Microbial Metabolism of the Fungicide 2,6-Dichloro-4-nitroaniline

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Escherichia coli B, *Pseudomonas cepacia*, and an unidentified bacterium were examined for their capacity to metabolize the fungicide 2,6-dichloro-4-nitroaniline (DCNA) in liquid culture using [14C]DCNA. *E. coli* B and *P. cepacia* both converted DCNA to 2,6-dichloro-*p*-phenylenediamine (DCPD) and 4-amino-3,5-dichloroacetanilide (ADCAA). Culture fluids of the unknown bacterium incubated with [14C]DCNA yielded ADCAA but no detectable amounts of DCPD. The organisms differed in their capacity to produce these metabolites. *Pseudomonas cepacia* completely metabolized DCNA within 48 hr, and accumulated ADCAA in the cultural fluid as the major metabolite. Similarly, ADCAA was the major DCNA metabolite accumulated by the unknown bacterium. *E. coli* B rapidly utilized DCNA but accumulated DCPD as the major DCNA metabolite. Conditions of low aeration favored conversion of DCNA to DCPD and ADCAA. At least four other unidentified DCNA metabolites were produced by the bacteria.

The fungicide 2,6-dichloro-4-nitroaniline (DCNA, Botran) is widely used for the postharvest control of *Rhizopus* rot on sweet potato roots and on fruits of cherries, nectarines, and peaches (Ogawa *et al.*, 1961; Martin, 1964; Wells, 1972). DCNA is also used on a number of vegetable crops for the control of foliage diseases caused by *Botrytis* spp. and *Sclerotinia* spp. (Beckman and Parsons, 1965; Higgons, 1961).

By such usage, the chemical is available for possible utilization by a variety of living organisms. In this regard, the fate of the chemical has been studied in plants such as lettuce and tomato which absorb DCNA through roots and translocate the chemical to leaves and stems where it is rapidly degraded (Lemin, 1965). Soybean plants are reported to metabolize DCNA to a compound tentatively identified as 4-amino-3,5-dichloromalonanilic acid (USDA, 1968). In rats most (70.4%) of the [14C]DCNA administered orally or intraperitoneally is recovered in the urine as 4-amino-3,5-dichlorophenol. A small amount (2.4%) is converted to 2,6-dichloro-*p*-phenylenediamine (Maté *et al.*, 1967). Bacteria have been isolated that metabolize DCNA to CO_2 (Groves and Chough, 1970). Microorganisms in soil under flooded conditions rapidly metabolize DCNA to a number of unidentified compounds. In sterile soil amended with nutrients, DCNA is utilized under aerobic as well as anaerobic conditions (Wang, 1972). Apart from CO_2 none of the microbial metabolites of DCNA have been identified.

The object of our study was to determine the manner in which microorganisms metabolize DCNA and to identify the major metabolic products of the chemical.

MATERIALS AND METHODS

Cultures. The bacteria used in this study were selected because they rapidly metabolize DCNA (Van Alfen, 1972). The isolates and their sources are *Escherichia coli* B (A. J. Clark, Department of Molecular Biology, University of California, Berkeley), *Pseudomonas cepacia* (J. W. Lorbeer, Department of Plant Pathology, Cornell University), and an unidentified bacterium that was isolated from the surface of a peach fruit (Van Alfen, 1972).

Chemicals. DCNA (2,6-dichloro-4-nitroaniline, Upjohn Co., Kalamazoo, Mich.) was recrystallized twice from benzene. Uniformly 14C-labeled DCNA, specific activity 0.98 μCi/μmol, was a gift of J. M. Ogawa, Department of Plant Pathology, University of California, Davis. The

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[14C]DCNA was found to be at least 99.7% pure by silica gel thin-layer chromatography (tlc) in benzene–diethyl ether (1:1, v/v). 2,6-Dichloro-*p*-phenylenediamine (DCPD) (mp 119.5–120.5°) was synthesized by reducing DCNA over zinc and acid (Roburn, 1961). 4-Amino-3,5-dichloroacetanilide (ADCAA) was synthesized by reacting 2.5 mmol of 2,6-dichloro-*p*-phenylenediamine, 3.0 mmol of sodium acetate, and 3.0 mmol of acetic anhydride in 30 ml of 0.001 *N* HCl at room temperature. The precipitate which formed within 1 min after mixing the reactants was collected by filtration and washed with 20 ml of water. The precipitate was recrystallized twice from acetone–water (43 mg, 77% yield, mp 205–207°).

