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Chemical Transformation of 4-Chloroaniline to a Triazene in a Bacterial Culture Medium

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A *Paracoccus* species in an anaerobic medium causes the transformation of 4-chloroaniline into a product identified as 1,3-bis(*p*-chlorophenyl)triazene. The rate at which this process takes place parallels the rate at which the *Paracoccus* species converts nitrate to nitrite and the transformation appears to involve the relatively rapid chemical reactions of diazotization and coupling.

Numerous pesticides such as phenylurea, phenylcarbamate, and acylanilide herbicides and certain acaricides release variously substituted aniline moieties on breakdown in the environment. However, our knowledge of the further transformation or fate of these intermediary products is still fragmentary. It appears that complex oligomers like azobenzenes (Bartha and Pramer, 1967) and diphenylamines (Briggs and Ogilvie, 1971) predominate at high concentrations of anilines, and at lower concentrations simpler products like acylated compounds (Tweedy et al., 1970; Bollag and Russel, 1976; Russel and Bollag, 1977) and oxidation products of the aromatic amine group (Kaufman et al., 1973) can be found. However, there are also many unidentified products reported in the literature which indicate that the biological and nonbiological transformation products from the aniline moiety have not yet been sufficiently clarified and require further attention if one is concerned with the possible impact of such chemicals on the environment.

The present paper reports the chemical transformation of 4-chloroaniline in a culture medium due to a change of pH and ion composition brought about by the metabolic activity of bacteria.

MATERIALS AND METHODS

The bacterium of the genus *Paracoccus* used in this study was isolated from soil by enrichment culture techniques with 4-chloroaniline in the growth medium (Bollag and Russel, 1976).

The bacteria were grown anaerobically in a Czapek–Dox medium containing: saccharose, 20 g; $NaNO_3$, 3 g; K_2HPO_4, 1 g; $MgSO_4$, 0.5 g; KCl, 0.5 g; $FeSO_4$, 0.01 g in 1 L of distilled water (final pH 7.4). After autoclaving sterilized 4-chloroaniline was added.

The 4-chloroaniline was purchased from Aldrich Chemical Co., Inc. (Milwaukee, Wis.) and recrystallized several times from hexane; its purity was established by TLC, melting point determination, and mass spectral analysis. Uniformly ^{14}C-labeled 4-chloroaniline was purchased from California Bionuclear Corporation (Sun Valley, Calif.) and had a sp. act. of 4.5 μCi/mmol. The labeled compound was diluted with sufficient unlabeled material to reach the desired concentration. The 4-chloroaniline was introduced into 50 mL of medium in 0.2 mL of ethanol solution after sterilization by membrane filtration (0.22-μm pore size Millipore filter) for a final concentration of 20, 50, 75, and 100 ppm and 10^{-3} μCi of radioactivity per mL.

To obtain anaerobic conditions, the flasks were flushed with helium until all air was removed (Bollag and Nash, 1974) and incubated at 28 °C.

The total number of cells of the *Paracoccus* sp. during growth in the Czapek–Dox medium was determined by the dilution plate method using nutrient agar. Plates were incubated at 28 °C for 24 h.

For experiments designed to study the chemical transformation, a modified Czapek–Dox medium was used. Instead of nitrate, nitrite was introduced into the medium and pH was adjusted to 6.0.

Nitrite was measured colorimetrically by the α-naphthylamine–sulfanilic acid method (American Public Health Association, 1971).

For all routine analyses, 5 mL of the growth medium or chemical reaction mixture (pH adjusted to 7.0) was extracted with an equal volume of diethyl ether. The organic phase was used for measurements of radioactivity and TLC analysis.

Radioactivity was determined with a Nuclear-Chicago Isocap-300 liquid scintillation counter. The samples were measured in a cocktail composed of 60 g of naphthalene, 100 mL of methanol, and 8 g of Omnifluor [(98% PPO (2,5-diphenyloxazole–2% Bis-MSB (*p*-bis(*O*-methylstyryl)benzene); New England Nuclear Corp., Boston, Mass.)] in 1 L of dioxane.

