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Microbial Metabolism and Cometabolism of Nitrophenols[1]

D. G. M. RAYMOND[2] AND M. ALEXANDER

Laboratory of Soil Microbiology, Department of Agronomy, Cornell University, Ithaca, New York 14850

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A bacterium able to use *p*-nitrophenol as a carbon and energy source was isolated from soil by enrichment culture. Nitrite was formed in stoichiometric amounts from *p*-nitrophenol by the organism, and chloroform-treated resting cells generated 4-nitrocatechol from the same substrate. Cells grown on *p*-nitrophenol oxidized *m*-nitrophenol to nitrohydroquinone, but the organism did not use *m*-nitrophenol as a carbon source for growth.

INTRODUCTION

Many pesticides important in agriculture contain nitro groups. The fungicide pentachloronitrobenzene (PCNB), the insecticides O,O-diethyl O-*p*-nitrophenyl phosphorothioate (parathion) and O,O-dimethyl O-*p*-nitrophenyl phosphorothioate (methyl parathion), and herbicides such as 3,5-dinitro-*o*-cresol (DNOC), 2-*sec*-butyl-4,6-dinitrophenol (DNBP), 2,5-dichloro-3-nitrobenzoic acid (Dinoben), and α,α,α-trifluoro-2,6-dinitro-N,N-di-*n*-propyl-*p*-toluidine (trifluralin) represent several of the most important pesticidal chemicals containing nitro groups. Nevertheless, surprisingly little is known about how such compounds are metabolized by soil and aquatic microorganisms, which are probably frequently the agents for the destruction of these compounds in nature.

A reduction of the nitro substituent of PCNB can be effected by certain actinomycetes with the corresponding formation of pentachloroaniline (1). Similarly, a soil pseudomonad has been found to bring about the successive reduction of both nitro groups of DNOC to yield first 3-amino-5-nitro-*o*-cresol and then, after cleavage of one amino group, 3-methyl-5-aminocatechol (2). On the other hand, a DNOC-utilizing *Arthrobacter* acted on the herbicide by releasing nitrite from the molecule (3).

Recent evidence has shown that microorganisms may modify pesticides either by using these chemicals as sources of energy, carbon, or nitrogen, bringing about extensive degradation in the process, or by a phenomenon known as *cometabolism* (4). In a cometabolic reaction sequence, the microorganism enzymatically transforms a compound which it cannot utilize as a source of energy or of one of the constituent elements in the substrate. The present study was designed to investigate the bacterial cometabolism of model nitroaromatic compounds and their metabolism when used as sources of carbon and energy for microbial growth.

MATERIALS AND METHODS

The nitrophenol utilizing bacterium was isolated from Honeoye silt loam by enrichment culture in a medium containing 5.5×10^{-4} M *p*-nitrophenol (PNP), 0.050 M phosphate buffer (pH 7.0), and 0.50 g $(NH_4)_2SO_4$, 0.10 g $MgSO_4 \cdot 7H_2O$, 20 mg $CaCl_2 \cdot 2H_2O$,

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[2] Deceased.

10 mg yeast extract, and 1.0 mg $FeCl_3$/l. of distilled deionized water. The soil was added to 100 ml of this medium contained in 250 ml Erlenmeyer flasks, and after 8 days of incubation at 30° on a rotary shaker, the growth was transferred to fresh medium. Because bacterial replication was not maintained after two such serial transfers, 0.1% soil extract was added to the medium, an addition which allowed for proliferation to proceed. Soil extract was prepared by autoclaving for 20 min a mixture containing 200 g Honeoye silt loam, 1.0 g Na_2CO_3, and 1000 ml water; the filtrate obtained after passing the cooled suspension through Whatman No. 42 filter paper was used. The enrichment was streaked on a medium containing the same ingredients plus 2.0% agar and 0.1% glucose that had been autoclaved separately. The colonies were then picked and the cultures purified by restreaking.

