Dulin, D.; Mill, T. *Environ. Sci. Technol.* **1982**, *16*, 815.
Glass, B. L. *J. Agric. Food Chem.* **1975**, *23*, 1109.
Green, A. E. S.; Cross, K. R.; Smith, L. A. *Photochem. Photobiol.* **1980**, *31*, 59.
Guy, R. D.; Narine, D. R. *Can. J. Chem.* **1980**, *58*, 555.
Hall, R. C.; Giam, C. S.; Merkle, M. G. *Weed Res.* **1968**, *8*, 292.
Hedlund, R. T.; Youngson, C. R. In "Fate of Organic Pesticides in the Aquatic Environment"; Faust, S., Ed.; American Chemical Society: Washington, DC, 1972; Adv. Chem. Ser. No. 111, pp 159–172.
Kenaga, E. E. *Down Earth* **1974**, *30*, 19.
Khan, S. U. *Environ. Lett.* **1973**, *4*, 141.
Mill, T.; Hendry, D. G.; Richardson, H. *Science (Washington, D.C.)* **1980**, *207*, 886.
Miller, G. C.; Miile, M. J.; Crosby, D. G.; Sontum, S.; Zepp, R. G. *Tetrahedron* **1979**, *35*, 1797–1800.
Miller, G. C.; Zepp, R. G. *Environ. Sci. Technol.* **1979a**, *13*, 860–863.
Miller, G. C.; Zepp, R. G. *Water Res.* **1979b**, *13*, 453.
Miller, G. C.; Zisook, R.; Zepp, R. *J. Agric. Food Chem.* **1980**, *28*, 1053.
Mosier, A. R.; Guenzi, W. D. *J. Agric. Food Chem.* **1973**, *21*, 83.
Nelson, N. H.; Faust, S. D. *Environ. Sci. Technol.* **1969**, *3*, 1186.
Osteryoung, J.; Whittaker, J. W. *J. Agric. Food Chem.* **1980**, *28*, 95.
Parris, G. E. *Environ. Sci. Technol.* **1980**, *14*, 1099.
Sinelnikov, V. E. *Tr., Inst. Biol. Vnutr. Vod, Akad. Nauk SSSR* **1976**, *33*, 65–73; *Chem. Abstr.* **1978**, *88*, 197317e.
Smith, R. C.; Calkins, J. *Limnol. Oceanogr.* **1976**, *21*, 746.
Strobel, H. A. "Chemical Instrumentation"; Addison-Wesley: Reading, MA, 1960; pp 14–33.
Wolfe, N. L., Environmental Research Laboratory, Athens, GA, personal communication, 1982.
Zepp, R. G. *Environ. Sci. Technol.* **1978**, *12*, 327.
Zepp, R. G.; Baughman, G. L.; Schlotzhauer, P. F. *Chemosphere* **1981**, *10*, 109.
Zepp, R. G.; Cline, D. M. *Environ. Sci. Technol.* **1977**, *11*, 359.
Zepp, R. G.; Schlotzhauer, P. F. *Chemosphere* **1981a**, *10*, 119.
Zepp, R. G.; Schlotzhauer, P. F. *Chemosphere* **1981b**, *10*, 479.
Zika, R. G. In "Marine Organic Chemistry"; Duursma, E. K.; Dawson, R., Eds.; Elsevier: Amsterdam, 1980; pp 299–326.

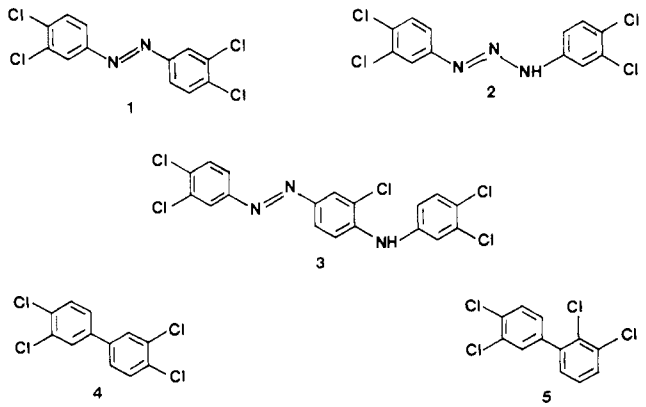
Received for review February 23, 1983. Revised manuscript received May 2, 1983. Accepted May 29, 1983. Mention of trade names or commercial products does not constitute endorsement or recommendation for use by the U.S. Environmental Protection Agency. This research was a part of Project 02.03-31, "Forms and Mechanisms by Which Pesticides and Chemicals are Transported", under the Bilateral Agreement on Environmental Protection between the United States and the Soviet Union.