Precoated thin-layer plates (silica gel F-254) with fluorescent indicator (layer thickness, 0.25 mm) were purchased from Brinkman Instruments, Inc. (Westbury, N.Y.). The following solvent systems were employed in all analyses: ether–hexane (4:1, v/v) and benzene–dioxane–acetic acid (90:25:4, v/v).

For routine TLC analysis, 1.0 mL of ether extract was concentrated to 0.1 mL and spotted on plates. All com-

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Table I. Mass Spectral Data for 1,3-Bis(4-chlorophenyl)triazene

m/e measd	Intensity	Composition	m/e calcd	m/e measd	Intensity	Composition	m/e calcd
269	0.4			102	2.7		
267	2.0			100.9927	8.0	$C_3H_2N_2Cl$	100.9906
265.0167	3.0	$C_{12}H_9N_3Cl_2$	265.0173	100	6.8		
241	0.5			98.9973	23.7	C_5H_4Cl	99.0002
239	2.1			92.0511	12.3	C_6H_6N	92.0500
237.0067	3.3	$C_{12}H_9NCl_2$	237.0112	91	8.6		
205	0.1			90	6.2		
203	1.0			77	22.8		
201.0345	1.8	$C_{12}H_8NCl$	201.0345	75.0234	5.2	C_6H_3	75.0236
167.0736	2.7	$C_{12}H_9N$	167.0734	66	30.9		
166	1.4			65	18.5		
141	24.7			64	8.6		
139.0068	61.7	$C_6H_4N_2Cl$	139.0063	63.5	4.3		
129	25.9			63	8.6		
127.0194	83.5	C_6H_6NCl	127.0188	62	4.9		
114	16.7			51	17.3		
113	36.4			50	22.2		
112	54.6			36	19.8		
111	100						

pounds were made visible with ultraviolet light at 254 nm. The 4-chloroaniline was further determined by spraying the plates with an aromatic amine specific reagent composed of 1 g of *p*-dimethylaminobenzaldehyde dissolved in 180 mL of 1-butanol, 30 mL of ethanol, and 3 mL of HCl. Radioactive zones on the plates were detected by autoradiography using an x-ray film (Kodak RD Royal X-Omat, Rochester, N.Y.). Radioactive spots were removed and the radioactivity was counted for quantitative evaluation.

For isolation of triazenes, 1 L of culture medium was extracted with 1 L of diethyl ether. The organic phase was then evaporated to dryness with an evaporator at room temperature. The resulting residues were dissolved in 2 mL of ether and separated by TLC. Triazenes were extracted from plates with ether and for further purification rechromatographed several times. After crystallization from ether and hexane, large, yellow, needle-shaped crystals were obtained. This material was used for chemical and spectral characterizations.

Low- and high-resolution mass spectra were obtained on an AEI MS-902 mass spectrometer with associated data system and at an ionization potential of 70 eV using the direct insertion probe. NMR spectra were taken on a JEOL PFT-100 instrument with a Nicolet 1080 Fourier transform system. Ultraviolet spectra were obtained with a Bausch and Lomb Spectronic 505 spectrometer.

RESULTS

During growth of a *Paracoccus* sp. in a Czapek–Dox medium under anaerobic conditions, a change of pH and the accumulation of nitrite could be observed (Figure 1). Continuous acidification of the growth medium occurred until a pH of 5.6 was reached and, concurrently, nitrite was formed and accumulated to about 35% of the originally introduced nitrate. These changes in the culture medium seem to be related to the growth of the bacterium. The dilution plate method showed that the bacterium was growing at a very fast rate for the first 24-h time period, and reached a maximum growth of 188×10^8 cells per mL; subsequently a small decrease in cell numbers was observed.