The bacteria were usually grown in the PNP medium described above with soil extract included. In some instances, Trypticase Soy Broth (BBL) fortified with 5.5×10^{-4} M PNP was used to obtain large cell yields. The medium was contained either in 500 or 2000 ml baffled Erlenmeyer flasks filled one-fifth with medium and incubated at 30° on a rotary shaker or for 36 hr at 30° in a 14-l. fermentor containing 12 l. of medium and aerated with 14 l. of air/min. The agitator in the fermentor was operated at a speed of 400 rev/min. The rate of growth was assessed turbidimetrically at 650 nm.

For the preparation of resting cell suspensions, bacteria in the logarithmic phase of growth were collected by centrifugation and washed three times with cold 0.05 *M* phosphate buffer, pH 7.0. Cells derived from cultures grown in the PNP-supplemented Trypticase Soy Broth were then incubated at 30° on a shaker with the PNP-salts medium for 3 to 6 hr, by which time the yellow color of PNP had disappeared; the organisms were then collected and washed again. The bacteria were suspended in 0.05 *M* phosphate buffer, pH 7.0, or in the salts solution minus the $(NH_4)_2SO_4$ but with 5.5×10^{-4} *M* PNP or *m*-nitrophenol (MNP).

Nitrite was estimated by the method of Montgomery and Dymoc (5), and ammonium and nitrate were determined by the Nessler and brucine methods, respectively (6). Nitrate estimations by the brucine method were corrected for nitrite in the sample. Oxygen uptake was assessed manometrically by standard methods (7) at 30° with air as the gas phase. The side arm contained an amount of cells equivalent to 0.1 mg dry wt. The recorded values for gas exchange are corrected for endogenous respiration.

Visible and ultraviolet spectra were obtained by means of a Beckman DB-G spectrophotometer. To prepare difference spectra, a 2.8 ml aliquot of the PNP or MNP reaction mixture was made alkaline by the addition of 0.2 ml of 40% NaOH, and the optical density was determined at 400 or 390 nm, respectively. An alkaline solution of either PNP or MNP was then prepared to have the same absorbancy at 400 or 390 nm, respectively, as the reaction mixture. This alkaline standard solution was then placed in the reference cell of the spectrophotometer, and the alkaline reaction mixture was placed in the sample cell. In this way, substances having absorbancies at different wavelengths from PNP or MNP were readily detectable.

To determine the concentrations of PNP or MNP in the presence of 4-nitrocatechol or nitrohydroquinone, the clarified reaction mixtures were made alkaline by the addition of 0.2 ml of 40% NaOH to a 2.8 ml aliquot or by dilution of the clarified reaction mixture with 1 N NaOH. The concentration of 4-nitrocatechol in the basic reaction mixture was determined by comparing the optical density at 510 nm of the unknown and standard solutions. To estimate the

PNP concentration in solutions containing 4-nitrocatechol, from the recorded optical density at 400 nm was subtracted 0.56 times the absorbancy of the solution at 510 nm. This correction was necessary because of the absorption by 4-nitrocatechol at 400 nm.

The concentration of MNP in the alkaline solution was established from the optical density (OD) at 390 nm. The level of MNP in the presence of nitrohydroquinone was estimated by determining the OD_{390} and OD_{540} of the clarified alkaline reaction mixture; because nitrohydroquinone has an absorbancy at 390 nm equal to 0.25 its absorbancy at 540 nm, a value equivalent to 0.25 times the OD_{540} was subtracted from the optical density at 390 nm to correct for nitrohydroquinone interference. The concentration of nitrohydroquinone was estimated directly from the OD_{540} of the clarified alkaline solution, a wavelength at which MNP does not interfere.

To extract microbial products from the reaction mixtures, the suspensions were clarified by centrifugation at 6000*g* for 15 min. The liquid was adjusted to pH 2 by the addition of 6 N HCl and extracted three times with dry diethyl ether. The aqueous phase was adjusted to pH 9 with 40% NaOH, and its absorption in visible and UV light were examined relative to water to ensure complete extraction; if unextracted products were present, the aqueous phase was acidified again with 6 N HCl and reextracted. The ether solutions were pooled, dried with anhydrous Na_2SO_4, and either concentrated or evaporated to dryness in a flash evaporator at a temperature not exceeding 50°.