Anal. Calcd for $C_8Cl_2H_8ON_2$: C, 43.85; H, 3.68; N, 12.79; Cl, 32.36. Found: C, 43.95; H, 3.71; N, 12.67; Cl, 32.22.

Instrumentation. Infrared and nuclear magnetic resonance (nmr) spectra were obtained on a Perkin-Elmer model 337 infrared spectrophotometer and a Hitachi Perkin-Elmer R-20 nmr spectrometer (60 MHz), respectively. Mass spectra were obtained on either an Associated Electrical Industries, Ltd., MS-7 or a Varian M-66 mass spectrometer. Radioactive samples were counted in a Nuclear Chicago model 6822 liquid scintillation counter. The scintillation solution consisted of 5.0 g of 2,5-diphenyloxazole and 0.1 g of dimethyl-*p*-bis(2-(5-phenyloxazolyl)benzene)/ l. of toluene. When aqueous solutions were counted, 1 part of Triton X-100 was used/2 parts of the toluene scintillation solution. The channels' ratio method was used to correct for quenching (Bush, 1963).

Detection of Products of DCNA Metabolism. Bacteria were cultured in 3 ml of liquid medium 523 (Kado and Heskett, 1970) containing 2 μg/ml of [14C]DCNA. After incubation for 2 days at 30° on a rotary shaker, the bacteria were pelleted by centrifugation of the cultures at 10,000 × *g*. The supernatant liquid was extracted with twice its volume of benzene, the benzene solutions were concentrated to approximately 50 μl under a stream of N_2, and the concentrated preparation was spotted on Eastman silica gel tlc plates. The plates were developed in an Eastman chromagram apparatus with benzene–diethyl ether (1:1, v/v). Autoradiograms then were prepared from the silica gel plates using Kodak no-screen medical X-ray film. Portions of the chromatograms bearing radioactive material were cut out (plastic backed plates), the pieces placed in 5 ml of scintillation counting solution, and the samples counted in the liquid scintillation counter.