Reductive Transformations of Nitrate with 3,4-Dichloroaniline and Related Compounds by *Escherichia coli*

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

The transformation of 3,4-dichloroaniline (3,4-DCA) in partly anaerobic cultures of *Escherichia coli* in the presence of nitrate ions affords tetrachloroazobenzene, tetrachlorobiphenyls, and dihydroxytetrachlorobiphenyls, all in yields <1%. Major products are 3,4-dichlorophenol and a bis(dichlorophenyl)triazene. All products are rationalized in terms of an intermediate diazonium cation. Labeling studies using $Na^{15}NO_3$ and [^{14}C]-3,4-DCA support this mechanism. When 3,4-dichloronitrobenzene is the substrate, it is reduced to tetrachloroazoxybenzene and tetrachloroazobenzene in both the absence and presence of added $NaNO_3$.

The fate of chlorinated anilines in the environment has been the subject of much interest because of the widespread use of herbicides containing a chloroaniline moiety. These anilines, principally 4-chloroaniline and 3,4-dichloroaniline (3,4-DCA), are formed in soil when the herbicides are hydrolyzed; they are then transformed further by microbial action. From 3,4-DCA the transformation products in soil include 3,3′,4,4′-tetrachloroazobenzene (1) (Bartha and Pramer, 1967; Bartha, 1968; Chisaka and Kearney, 1970; Sprott and Corke, 1971), 1,3-bis(3,4-dichlorophenyl)triazene (2) (Plimmer et al., 1970), and 3,3′,4′-trichloro-4-(3,4-dichloroanilido)azobenzene (3) (Linke, 1970). Interest in these systems revived when Poland et al. (1976) noted the structural similarity of azo compound 1 and 2,3,7,8-tetrachlorodibenzodioxin and reported that both compounds are potent inducers of liver aryl hydrocarbon hydroxylase [see also Sundström et al. (1978) and Bunce et al. (1979)].



Pure cultures of bacteria transformed 3,4-DCA into **1**, **2**, and the isomeric tetrachlorobiphenyls **4** and **5**, in addition to other unidentified materials. These processes took place only when nitrate was present and when the bacteria possessed a nitrate reductase enzyme (Corke et al., 1979; Shushan et al., 1981). It was proposed that these transformations involve the microbial reduction of NO_3^- to NO_2^- and that diazotization of the aniline then occurs [Corke et al., 1979; see also Minard et al. (1977)]. This was shown by intercepting the diazonium ion with 2-naphthol, and the formation of products **1**, **2**, **4**, and **5** was suppressed. Diazotization appears to be a purely chemical, as opposed to a biological, process because the rate of diazotization from preformed NO_2^- and the aniline is the same whether or not the cells are present (Lammerding et al., 1982).

In this paper we present further studies on the mechanism of the transformation of 3,4-DCA by a representative bacterial species (*Escherichia coli*) used in the previous

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

0021-8561/83/1431-1071$01.50/0

work. Our rationale for using *E. coli* for these model studies is that we had previously found (Corke et al., 1979) that maximum yields of the products derived from diazonium ions were obtained with this organism. We speculate that the reason for this is that *E. coli* is unusual in being able to reduce NO_3^- to NO_2^- but is inefficient at reducing NO_2^-. Consequently, the use of the organism is advantageous for model studies designed to probe the diazotization pathway. In addition to quantitation of the products, we have used ^{15}N-labeling studies to confirm the diazotization step proposed before and have extended our studies to 3,4-dichloronitrobenzene (3,4-DCNB).

EXPERIMENTAL SECTION

Solvents were from Caledon Laboratories, pesticide grade. Chlorinated anilines were purified by chromatography over alumina, eluting with benzene–hexane, and recrystallized. Both the anilines and 3,4-DCNB were free of impurities to better than the levels of the minor products analyzed, as shown by GLC.

For gas chromatography a Varian Model 200 gas chromatograph equipped with a tritium foil electron capture detector was used. The column was 1.5 mm o.d. × 10 ft glass, packed with 2.5% SE-30 on Chromosorb W, 80–120 mesh, HMDS treated, operated isothermally at 200 °C (injector 235 °C, detector 220 °C) with N_2 (50 mL min^{-1}) as the carrier gas.