Thin-layer chromatography was carried out using 20 × 20 cm plates of silica gel F-254 on aluminum and cellulose Microcrystalline Avicel on glass (Brinkmann Instruments, Westbury, N. Y.). A 2–5 μl aliquot of the concentrated ether extract was used. Preparative thin-layer chromatography was carried out using silica gel HF-254 (Brinkmann) plates having a coating thickness of 1.0 mm; the product was isolated by scraping off the silica gel band containing the compound, suspending the gel in 6 N HCl, and extracting the suspension with diethyl ether. The ether was then treated as described above.

Gas chromatography was accomplished with an Aerograph model 200 chromatograph (Wilkins Instrument and Research, Walnut Creek, Calif.) equipped with a flame ionization detector. The column was a 286 cm long, 2.4 mm internal diam coiled stainless steel tube containing 1:9 (w:w) DC 200 silicone oil on 60/80 mesh Chromosorb W, hexamethyldisilazane-treated. All compounds were chromatographed as the trimethylsilyl (TMS) derivatives. To prepare the derivatives, an excess of N,O-bis(trimethylsilyl)-acetamide (Pierce Chemical Co., Rockford, Ill.) was added to the residue remaining after an aliquot of the concentrated extract was freed of ether. The sample was then diluted with CS_2 before injection into the chromatograph. The column, injector, and detector temperatures were 200°, and the flow rate of the carrier gas, N_2, was 40 ml/min. Infrared spectra were obtained with a Beckman IR-10 spectrophotometer. Solid samples were inserted in the mass spectrometer, Perkin-Elmer (Norwalk, Conn.) model 270, by a direct inlet device and subjected to an ionization potential of 70 eV.

Analytical grade nitrophenols obtained from Eastman Organic Chemicals (Rochester, N. Y.) were recrystallized before use. Nitrohydroquinone and 3-nitrocatechol were prepared by the Elbs persulfate oxidation method (8) and purified by the procedures of Forrest and Petrow (9). 4-Nitrocatechol and some 3-nitrocatechol were prepared by the method of Weselsky and Benedikt (10), and 4-nitrocatechol was also obtained from Aldrich Chemical Co., Milwaukee, Wisc.

RESULTS

The organism isolated from the enrichments was a small, gram-negative, motile, peritrichous rod which, on the basis of standard diagnostic tests, appeared to be a strain of either *Flavobacterium* or a closely related bacterium (11, 12). The isolate was catalase-positive but negative in the Voges-Proskauer, methyl red, and oxidase tests. It was unable to form indole or H_2S, hydrolyze urea or starch, reduce nitrate anaerobically, or ferment several carbohydrates. It could hydrolyze gelatin, use nitrate as sole nitrogen source and citrate as sole carbon source, and it produced a greenish-yellow, water-insoluble pigment on potato slices.

As the isolate grew, it brought about a destruction of PNP, and the yellow color in the PNP-containing medium disappeared. Some component of soil extract was necessary for its multiplication in the nitrophenol medium. Multiplication of the isolate in this medium, as measured turbidimetrically, paralleled the loss of PNP and the formation of nitrite, the conversion of the organic nitro group to nitrite being essentially quantitative. Samples were taken from the culture during the period when growth and PNP utilization were proceeding rapidly and also during the early part of the stationary phase, at which time no PNP was present, and difference spectra were determined from 200 to 750 nm relative to alkaline PNP at a concentration corresponding to the PNP concentration of the sample at that time, as estimated at 400 nm. The difference spectra so obtained revealed no peaks, suggesting that light-absorbing products had not accumulated.