Isolation of DCNA Metabolites. Ten-liter jars with 5 l. of liquid medium 523, amended with 20 μg of DCNA/ml, were autoclaved for 45 min at 121°. The medium was inoculated with 10 ml of a 12-hr culture of bacteria and incubated at room temperature (approximately 23°) with good
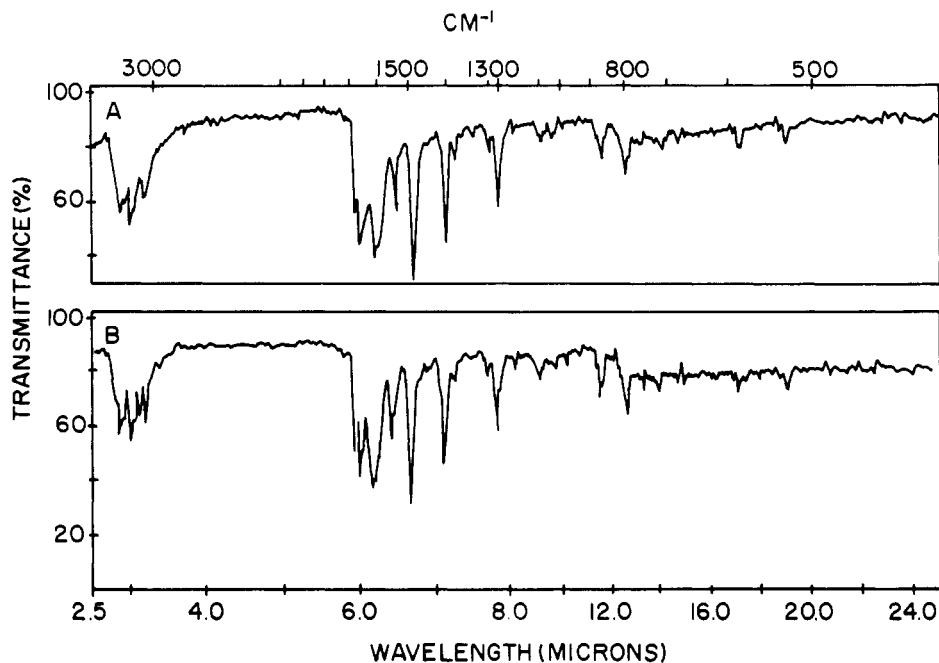
Figure 1. The infrared spectra of synthetic 4-amino-3,5-dichloroacetanilide (A) and compound M3 (B).
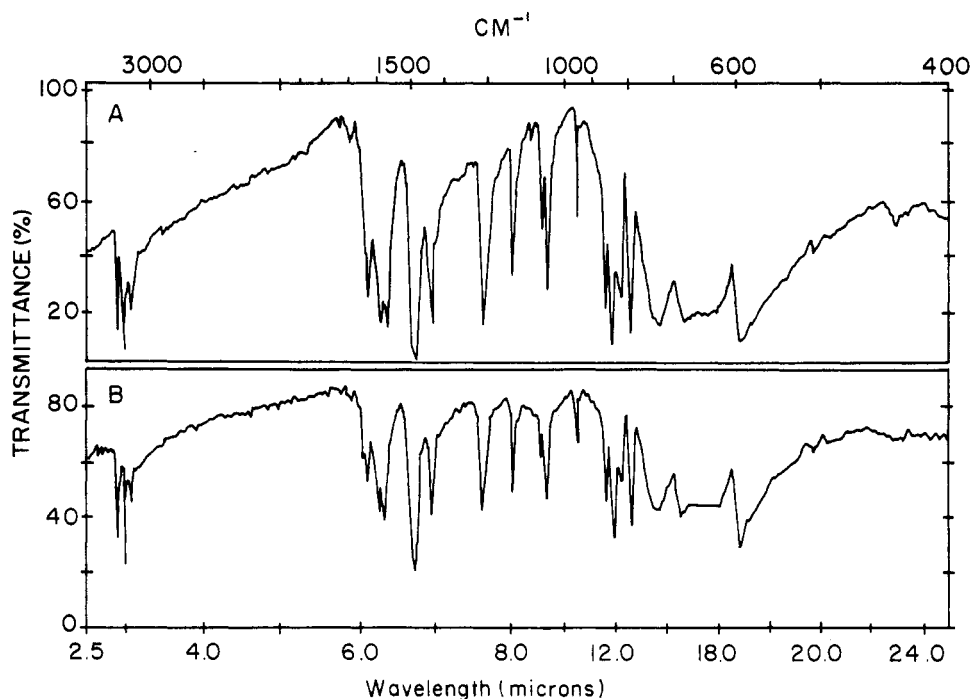

Figure 2. The infrared spectra of synthetic 2,6-dichloro-p-phenylenediamine (A) and compound M5 (B).

Table I. DCNA Metabolites Produced by Bacteria

Bacteria	M1[a]	M2	M3	M4	M5	M6
P. cepacia	0.6[b]	1.1	76.4	0	0.3	0.3
E. coli B	1.0	0.6	2.0	3.2	52.4	16.2

[a] Benzene-soluble, radioactive compounds isolated from 48-hr cultures of the bacteria in 3 ml of 523 liquid medium amended with 2 μg of [^{14}C]DCNA/ml, specific activity 0.96 μCi/μmol. The metabolites (M1, M2, etc.) refer to those detected by thin-layer chromatography of the benzene extract of the bacterial culture fluid in benzene–diethyl ether (1:1, v/v). R_f values were: M1, 0; M2, 0.14; M3, 0.35; M4, 0.45; M5, 0.70; amd M6, 1.0. [b] Calculated by (dpm in the metabolite/dpm [^{14}C]DCNA administered) × 100.

