of the azonaphthol coupling product was followed after incubation of the aniline, sodium nitrate, 2-naphthol, and mid-log phase cells are shown in Table I. In no case was color observed to develop in the chemical control system.

The results in Table I are interpreted as follows: The microbial conversion of an aniline to the azonaphthol is a general reaction, which fails only under certain conditions. (1) The first is steric hindrance at the position ortho to the amino group: one ortho substituent is sufficient to reduce the reactivity from highly positive to positive. Substitution in both the 2 and 6 positions inhibits the reaction entirely. (2) The second is due to electronic factors: strongly electron withdrawing substituents such as CF_3, NO_2 or three chloro substituents inhibit the transformation of the aniline to the azonaphthol. The electron-releasing substituent OCH_3 is able to counterbalance its own steric hindrance in the case of 2-methoxyaniline and 2,5-dimethoxyaniline.

These results were interpreted as being due to the effect of the substituent on the rate of diazotization. For the reaction sequence

$$ArNH_2 \xrightarrow{(a)} ArN^+{\equiv}N \xrightarrow{(b)} ArN{=}N{-}C_{10}H_6OH \quad (1)$$

steric hindrance would be expected to interfere with the diazotization step (a) more than the coupling step (b). The electronic factors are also consistent with step (a) being affected, since diazotization involves a nucleophilic attack by the amino-nitrogen atom on the nitrosating agent. Electron-releasing groups increase the nucleophilicity of the amino group, while electron-withdrawing groups have

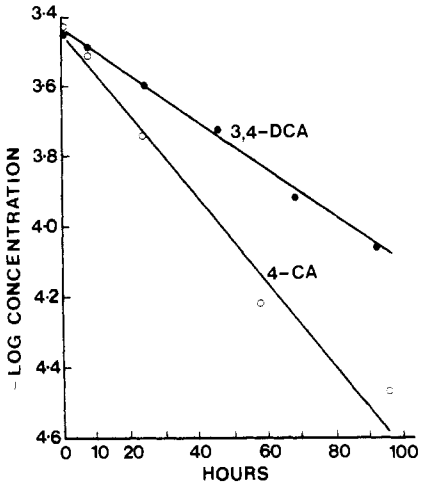

Figure 1. Disappearance of 4-CA and 3,4-DCA in chemical and bacterial experimental systems plotted as log concentration vs. time. Concentration was expressed in moles per liter.

Table III. Pseudo-First-Order Rate Constants for Aniline Disappearance in the Chemical and Bacterial Systems

	biological		chemical	
aniline	k_{obsd}, h^{-1}	r	k_{obsd}, h^{-1}	r
aniline	0.010	0.986	0.009	0.986
4-CA	0.012	0.986	0.009	0.999
4-MeA	0.012	0.984	0.009	0.946
4-MeOA	0.010	0.993	0.009	0.986
3,4-DCA	0.007	0.998	0.007	0.998

the opposite effect. The substituent effects are inconsistent with coupling (b) being the process affected, since coupling is an electrophilic attack of the diazonium cation upon 2-naphthol. Consequently electron-withdrawing substituents should accelerate this process and electron-releasing ones impede it.

In the experiments on rates of disappearance of five representative anilines, the microbial system contained the aniline, sodium nitrate, and 2-naphthol. Nitrate was converted to nitrite within the first 10 h of incubation with a simultaneous change in pH of the medium from 6.9 to 6.3. The chemical system consisted of the aniline, sodium nitrite, and 2-naphthol at pH 6.3.

Typical chemical and microbial reactions are illustrated in Figure 1, and pseudo-first-order rate constants are reported in Table III. For the five anilines studied, the rates of the chemical and microbial reactions were identical. This indicates that diazotization itself is not a biologically mediated process. It is chemical, taking place outside the bacterial cells, whose function is to simply reduce nitrate to nitrite.