For gas chromatography–mass spectrometry (GC–MS) a similar column was used with a Perkin-Elmer Sigma III gas chromatograph interfaced to a VG 7070-F double-focusing mass spectrometer.

Procedures. The procedures described below, and the results obtained, were those from typical experiments, which in all cases were performed in duplicate. Owing to the nature of the experiments, minor variations in product yields were observed from run to run.

Quantitation of Products. Cells of *E. coli* no. 263 from the culture collection of the Microbiology Department, University of Guelph, were grown in 200-mL volumes of the previously reported (Lammerding et al., 1982) glucose–phosphate medium. Also present were either 3,4-DCA (6×10^{-4} M, 100 ppm) or 3,4-DCNB (2.6×10^{-4} M, 50 ppm). For the quantitative experiments, the duplicated treatments were as follows. (a) 3,4-DCA plus 100 ppm of $NaNO_3$; (b) 3,4-DCA plus 100 ppm $NaNO_3$ plus 100 ppm of 2-naphthol; (c) 3,4-DCNB alone; (d) 3,4-DCNB plus 100 ppm of $NaNO_3$; (e) 3,4-DCNB plus 100 ppm of $NaNO_3$ plus 100 ppm of 2-naphthol. At the concentrations indicated, 2-naphthol did not inhibit the growth of the microorganisms. It had previously (Corke et al., 1979) been established that the reactions under study did not take place in the absence of nitrate (or equivalently nitrite). The procedure below was also used with minor modification for the experiments with 4-chloroaniline.

Cells of a 24-h culture were used to inoculate the medium at the rate of 2% v/v and were incubated in a cotton-stoppered 500-mL Erlenmeyer flask at 37 °C for 6 h with shaking by using a New Brunswick SCI Model G25 rotary-drive incubator operated at 125 oscillations/min. After this period, shaking was discontinued and the cells were incubated undisturbed for 5 days at 37 °C.

The orange mixture was then transferred aseptically into 250-mL polycarbonate centrifuge tubes and centrifuged (Sorvall Model RC-5B centrifuge fitted with a GSA head and prechilled to 4 °C) for 10 min at 8000 rpm (10400*g*). The supernatant fluid was decanted and extracted with 100 mL of hexane. The deep orange pellet was suspended in 30 mL of acetone, sonicated for 15 min to aid extraction, and centrifuged again. A second identical acetone extraction was performed on the resulting pellet. Even after these extractions, the pellet remained strongly colored. The extracts were dried and evaporated separately at 45 °C under vacuum, and the residue was redissolved in hexane (10 mL).

To aid in the analysis, the crude extracts were fractionated into simpler mixtures by chromatography over alumina (neutral alumina, Brockman activity I, 80–200 mesh, from Fisher Scientific). A 7 × 146 mm o.d. disposable Pasteur pipet plugged with glass wool was charged with alumina (2 g), and 2 mL of the crude hexane extract was added. Elution was by means of 2:1 v/v hexane–benzene (3 × 2 mL) followed by 2:1 v/v benzene–acetone (2 × 2 mL) and finally methanol (2 mL). Each of the six fractions was then analysed by gas chromatography and also by GC–MS. Both GLC retention times and mass spectra were compared with those of reference compounds; quantitation of products was done by comparison of gas chromatographic peak areas with those of reference standards of known concentration. In the case of the dihydroxytetrachlorobiphenyl, for which no standard was available, the response of the GC detector was assumed to be the same as for the tetrachlorobiphenyls. Residual aniline was determined by the method of Pease (1962), DCNB by GLC quantitation using a known standard. The production of 3,4-dichlorophenol was determined by the following modification of the standard method ("Standard Methods for the Examination of Water and Wastewater", 1976). To 1 mL of the medium (or medium plus cells) were added 3.5 mL of 0.1 M ammonia and 2.0 mL of phosphate buffer (K_2HPO_4, 0.12 M; KH_2PO_4, 0.11 M). This final solution had pH 7.9 ± 0.1. To this was added 0.1 mL of each of the following solutions: 4-aminoantipyrine (2 g/100 mL) and $K_3Fe(CN)_6$ (8 g/100 mL). The volume was brought to 10 mL and the absorbance was read at 510 nm after 15 min. A calibration curve was made up with known concentrations of 3,4-dichlorophenol.