Resting cell suspensions (2 mg cells/ml) were prepared from a 36 hr culture grown on PNP. These were incubated with 5×10^{-4} M PNP in the ammonium-free salts solution, either in the presence or absence of 0.1% chloroform. As shown in Fig. 1, the nitro of PNP was converted quantitatively to nitrite by untreated cells, but little nitrite was liberated by cells treated with chloroform. On the other hand, an unknown aromatic compound appeared in the solution. This compound, when the reaction mixture was clarified and made alkaline, had a broad prominent peak with a maximum at 510 nm. For reasons cited below, the concentration of this product is plotted as if it were 4-nitrocatechol, using the absorbancy at 510 nm to calculate the nitrocatechol concentration. The level of the presumed 4-nitrocatechol rose as PNP was metabolized, and the yield of the former was quite similar to the quantity of the latter that had been destroyed. No such absorbancy appeared in

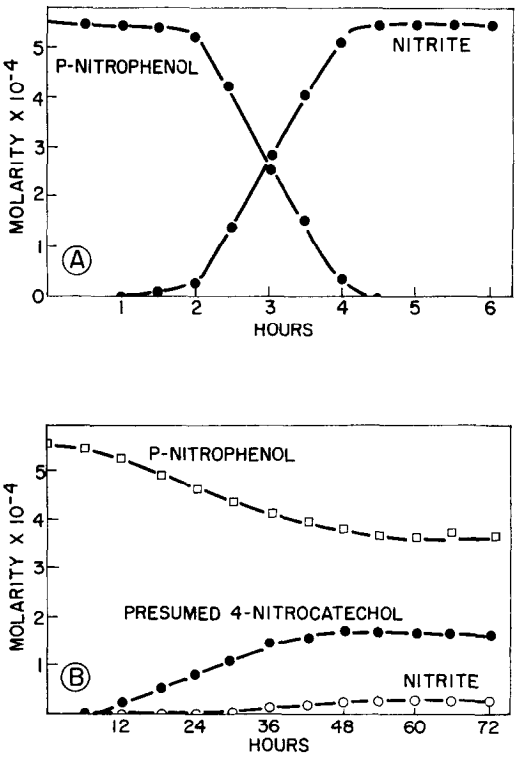

FIG. 1. *Metabolism of p-nitrophenol and accumulation of nitrite and a product absorbing at 510 nm when PNP was incubated with untreated cells (A) and with cells treated with chloroform (B).*

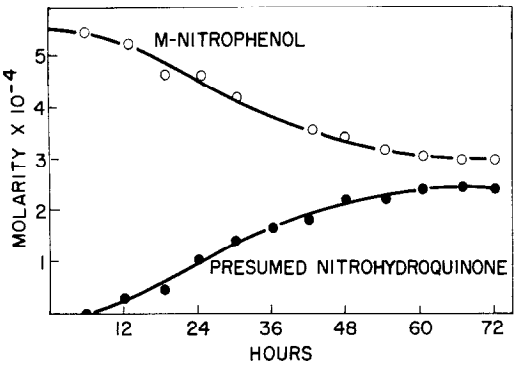

FIG. 2. *Bacterial conversion of m-nitrophenol to a product, presumably nitrohydroquinone, absorbing at 540 nm.*

reaction mixtures containing the cells which had not been treated with chloroform.

The bacterium did not grow when MNP was used in place of PNP in the culture medium and at the same concentration. Nevertheless, PNP-grown cells did metabolize MNP, and hence this substrate is subject to cometabolism by the bacterium. Thus, a product appeared in the solution when resting cells (2 mg/ml) prepared from a 36 hr culture grown in PNP-supplemented Trypticase Soy Broth were incubated at 30° with 5.5×10^{-4} M MNP in the ammonium-free basal medium. When the reaction mixture was clarified by centrifugation, the supernatant made alkaline, and a difference spectrum prepared relative to an alkaline solution of MNP at the initial substrate concentration used, a broad absorption band with a maximum at 540 nm was observed. Nitrite could not be detected, however. The time course of this reaction is shown in Fig. 2. The substance with an absorption maximum at 540 nm is plotted as if it were nitrohydroquinone, for reasons which are presented below. The data suggest a quantitative conversion of MNP to the presumed nitrohydroquinone.