With the exception of 3,4-dichloroaniline which reacted a little slower, the rates of disappearance of the other four anilines were almost identical. Steric hindrance is not a problem with the five anilines selected for this study. The apparent lack of an electronic substituent effect indicates that the rate-limiting step under the weakly acidic conditions is the formation of the nitrosating species (Hegarty, 1978); only 3,4-dichloroaniline with two chloro substituents is sufficiently deactivated to react more slowly.

The formation of azonaphthol from 4-CA and 3,4-DCA was determined quantitatively. The rates of formation were quite different in the chemical and microbial systems (Figure 2; Table IV), and in addition, the final yield of azonaphthol was higher in the chemical system. To examine possible reasons for these differences, cells of *E. coli* were grown in the medium with nitrate but in the absence

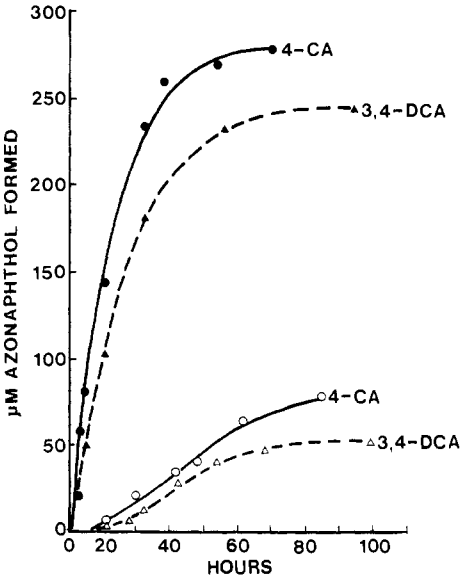

Figure 2. Formation of azonaphthols from 4-CA and 3,4-DCA in chemical and bacterial experimental systems: open symbols, biological system; closed symbols, chemical controls.

Table IV. Pseudo-First-Order Rate Constants for Azonaphthol Formation in Chemical and Microbial Systems

	biological		chemical	
aniline	k_{obsd}, h^{-1}	r	k_{obsd}, h^{-1}	r
aniline	0.005	0.761	0.008	0.906
4-CA	0.011	0.946	0.019	0.900
4-MeA	0.008	0.892	0.007	0.871
4-MeOA	0.008	0.972	0.008	0.872
3,4-DCA	0.015	0.922	0.007	0.882

of 4-chloroaniline and 2-naphthol. After 10 h of incubation, all the nitrate had been converted to nitrite and the pH of the medium was 6.3. The medium was then filtered through Falcon disposable filters (pore size 0.45 μm), and under aseptic conditions, sterile solutions of 4-CA and 2-naphthol were added to bring the final concentrations of these two substances to 50 μg (mL of medium)$^{-1}$. The tubes were then incubated at 37 °C, and when 4-CA was no longer detectable, the concentrations of 1-(4-chlorophenylazo)-2-naphthol were determined. A parallel set of the above experimental tubes was heated at 65 °C for 20 min to denature any enzymes present before adding 4-CA and 2-naphthol. The yields of azonaphthol in the three experimental systems were chemical, 65%, biological (filtered), 42%, and biological (unfiltered), 18%. However, the filtered biological samples contained the same amounts of azonaphthol whether or not they were heat treated; this excludes enzymic participation in the coupling reaction. These results suggest that filterable cellular components excreted by the cells or released upon cell lysis may compete with the 2-naphthol for trapping the diazonium ion. It is consistent with this interpretation that the azonaphthol is produced faster in the chemical system than in the biological systems over the time period where pseudo-first-order behavior is observed.

A final difference between the microbial and chemical reactions is the obvious lag in the microbial system in appearance of azonaphthol; this is not evident in the chemical system. This lag presumably occurs because in the biological system, the reaction cannot begin until the bacteria have reduced nitrate to nitrite.