When radioactive 4-chloroaniline was introduced to the culture medium at a concentration of 100 ppm, only a small amount of the total radioactivity disappeared after 48 h of incubation (Figure 1). TLC analysis of the growth medium showed that, at this time, all added 4-chloroaniline was completely transformed. Concurrent with the transformation of the substrate, it was possible to observe

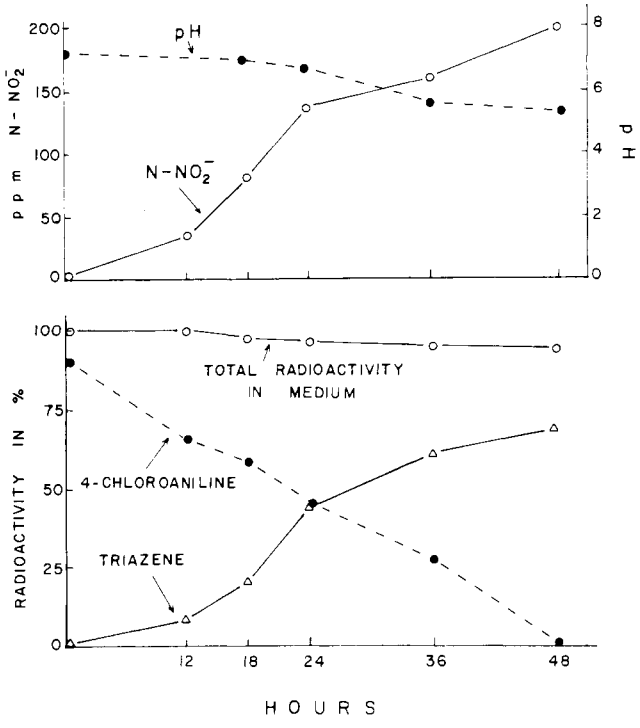


Figure 1. Change of pH, formation of nitrite, and transformation of 4-chloroaniline (100 ppm) during growth of a *Paracoccus* sp. under anaerobic conditions.

the formation of new compounds. One, in particular, accumulated in large amounts. On plates, it gave yellow spots which, after a few hours, changed to red ones. This compound, accounting for 80% of all the radioactivity in the growth medium, was isolated from the plate (R_f 0.74) and, after rechromatography and crystallization from ether and hexane, yielded large yellow needles. This material was identified as 1,3-bis(*p*-chlorophenyl)triazene (I), mp 127–128 °C (lit. mp 129 °C; Day et al., 1951; Mitsuhashi and Simamura, 1970).

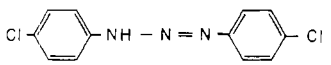
The infrared spectrum (broad absorption due to N–H at 3420–3460 cm^{-1}) and UV–vis spectrum (λ_{max} 356, 298, 238 nm; $\epsilon \times 10^{-4}$ = 2.23, 1.02, 1.74) are in agreement with values reported for this compound in the literature (Day et al., 1951; Plimmer et al., 1970). The mass spectral data (Table I) substantiate this identification. Of particular significance is the lack of an ion at *m/e* 154 which would be present if the compound were the isomeric 2-amino-

Table II. Effect of pH on the Chemical Reaction of Nitrite and 4-Chloroaniline to Form 1,3-Bis(4-chlorophenyl)triazene[a]

	Radioactivity, dpm, at pH			
	5.0	6.0	7.0	8.0
4-Chloroaniline	83	152	3853	3821
1,3-Bis(4-chlorophenyl)triazene	3325	3234	59	63

[a] Initial concentration of 4-chloroaniline was 100 ppm and the quantitative evaluation was made by measurement of the radioactivity in the corresponding R_f area of 4-chloroaniline and the triazene upon TLC of a sample after 24 h.

4′,5-dichloroazobenzene. The NMR spectrum [4.70 (br s, N–H), 7.44 (d, J = 8 Hz, 4 H), 7.5–7.75 (m, 2 H), 7.83 ppm (d, J = 8 Hz, 2 H)] is also in agreement with the triazene structure (I).



I

This compound appears to be the main transformation product of 4-chloroaniline under the described conditions. Of the other compounds which were isolated, it is worthwhile to mention 4-chloroacetanilide which was identified by melting point determination and mass spectrometry. The acetylated compound accounted for approximately 5% of the originally applied 4-chloroaniline.