Control experiments were done in which the diazonium salt (formed chemically from 3,4-DCA) was allowed to decompose in a sterile medium at 37 °C and pH 6.9. The yield of 3,4-dichlorophenol was 3.1%.

The results of these experiments are reported in Table I.

Isotopic Tracer Studies and Quantitation of 3,4-Dichlorophenol. Uniformly ring ^{14}C labeled 3,4-DCA plus unlabeled 3,4-DCA to a total concentration identical with that of experiment 1 was used in the following experiments. The [^{14}C]DCA was obtained from Amersham Corp. and was used at a concentration of 1 μCi mL^{-1}, with 1 mL of solution used/flask. (a) 3,4-DCA plus 100 ppm of $NaNO_3$ plus *E. coli* (chemical control and correction for background counts); (b) [^{14}C]-3,4-DCA plus 100 ppm of $NaNO_3$ plus *E. coli*; (c) [^{14}C]-3,4-DCA plus 100 ppm of $NaNO_2$, sterile control, pH 6.9; (d) [^{14}C]-3,4-DCA plus 100 ppm of $NaNO_2$, sterile control, pH 6.0. Also included was the following control: (e) 3,4-dichlorophenol (50 ppm) plus *E. coli*, pH 6.9.

The incubation conditions were identical with those in experiment 1. Periodic analyses were performed on these solutions to monitor (i) pH change, (ii) 3,4-DCA disappearance [method of Pease (1962)], (iii) the presence of 3,4-dichlorophenol in the medium using the 4-aminoantipyrine method (see above). Table II gives these results.

Scintillation counting was carried out by the following methods using a Beckman Model LS-3150T counter. (1) Phenethylamine was diluted to 10 mL with a toluene-based cocktail (0.3 g of POPOP and 5.0 g of PPO plus 100 mL of Triton X-100 per L of toluene). (2) Aqueous samples

Table I. Conversion of 3,4-DCA (3,4-DCNB) to Products by Microbial Transformation[a,c]

products/reactants[b]	DCA/NO_3^-	DCA/NO_3^-/ $C_{10}H_7OH$	DCNB	DCNB/NO_3^-	DCNB/NO_3^-/ $C_{10}H_7OH$
DCA or DCNB, initial	120 (100)	120 (100)	52 (100)	52 (100)	52 (100)
DCA or DCNB, recovered	7.3 (6.1)	65.6 (55)	<0.05	<0.05	<0.05
$C_{12}H_6Cl_4$, 4 and 5	0.08 (0.1)	tr		0.012 (0.05)	tr
$C_{12}H_6Cl_4N_2$, 1	0.06 (0.1)	0.007 (0.01)	0.15 (0.06)	0.010 (0.04)	0.004 (0.02)
$C_{12}H_6Cl_4N_2O$			0.27 (1.0)	0.58 (2.2)	0.19 (0.7)
$C_{12}H_4Cl_4(OH)_2$	0.07 (0.1)			n.d.	n.d.
bis-substituted triazene, 2	4.6 (7.7)	0.20 (0.3)		0.30 (1.2)	0.15 (0.6)
3,4-dichlorophenol[d]	38.0 (31.7)	n.d.		n.d.	n.d.
azonaphthol		18.2 (15.2)			2.7 (5.2)
total dichlorophenyl groups recovered	54.9 (45.8)				

[a] All quantities in micromoles. [b] Numbers in parentheses are percentages. [c] Abbreviations: $C_{10}H_7OH$ ≡ 2-naphthol; $C_{12}H_6Cl_4$ ≡ tetrachlorobiphenyl, total of both isomers; $C_{12}H_6Cl_4N_2$ ≡ tetrachloroazobenzene; $C_{12}H_6Cl_4N_2O$ ≡ tetrachloroazoxybenzene; $C_{12}H_4Cl_4(OH)_2$ ≡ tetrachlorodihydroxybiphenyl; bis-substituted triazene ≡ 1,3-bis(dichlorophenyl)triazene; azonaphthol = 1-dichlorophenylazo-2-naphthol; n.d. = not determined; tr = trace. [d] See also Table II.