A separate experiment was run to provide further evidence in support of the diazotization pathway. The mass

spectrum of the azonaphthol shows a prominent molecular ion at m/e 316. When the experiment was run by using ^{15}N labeled sodium nitrate, the molecular ion was then observed at m/e 317. A substantial peak at M - 1 indicated that hydrogen loss is a major route for the molecular ion of the azonaphthol, but after correction for this, the azonaphthol was found to incorporate one ^{15}N essentially quantitatively. This shows that one nitrogen atom in the azonaphthol originates with the $NaNO_3$. Since the bacteria were shown to reduce nitrate to nitrite, this result provides additional confirmation for the diazonium ion pathway proposed in our previous paper (Corke et al., 1979) for the microbial transformations of anilines under anaerobic conditions.

Further studies are in progress to determine (1) the genesis of the other compounds (triazenes, azobenzenes and biphenyls) and (2) whether these model systems are operative in soils.

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Residues of Dibromochloropropane in Fresh and Preserved Peaches

George E. Carter, Jr.,* and Melissa B. Riley

1,2-Dibromo-3-chloropropane (DBCP) was found at a level of 24.7 ppb in ripened peaches from orchards which had been fumigated 144 days prior to harvest. Peaches from trees in soil that was fumigated 270 days prior to harvest contained DBCP at 0.32 ppb. Peaches from nonfumigated orchards were found to contain either DBCP or a compound which could not be distinguished from DBCP at levels of 0.13–0.26 ppb. Peaches preserved in 1948, prior to the release of DBCP, were found to contain 0.25 ppb of DBCP. The compund present in the preserved peaches could not be differentiated from authentic DBCP by either gas chromatography or mass spectrometry. Background levels of this compound complicate determination of DBCP residues in fresh peach fruit.

1,2-Dibromo-3-chloropropane (DBCP) was used as a soil fumigant for nematode control in South Carolina peach orchards until its use was suspended pending cancellation hearings in Oct 1979. DBCP use was voluntarily canceled in March 1981 for all uses except pineapples in Hawaii. Voluntary cancellation was based on the toxic and carcinogenic nature of DBCP and preliminary findings that DBCP use could result in potential exposure from drinking water, potential exposure from food residues, and potential occupational exposure. Due to voluntary cancellation, reregistration of DBCP is a possibility based on further research presently being conducted by the EPA and other interested parties. After peach trees are planted, a nematicide such as DBCP is required in sandy soils of South Carolina to prevent decimation of peach orchards by peach tree short life (Chandler et al., 1962). Peach tree short life is less severe when soil fumigation is used before and after planting (Zehr et al., 1976), and DBCP was the only chemical which was labeled for fumigation after planting. The relative lack of reported information on DBCP residues in peach fruits as well as other crops where DBCP has been used (Newsome et al., 1977) led to the present study conducted to determine presence and persistence of DBCP in peach fruit.

Department of Plant Pathology and Physiology, Clemson University, Clemson, South Carolina 29631.

MATERIALS AND METHODS

Sample Collection. Samples of fresh peaches were colected from orchards where DBCP (a) had been used 14, 77, 144, and 270 days prior to harvest, (b) had been used but not within 365 days, and (c) had never been used. Peaches were collected in wide-mouth canning jars rinsed with ethyl acetate. Ethyl acetate rinsed aluminum foil was placed between the jar and lid. Samples were placed on dry ice immediately and kept frozen until extracted. Preserved peaches including those which predated the release of DBCP were obtained by asking South Carolina county extension agents to assist. County agents requested, through personal contact and organized meetings attended, that peach fruit documented as to the date of preservation be forwarded to this laboratory for study.

Extraction Procedure. The procedure obtained from the California Department of Food and Agriculture for extraction of DBCP was used with only minor modifications (Jackson and Fredrickson, 1978). The peaches (pits removed) were mixed with dry ice in a Waring blender and ground until a homogeneous friable mixture was obtained.

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