The effect of pH on the chemical formation of triazene in a noninoculated Czapek–Dox medium whose pH was obtained by addition of buffer is shown in Table II. The 4-chloroaniline at a concentration of 100 ppm was incubated for 24 h at pH values between 5 and 8 in the presence of 200 ppm of nitrite nitrogen. No triazene was observed in the reaction mixture at pH 7.0 and above, but below a neutral pH triazene was produced and all the added 4-chloroaniline was transformed to the corresponding triazene.

In Table III the effect of various concentrations of 4-chloroaniline with respect to triazene formation is compared during the growth of the *Paracoccus* sp. and during incubation in a sterile medium amended with 200 ppm of nitrite nitrogen. It is an interesting observation that at a concentration of 20 ppm of 4-chloroaniline, no triazene was formed in the bacterial culture medium, whereas in the chemical reaction mixture almost all aniline was converted to the corresponding triazene. Concurrently it was observed that during the growth of the bacteria with 20 ppm of 4-chloroaniline a loss of approximately 60% of the radioactivity occurred. The absolute amount of radioactivity lost from the biological medium remained

Scheme I

a. $NO_3^- \xrightarrow[\text{slow}]{\textit{Paracoccus}\ \text{sp.}} NO_2^-$ biologically mediated

b. $H^+ + NO_2^- \overset{\text{fast}}{\rightleftharpoons} HONO$ affected by pH

c. $H^+ + HONO \overset{\text{fast}}{\rightleftharpoons} H_2ONO^+$ affected by pH

d. $H_2ONO^+ + NO_2^- \overset{\text{slow}}{\rightleftharpoons} NONO_2 + H_2O$

e. $NONO_2 + ArNH_2 \xrightarrow[\text{fast}]{} ArNH_2NO^+ + NO_2^-$

f. $ArNH_2NO^+ \xrightarrow[\text{fast}]{} ArN_2^+ + H_2O$

g. $ArN_2^+ + ArNH_2 \longrightarrow ArN{=}NNHAr + H^+$

essentially constant with increasing concentration of 4-chloroaniline and, simultaneously, an almost linear increase of triazene took place.

DISCUSSION

Pesticides are exposed to numerous biological, chemical, and physical factors, any of which can cause their transformation. It is often not easy to distinguish which factor is responsible for a given structural transformation especially since all three factors are so closely interdependent. It is well known that the chemical or physical environment can be changed by the metabolic activity of microorganisms, but these secondary effects on the transformation of pesticides have not usually received adequate attention.

In the present investigation there appears to be experimental evidence for such a secondary effect stemming from two biologically mediated activities: (1) the transformation of nitrate to nitrite and (2) the lowering of the pH of the medium. This leads to the proper physicochemical conditions which cause the conversion of 4-chloroaniline to 1,3-bis(*p*-chlorophenyl)triazene.

From the thorough investigations of Hughes et al. (1958) on the kinetics and mechanism of nitrosation and diazotization, one can outline the probable course of the chemical transformation as shown in Scheme I. The equilibrium concentrations of nitrous acid (reaction b) and nitrous acidium ion (reaction c) both increase as the pH decreases. In the pH range used in these experiments (~5–7) the concentration of nitrous acid, and thus the concentration of nitrous acidium ion, are quite low.

Under similar conditions, Hughes et al. (1958) found that for unsubstituted aniline, the formation of the nitrosating species, nitrous anhydride (step d), is slower than the nitrosation reaction (step e) and therefore the overall rate of diazotization (steps b through f) is independent of the amine concentration. Anilines with electronegative substituents such as bromo or chloro are less basic and therefore less reactive in step e. Nevertheless, at the concentrations of 4-chloroaniline used in these experi-

Table III. Effect of Different Concentrations of 4-Chloroaniline on the Formation of 1,3-Bis(4-chlorophenyl)triazene during Anaerobic Incubation in a Sterile Medium Supplied with Nitrite (Incubation 24 h) and in the Medium of a *Paracoccus* sp. Grown under Anaerobic Conditions (Incubation 72 h)