Table II. pH Change, 3,4-DCA Disappearance, and 3,4-Dichlorophenol Formation with Time[a]

	time, h						
	0	6	24	48	72	144	168
experiment 1							
pH	6.9	6.7	6.25	6.3	6.3	6.3	6.3
DCA	100	98	75	61	50	29	24
DCP[b]	0	10	31	25	10	9	10
experiment 2							
pH	6.9	6.7	6.25	6.25	6.3	6.3	6.3
DCA	102	98	76	60	50	30	23
DCP	0	15	31	24	8	8	8
experiment 3							
pH	6.9	6.9	6.9	6.9	6.9	6.9	6.9
DCA	103	102	96	97	98	95	92
DCP	0	0	10	4	3	4	2
experiment 4							
pH	6.0	5.9	6.0	6.0	6.0	6.0	6.0
DCA	103	45	26	21	23	15	13
DCP	0	30	36	33	22	23	19

[a] Average of duplicate flasks. All quantities in $\mu g\ mL^{-1}$. [b] DCP = 3,4-dichlorophenol.

Table III. Disintegrations per Minute from Fractions Obtained on Incubation of [^{14}C]-3,4-DCA with *E. coli* at 37 °C[a]

experiment	volatiles	pellet	supernatant:whole	extract	residue	total
2b	228 (10)[b]	401 (18)	1031 (47)	476 (22)	518 (24)	1937 (87)
2c	50 (2)	0 (0)	1727 (79)	1662 (76)	27 (1)	2055 (93)
2d	160 (7)	559[c] (25)	1111 (51)	476 (22)	604 (27)	2107 (95)

[a] All dpm in thousands; sampling losses of 277 000 assumed for each experiment; total dpm introduced 2.22×10^6. [b] Numbers in parentheses are percentages based on the total dpm introduced. [c] A precipitate formed at pH 6.0 in this chemical control.

were counted by solubilizing 0.1 mL in 1 mL of Scintigest (Fisher) and then diluting to 10 mL with the cocktail. (3) Ether extracts were diluted directly to 10 mL with the cocktail. The following fractions were analyzed. (1) Volatile material; (2) cell pellet, after extraction; (3) supernatant liquid after centrifugation; (4) organic extract of supernatant liquid after extraction with hexane–ether (1:2 v/v); (5) aqueous medium after extraction with solvent. Table III gives the data.

In the case of the volatile material, the analysis was performed by using a vial of phenylethylamine (2 mL) suspended over the medium. Under these conditions CO_2 and volatile dichlorophenol are counted together. They were distinguished by dividing the sample, counting 1 mL directly, and determining phenol in the other 1 mL colorimetrically and then extracting the phenol into ether and counting again. Differences in counts were attributed to $^{14}CO_2$ (Table IV). Losses occurred during the base exchange (compare Tables III and IV). This causes the percentages of CO_2 reported in Table IV to be overestimates. The details of the base exchange were that 1 mL of the phenethylamine solution was acidified with 5 mL of 1 M HNO_3 and extracted into ether (5 mL). The ether layer was then extracted into 2 mL of 1 M NaOH. The ether layer was saved, and the NaOH extract was counted. Then the aqueous material was acidified and extracted back into the saved ether and counted again.

Table IV. Attribution of Volatile Material to $^{14}CO_2$ and to [^{14}C]-3,4-Dichlorophenol at the End of the Experiment (9 Days)

experiment	$^{14}CO_2$[a]	[^{14}C]-3,4-DCP[a]	% CO_2[b]
2b	44 317	73 504	2.0
2c	16 998	9 277	0.8
2d	31 944	48 523	1.4

[a] $^{14}CO_2$ plus [^{14}C]-3,4-DCP dpm are approximately half those reported as "volatiles" in Table III because half the volume was counted. [b] Percent of initial 3,4-DCA.

^{15}N Isotopic Labeling Studies. The compounds 3,4-DCA or 3,4-DCNB were used at the concentrations of experiment 1. Five duplicate treatments were carried out as follows. (a) 3,4-DCA plus 100 ppm of $Na^{14}NO_3$; (b) 3,4-DCA plus 100 ppm of $Na^{15}NO_3$; (c) 3,4-DCNB alone;

(d) 3,4-DCNB plus 100 ppm of $Na^{14}NO_3$; (e) 3,4-DCNB plus 100 ppm of $Na^{15}NO_3$. After extraction and chromatographic separation as before, the products were analyzed by GC–MS. As expected, no change was observed in the mass spectrum of any compound lacking nitrogen. We consider the various nitrogenous products case by case. *Tetrachloroazobenzene*, 1. Experiment a is compared with b and d with e. The 1 in experiment b contains one ^{15}N per molecule, but that from experiment e contains no ^{15}N. This was shown by measuring the intensity ratios of the ions at m/e 318–324. In the case of experiment b this whole molecular ion cluster was shifted to m/e 319–325 and with the intensities of the even mass ions at m/e 318–324 less than 10% of the odd mass ions at m/e 319–325 after correcting for the contributions of ^{13}C isotopes. *Tetrachloroazoxybenzene*. No ^{15}N incorporation was observed. *Bis(dichlorophenyl)triazene*, 2. Experiment a is compared with b and d with e. Both experiments b and e contain one ^{15}N (m/e 334 compared with 333).