aeration (5 l./min) for 3 days. The cultures were centrifuged at 10,000 × g and the bacterial pellet was discarded. The culture fluid, 5 l., was extracted with 1 vol of benzene, the benzene solution concentrated by evaporation at 45° to approximately 2 ml, and the concentrated preparation streaked onto Merck preparative silica gel tlc plates (2 mm thick). After the plates were developed in benzene–diethyl ether (1:1, v/v), the DCNA metabolites were eluted from the silica gel with acetone. The acetone solution was concentrated by evaporation at 45° and chromatographed on a second Merck preparative silica gel tlc plate in chloroform–acetone (7:3, v/v). The bands of silica gel which contained DCNA metabolites were removed and the metabolites eluted from the gel with acetone. The ac-
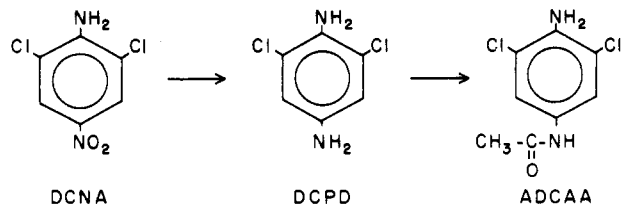
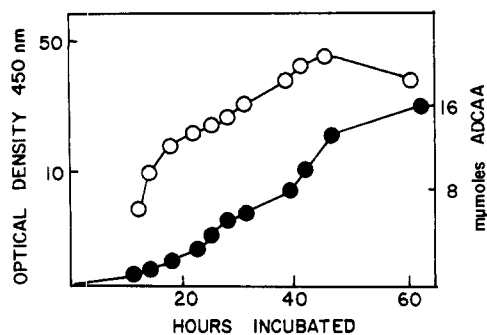**Figure 3.** Proposed pathway of metabolism of DCNA by bacteria in liquid culture.

Figure 4. Time–course study of growth and DCNA metabolism of *Pseudomonas cepacia.* (O———O), growth of the bacterium in liquid medium 523 as measured by optical density at 450 nm; (●———●), mμmol of ADCAA formed in the culture.

etone solutions were evaporated to dryness and the precipitates recrystallized twice from an acetone–water solution.

Time-Course Studies. Portions (1 ml) of a culture in 15 ml of medium 523 containing [^{14}C]DCNA (4.5 μg/ml) were removed at various time intervals after inoculation. The OD$_{450}$ (1-cm pathlength) of the samples was determined using a Zeiss PMQ II spectrophotometer. The bacteria then were pelleted and radioactive compounds in the culture fluids were detected by tlc as previously described.

RESULTS AND DISCUSSION

Products of DCNA Metabolism. After a 48-hr incubation of the bacterial isolates in liquid medium 523 amended with [^{14}C]DCNA, at least 95% of the radioactivity administered as [^{14}C]DCNA was recovered in the culture fluids of all three bacteria. In each case over 80% of the radioactivity from the bacterial culture fluid was extracted into benzene. Autoradiograms of the tlc chromatograms of the benzene extract of the *E. coli* B cultures showed six radioactive compounds other than DCNA (Table I). These compounds, labeled as M1, M2, M3, M4, M5, and M6 for convenience, had R_f values of 0.0, 0.14, 0.35, 0.45, 0.70, and 1.0, respectively. However, more than one DCNA metabolite may occur in certain radioactive areas on the chromatogram, particularly in regions occupied by M1 and M6. In this system DCNA has an R_f value of 0.87. With the exception of M4, the same metabolites were detected in DCNA-amended cultures of *P. cepacia.* Although M3 was the most abundant DCNA metabolite found in cultures of *P. cepacia,* M5 occurred in greatest quantities in *E. coli* B cultures (Table I). In cultures of bacterium F-8 (data not shown), 90% of the DCNA had been converted to M3. M1 and M2, the only other DCNA metabolites detected in the cultures of bacterium F-8, accounted for less than 5% of the DCNA initially present in the medium.