	Sterile medium (pH 6.0)				Growth medium (pH 5.6)			
4-Chloroaniline, ppm	Nitrite nitrogen, ppm	Transformation of 4-chloroaniline, %	Loss of radioact., %	Formation of triazene, %	Nitrite nitrogen, ppm	Transformation of 4-chloroaniline, %	Loss of radioact., %	Formation of triazene, %
0	183				195			
20	180	100	3.3	91.3	193	100	60.1	0
50	179	100	3.0	90.0	198	100	50.8	42.7
75	174	100	2.9	86.3	192	100	18.3	50.1
100	174	100	6.0	84.0	187	100	16.4	78.5

ments, the rate of step e should be comparable to step d at pH 5; at a higher pH, step d would undoubtedly be rate limiting.

The rate of triazene formation by coupling of the diazonium ion with free amine (step g) is slower than the rate of diazotization. Although Hughes et al. (1958) did not study the kinetics of this particular reaction, they do mention that the concentration of the diazonium ion of aniline increased linearly with time; coupling or hydrolysis did not lead to a significant loss of this species even after 90% of the aniline was diazotized. However, in the case of anilines bearing electronegative substituents, a nonlinear increase in diazonium ion concentration occurred after about 75% reaction due to coupling to the triazene. Thus, for 4-chloroaniline, the coupling reaction (step g) takes place at a rate competitive with the overall rate of diazonium ion formation. Therefore, triazene formation, like diazotization, is rate limited by the availability of a nitrosating reagent which in turn is limited by the nitrite and hydrogen ion concentrations. The metabolic activity of *Paracoccus* increases the concentrations of both.

Figure 1 shows that the rate of triazene formation essentially parallels the rate of nitrite formation and this is what one would expect at pH 5–6. By comparison with the kinetic data for the diazotization of *m*-bromoaniline at pH 5 (Hughes et al., 1958) it can be calculated that at a nitrite concentration of 30 ppm, at least 50% of the 4-chloroaniline would be diazotized within 10 min, i.e. the reaction would occur about as rapidly as the nitrite is formed. However, initially the medium is about pH 7, not pH 5, and remains above pH 6 for about 18 h. In this pH range, the equilibrium concentration of nitrous acid is calculated to be so low that the rate of triazene formation should be imperceptible. Indeed, for a strictly chemical reaction (no *Paracoccus* present), no triazene is formed at pH 7 after 24 h (Table II). It is difficult to explain why the reaction proceeds as readily as it does at pH 7 in the presence of *Paracoccus*. Perhaps the nitrosating reagent is not nitrous anhydride, but an organic nitrosating species derived from the *Paracoccus* and nitrite. Alternately, nitrous anhydride may still be the predominant nitrosating species, but its formation may be catalyzed in the presence of *Paracoccus* in a manner similar to its anion- (acetate, phthalate, or phosphate) catalyzed formation as described by Hughes et al. (1958).

A number of condensation products arising from substituted anilines have been found in soils and in microbial cultures receiving high dosages of anilines or aniline-based pesticides (Bollag, 1974). Most reports describe the isolation of azobenzenes, but in soil it was also found that a diphenylamine (Briggs and Ogilvie, 1971) or a triazene derivative (Plimmer et al., 1970) can be formed. The actual cause of condensation or polymerization could not unequivocally be elaborated. It appears that the concentration of the available aniline is in itself an important factor and that peroxidases (Bordeleau et al., 1972) and photochemical effects (Rosen et al., 1970) represent the main factors in the formation of azobenzenes.

Plimmer et al. (1970), who isolated 1,3-bis(3,4-dichlorophenyl)triazene from propanil (3,4-dichloropropionanilide) in soils, proposed that soil nitrite reacts with 3,4-dichloroaniline to form an intermediate diazonium cation, which subsequently couples with free 3,4-dichloroaniline resulting in the formation of the triazene.

Although the formation of a triazene derivative from an aniline compound under the described conditions is a well-established chemical reaction (Hartman and Dickey, 1943), the occurrence of this reaction under specific natural conditions must always be considered a possible dangerous environmental factor since triazenes are known for their multiple toxic effects.

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