RESULTS AND DISCUSSION

The yields of the various products are given in Table I. Taking the reaction of 3,4-DCA in the presence of NO_3^- as an example (first column of Table I), it is clear that the material balance is very poor; compounds 1–5 plus unreacted 3,4-DCA account for very little of the starting material. Thus, although the microbial transformation of 3,4-DCA in pure culture gives products, e.g., 1, 4, and 5, that are highly toxic, the actual amounts of such compounds formed are likely to be extremely small. To the extent that these results in pure culture have relevance to the field, they are consistent with our previous comments (Bunce et al., 1979) that the rather large amounts of tetrachloroazobenzene 1 previously found in fields treated with chloroaniline-based herbicides likely result from contamination of the original herbicide by 1 rather than from biological action.

A search by gas chromatography and by GC/MS for additional products turned up a probable dihydroxytetrachlorobiphenyl (m/e 324) and either an aminotetrachlorobiphenyl or a tetrachlorodiphenylamine (m/e 307). Both of these are minor products, however. Of them, the first most probably results from microbial oxidation of either 4 or 5 and hence is a first step in mineralization of the biphenyl derivative [cf. Tulp et al. (1978)]. The compound of mass 307 is more likely an aminotetrachlorobiphenyl on the following grounds: it is not identical with an authentic sample of 3,3′,4,4′-tetrachlorodiphenylamine, and Tanaka et al. (1981) have observed a pair of aminodichlorobiphenyl derivatives upon photolysis of Monuron [3-(*p*-chlorophenyl)-1,1-dimethylurea] in aqueous solution.

Given that diazonium ion cations were already believed to be present as intermediates in these reactions, an obvious product to seek was 3,4-dichlorophenol, which would form by decomposition of the diazonium salt in water. Considerable but not reproducible amounts of this product were indeed observed. In addition, the dichlorophenol was found to volatilize, for it could be detected in the phenethylamine solution that had been used for the purpose of trapping volatilized CO_2. In fact, most of the counts representing volatile material when [^{14}C]-3,4-DCA was the substrate could be attributed to 3,4-dichlorophenol rather than to CO_2. Very little mineralization of 3,4-dichlorophenol occurred during the time span of our experiments [cf. Baker and Mayfield (1980a,b)]. At 100 ppm of the phenol, bacterial growth was inhibited, but growth took place at concentrations of 25 and 50 ppm. The 3,4-dichlorophenol slowly disappeared from the medium in these experiments. However, entrapment in a vial of phenethylamine suspended in the headspace above the medium showed that the dichlorophenol was being lost mainly by volatilization rather than by metabolism. Similar results were obtained with sterile controls.

Returning to the question of the material balance, the uncertainty in the amount of diazonium salt decomposing via the phenol route makes it impossible to determine this quantity confidently. It is significant that the presence of 7×10^{-4} M 2-naphthol reduces the yields (based on DCA consumed) of the various products by >90% and correspondingly 34% of the azo coupling product forms. Consequently, we can be reasonably sure that a major proportion of the 3,4-DCA is converted to diazonium salt and ultimately to the phenol and whatever other products that form along with the phenol.

In a different approach to discovering the fate of the initial 3,4-DCA, uniformly ring labeled [^{14}C]-3,4-DCA was subjected to the microbial reactions. The following were examined: (i) volatile material; (ii) the cell pellet, as a measure of 3,4-DCA actually incorporated and unextractable; (iii) the supernatant liquid following centrifugation. This last was subdivided into material extractable and not extractable into an organic solvent (hexane–ether, 1:2). These results are in Table III. This time, a good (~90%) material balance was obtained. The greatest proportion of the radioactivity is found in the extractable products, with lesser amounts remaining in the cell pellet or having volatilized. Since the results of the quantitation experiments (Table I) refer to the material obtained from the organic extract of the supernatant liquid together with that extracted from the pellet with acetone, it is seen why the material balance in the previous experiment is so poor; a great deal of the starting material does not show up as extractable organic products.