Identification of Metabolites. Compound M3 was isolated from a culture of bacterium F-8 by procedures described in the Methods section. The R_f values in chloroform–acetone and benzene–diethyl ether were 0.1–0.2 and 0.3–0.5, respectively. Eighteen milligrams of crystalline M3 (mp 207–209°) was recovered from a culture that had
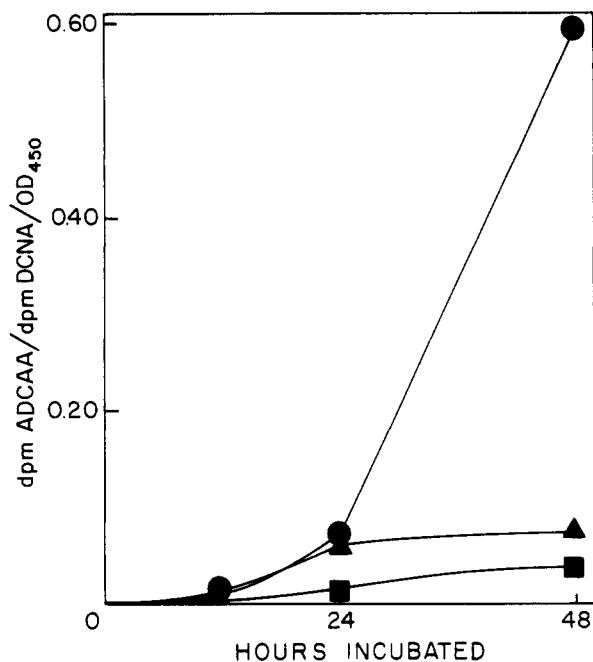

Figure 5. Formation of 4-amino-3,5-dichloroacetanilide from DCNA by *P. cepacia.* Ratio of dpm ADCAA/dpm DCNA per unit of optical density at 450 nm of the bacterial culture is plotted as a function of incubation time in hours. The bacteria were grown at 28° in medium 523 that was aerated with water-washed air. The rates of aeration were: 1 ml/min (●———●); 13 ml/min (▲———▲); and 120 ml/min (■———■).
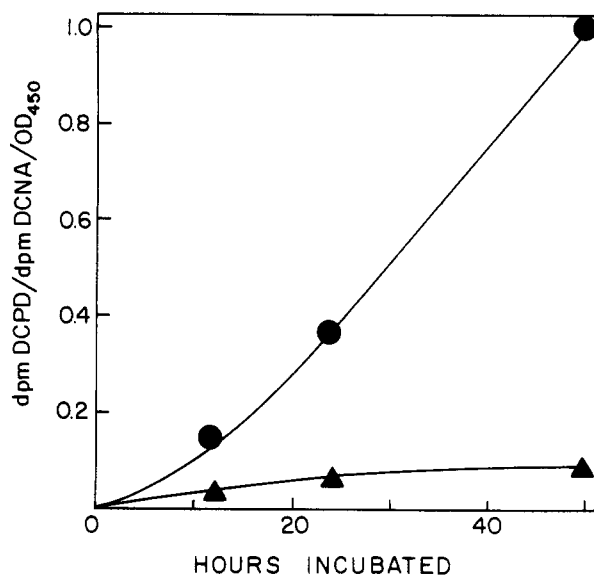

Figure 6. Formation of DCPD from DCNA by *E. coli* B (defined as the ratio of dpm DCPD/dpm DCNA per unit of optical density at 450 nm of the bacterial culture) is plotted as a function of incubation time in hours. The cultures were incubated at 30° and gassed with air (▲———▲) or with N$_2$ (●———●).

been amended with 125 mg of DCNA. By mass spectrometry, compound M3 showed a parent peak of m/e 218 which corresponds to the molecular weight of ADCAA. The spectrum also showed P + 2 and P + 4 peaks with intensities of 62 and 10% relative to the parent peak, indicating the presence of two chlorine atoms. By the same criteria, the fragment which produced the base peak (m/e 176) also contained two chlorine atoms. This fragment is the result of a rearrangement to give NH$_2$PhCl$_2$NH$_2^+$. The only other fragments with relative intensities greater than 10% of the major fragment were m/e 43 which corresponds to CH$_3$C$^+$=O, and m/e 175 (NH$_2$(Cl)$_2$PhN$^+$H),

both of which are expected fragments of ADCAA. The nmr data indicated the presence of eight hydrogens with the following chemical shifts: 9.8 (s, 1 H,–CNH), 7.5 (s, 2 H, aromatic protons), 5.19 (s, 2 H, NH_2), 2.02 (s, 3 H, CH_3). Infrared spectra of chemically synthesized ADCAA and the isolated metabolite were identical (Figure 1).