Of the volatile material, most is represented by 3,4-dichlorophenol rather than by CO_2. This confirms that rather little mineralization of these compounds occurs during the time of our experiments. Both CO_2 and 3,4-dichlorophenol are trapped by phenethylamine, because both H_2CO_3 and 3,4-dichlorophenol have comparable pK_a values.

GC–MS Results. Of the various metabolites encountered, the chlorobiphenyls and the chlorohydroxybiphenyls contain no nitrogen. No shift in their molecular ion peaks would be anticipated when $^{15}NO_3^-$ was used in place of $^{14}NO_3^-$, and none was observed.

The 1,3-diaryltriazene is commonly accepted (Plimmer et al., 1970; Minard et al., 1977; Corke et al., 1979) as arising by reaction 1. Since the nitrogen atom derived

$$ArNH_2 \xrightarrow{HNO_2} ArN_2^+ \xrightarrow{ArNH_2} ArN{=}NNHAr \quad (1)$$

from nitrite (and thus from nitrate) occupies almost exclusively (Zollinger, 1961) the terminal position of the diazonium cation, this nitrogen becomes the central nitrogen atom of the triazene. When the experiment is run with $^{15}NO_3^-$, it is expected that the triazene should incorporate one ^{15}N atom. In accord with this expectation, the ^{35}Cl molecular ion of the triazene shifts almost entirely from m/e 333 to m/e 334 when $^{15}NO_3^-$ is used. This is true for the runs with both 3,4-DCA and 3,4-DCNB, although the actual amount of the triazene is much smaller in the latter case. Correspondingly, the azonaphthol that is formed in the experiments run in the presence of 2-naphthol also incorporates one ^{15}N from $Na^{15}NO_3$ (Lammerding et al., 1982).

The genesis of tetrachloroazobenzene 1 from 3,4-DCA in soil has not previously been settled decisively. Bartha (1968) suggested an oxidative mechanism (eq 2); our

$$2ArNH_2 \xrightarrow{2O} ArN{=}NAr \quad (2)$$

trapping experiments with 2-naphthol (Corke et al., 1979) indicated that under the very different conditions of pure microbial culture, 1 arose from a diazonium ion (eq 3).

$$2ArNH_2 \xrightarrow{HNO_2} 2ArN_2^+ \xrightarrow[-N_2]{\text{reduction}} ArN{=}NAr \quad (3)$$

Although the details of eq 3 remain obscure, the labeling experiments clearly favor this latter pathway. For both 4-chloroaniline and 3,4-DCA, a shift to 1 higher mass unit is observed when the aniline is transformed by the microorganisms in the presence of $^{15}NO_3^-$. We cannot say whether this mechanism also operates under field conditions.

A compound that has been observed regularly in the GC–MS runs from 3,4-DCA is a substance having M^+ 307, and a chlorination pattern corresponding to four chlorine substituents. Such a mass is possible for either a bis(dichlorophenyl)amine or an aminotetrachlorobiphenyl. This compound did not incorporate ^{15}N from $Na^{15}NO_3$.

From the experiments presented here and our previous results, we conclude that in suspensions of *E. coli* under partly anaerobic conditions, the microbial transformation of 3,4-dichloroaniline (and most likely, other anilines) proceeds in three phases, as follows. (1) Reduction of NO_3^-. This is a purely biological reaction as shown by the requirement for a nitrate reductase enzyme system in the organism. (2) Diazotization of $ArNH_2$. This is a purely chemical reaction. This is shown by the agreement between the rate of disappearance of the aniline in the biological process and that of the chemical controls using NO_2^-. (3) Conversion of ArN_2^+ to products. This is a combination of chemical and biological processes. The major product (3,4-dichlorophenol) is formed by the decomposition of ArN_2^+ in water. The intervention of the organisms is shown by the formation of other products that are not produced chemically and by the incorporation of ^{14}C into the cell pellet.

Experiments with 3,4-DCNB. The microbial reduction of chlorinated nitrobenzenes is not a new process; pentachloronitrobenzene, for example, is known (Buser and Bosshardt, 1975) to be reduced to pentachloroaniline in soil. Just as nitrate ion can act as an electron acceptor, so also can aromatic nitro compounds (compare the structures $^-O–NO_2$ and $Ar–NO_2$). Chemically, aromatic nitro compounds $ArNO_2$ can be reduced to a wide variety of products: anilines $ArNH_2$, azoxy compounds ArN=N(O)Ar, and azo compounds ArN=NAr among others. Exactly these processes are seen to occur with 3,4-DCNB.

In the absence of added nitrate, small amounts of tetrachloroazobenzene 1 and the corresponding azoxy compound are formed. Under these conditions, with no added nitrate, these two compounds cannot incorporate ^{15}N.

When nitrate is also present in the medium the 3,4-DCA that is formed by reduction of the nitro compound can react further, by the diazonium salt route. Accordingly, the whole spectrum of products usually formed from 3,4-DCA is now observed and 2-naphthol intervenes in a manner similar to that observed with 3,4-DCA (Table I). An interesting result is found with added $Na^{15}NO_3$, however; most of the tetrachloroazobenzene and tetrachloroazoxybenzene are produced by direct reduction and not by the diazotization pathway. This is shown because neither of these products incorporates any appreciable amount of ^{15}N under these reaction conditions.

Registry No. 1, 14047-09-7; 2, 14581-48-7; 4, 32598-13-3; 5, 41464-43-1; 3,4-DCA, 95-76-1; 3,4-DCNB, 699-54-7; 3,4-dichlorophenol, 95-77-2; tetrachloroazoxybenzene, 21232-47-3; tetrachlorodihydroxybiphenyl, 86374-32-5; 1-dichlorophenylazo-2-naphthol, 86374-33-6; nitrate, 14797-55-8.

LITERATURE CITED

Baker, M. D.; Mayfield, C. I. *Water Res.* **1980a**, *14*, 1765.
Baker, M. D.; Mayfield, C. I. *Water, Air, Soil Pollut.* **1980b**, *13*, 411.
Bartha, R. *J. Agric. Food Chem.* **1968**, *16*, 602.
Bartha, R.; Pramer, D. *Science (Washington, D.C.)* **1967**, *156*, 1617.
Bunce, N. J.; Corke, C. T.; Merrick, R. L.; Bright, J. H. *Chemosphere* **1979**, *8*, 283.
Buser, H. R.; Bosshardt, H. P. *Pestic. Sci.* **1975**, *6*, 35.
Chisaka, H.; Kearney, P. C. *J. Agric. Food Chem.* **1970**, *18*, 854.
Corke, C. T.; Bunce, N. J.; Beaumont, A. L.; Merrick, R. L. *J. Agric. Food Chem.* **1979**, *27*, 644.
Lammerding, A. M.; Bunce, N. J.; Merrick, R. L.; Corke, C. T. *J. Agric. Food Chem.* **1982**, *30*, 644.
Linke, H. A. B. *Naturwissenschaften* **1970**, *57*, 307.
Minard, R. D.; Russel, S.; Bollag, J. M. *J. Agric. Food Chem.* **1977**, *25*, 841.
Pease, H. L. *J. Agric. Food Chem.* **1962**, *10*, 279.
Plimmer, J. R.; Kearney, P. C.; Chisaka, H.; Yount, J. B.; Klingbeil, U. I. *J. Agric. Food Chem.* **1970**, *18*, 859.
Poland, A.; Glover, E.; Kende, A. S.; DeCamp, M. R.; Giandomenico, C. M. *Science (Washington, D.C.)* **1976**, *194*, 627.
Shushan, B.; Bunce, N. J.; Boyd, R. K.; Corke, C. T. *Biomed. Mass. Spectrom.* **1981**, *8*, 225.
Sprott, G. D.; Corke, C. T. *Can. J. Micro.* **1971**, *17*, 235.
"Standard Methods for the Examination of Water and Wastewater", 14th ed.; American Public Health Association: Washington, DC, 1976; p 582.
Sundström, G.; Jansson, B.; Renberg, L. *Chemosphere* **1978**, *7*, 973.
Tanaka, F. S.; Wien, R. G.; Hoffer, B. L. *J. Agric. Food Chem.* **1981**, *29*, 1153.
Tulp, M. T. M.; Schmitz, R.; Hutzinger, O. *Chemosphere* **1978**, *7*, 103.
Zollinger, H. "Azo and Diazo Chemistry"; Interscience: London, 1961; pp 40–41.

Received for review July 13, 1982. Revised manuscript received February 7, 1983. Accepted May 23, 1983. This research was supported by funds from the Ontario Ministry of Agriculture and Food.