Another metabolite extracted into benzene (M5) was isolated from cultures inoculated with *E. coli* B. The isolation procedures described in the Methods section were followed except that the cultures were incubated 7 days at 23° in standing cultures without aeration. By tlc in the benzene–diethyl ether and the chloroform–acetone solvent systems M5 and R_f values of 0.65–0.7 and 0.45–0.6, respectively. The purification procedure yielded 39 mg of crystalline M5 (mp 119.5–120.5°) from a culture that initially contained 200 mg of DCNA. Mass spectrometry showed that the base and parent peak of compound M5 was m/e 176, which is the molecular weight of DCPD. The parent peak had P + 2 and P + 4 peaks with intensities of 72 and 12%, respectively, indicating the presence of two chlorine atoms. There were no other fragmentation peaks with intensities greater than 10% relative to the major fragment. The nmr data indicated the presence of six hydrogens with the following chemical shifts: 6.62 (s, 2 H, aromatic protons) and a broad peak at 4.2 (s, 4 H, NH_2, NH_2). The infrared spectra of the chemically synthesized DCPD and the isolated metabolite were identical (Figure 2).

Thus the initial step in the metabolism of DCNA by the bacteria is a reduction of the nitro group to an amine yielding DCPD (Figure 3). The latter then is acetylated to form ADCAA. The reduction appears to be the rate-limiting step since, in media amended with DCNA, some bacteria that are capable of forming ADCAA do not accumulate DCPD. In a test of 18 different bacterial isolates that were incubated with DCNA, Van Alfen (1972) observed that 17 synthesized DCPD and 10 accumulated detectable amounts of ADCAA. Thus, the series of reactions shown in Figure 3 may represent a general route of DCNA metabolism in bacteria. Many fungi also have the capacity to form DCPD and ADCAA from DCNA (Van Alfen, 1972). However, fungi produce other products, as yet unidentified, as major DCNA metabolites. Acetylation of an aniline compound by microorganisms has been reported by Tweedy *et al.* (1970a,b), who showed that four soil microorganisms acetylate *p*-bromoaniline, a metabolite of the herbicide 3-(*p*-bromophenyl)-1-methoxy-1-methylurea (metobromuron).

We were unable to detect an azobenzene or other oxidation product of DCPD similar to that which has been reported for 3,4-dichloroaniline, a metabolite of the herbicide 3,4-dichloropropionanilide (propanil) (Bartha and Pramer, 1970) and for 3-chloro-4-methoxyaniline (Briggs and Ogilvie, 1971). Tweedy *et al.* (1970a,b) also failed to

detect azobenzene formation from metobromuron. However, their experiments as well as ours were carried out under conditions quite different from these reported by Bartha and Pramer (1970) and by Briggs and Ogilvie (1971).

Effects of Cultural Conditions on the Metabolism of DCNA. Figure 4 illustrates data of an experiment that relate growth of *P. cepacia* to the metabolism of DCNA to ADCAA by the bacterium. Under the conditions of this experiment, the DCNA was not completely metabolized. The bacterium appears to continue to convert DCNA to ADCAA as long as it grows.

Since the rate and degree of conversion of DCNA to ADCAA seemed to vary from one experiment to another, the effect of rate of aeration on DCNA metabolism by *P. cepacia* was studied. The results of this experiment (Figure 5) show that the greatest rate of conversion of DCNA to ADCAA occurred when the rate of aeration was lowered.

To confirm that the reduction of DCNA to DCPD was enhanced by conditions of low aeration, *E. coli* B, which accumulates DCPD in the culture fluid, was cultured under air and under N_2. The results show (Figure 6) that the rate of conversion of DCNA to DCPD is significantly greater under N_2 than the rate of conversion under air. This is to be expected for such a reductive reaction. The results of these experiments substantiate the report by Wang (1972) that DCNA in soil is more rapidly degraded under flooded (anaerobic) than upland (aerobic) conditions and suggest that the initial step in the metabolism of DCNA in soil is its reduction to DCPD.

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