Concentrated ether extracts of the reaction mixtures made after a 50 hr incubation were examined by thin-layer chromatography. The developed plates were viewed under UV light (254 nm), and the dark regions on the plates were circled. The plates were then sprayed with either a solution of 0.04% bromcresol purple in 50% (v/v) aqueous ethanol adjusted to pH 10 with NaOH or with 1 N NaOH. The NaOH solution made the authentic nitrophenols yellow, the 4-nitrocatechol and the product of PNP metabolism red, and 3-nitrocatechol, nitrohydroquinone, and the product of MNP metabolism purple. No fluorescence-quenching spots other than those which later became colored with the NaOH spray were detected, nor were any carboxylic acids observed since the bromcresol purple did not become yellow on the plates. The R_f values and the various color reactions given in Table 1 indicate that 3-nitrocatechol was not a product of the metabolism of either PNP or MNP, and that 4-nitrocatechol and nitrohydroquinone were possible products of PNP and MNP oxidation, respectively. In the ethanol: NH_4OH:-water solvent system, the only one that effectively separated MNP from its product, both the authentic nitrohydroquinone and the metabolite formed from MNP exhibited streaking.

Gas chromatography was employed to provide further evidence for the identity of the metabolites. For this purpose, resting cells (2 mg/ml dry wt) were incubated for 50 hr with 5.5×10^{-4} M PNP in the presence of 0.1% chloroform or with 5.5×10^{-4} M MNP, and the reaction mixtures were then extracted with ether. TMS derivatives of the authentic chemicals and of the ether extracts were examined by means of gas chromatography. The retention times observed were: PNP, 179 sec; authentic 4-nitrocatechol, 370 sec; ether extract of the reaction mixture containing cells and PNP, 179 and 370 sec; MNP, 128 sec; authentic nitrohydroquinone, 253 sec; and an ether extract of the reaction mixture containing

TABLE 1

Thin-layer chromatography of nitrophenols, nitrocatechols, and products of bacterial nitrophenol metabolism

Compound	R_f value				Color[b]
	Solvent A[a]	Solvent B	Solvent C	Solvent D	
p-Nitrophenol	0.89	0.95	0.84	0.85	Yellow
m-Nitrophenol	0.83	0.84	0.73	0.72	Yellow
3-Nitrocatechol	0.55	0.84	0.78	0.58	Purple
4-Nitrocatechol	0.68	0.81	0.74	0.65	Red
Nitrohydroquinone	0.83	0.84	0.72	0.55	Purple
Product formed from *p*-nitrophenol	0.68	0.81	0.74	0.65	Red
Product formed from *m*-nitrophenol	0.83	0.84	0.73	0.55	Purple

[a] Solvent system A was benzene:*p*-dioxane:acetic acid (90:25:4); B was chloroform; C was ethanol (95%); and D was ethanol:NH_4OH:water (80:4:16). Solvent systems A, B, and C were used with a silica gel stationary phase and D was used with a cellulose stationary phase.

[b] The color that the spot assumed when the plates were sprayed with 1 N NaOH solution.

cells and MNP, 128 and 253 sec. No peaks other than those listed were detected. Cochromatography of 1.0 μl of the TMS derivatives of 4-nitrocatechol or nitrohydroquinone with the extracts of the PNP or MNP reaction mixtures, respectively, increased the area of the slower-moving peaks in both instances, but the retention time of these peaks was unchanged. These data provide further evidence that the product generated from PNP is indeed 4-nitrocatechol and the metabolite formed from MNP is nitrohydroquinone. Both thin-layer and gas chromatography suggest that these were the only organic compounds excreted in the metabolic process.

The concentrated ether extract made from the reaction mixture containing chloroform-treated resting cells incubated 50 hr with PNP was examined by preparative thin-layer chromatography using benzene:*p*-dioxane:acetic acid (90:25:4) as the development solvent. A substance was observed with the same R_f value (0.65) as 4-nitrocatechol. The infrared spectrum of this compound was essentially the same as the spectrum of authentic 4-nitrocatechol, with the exception of a couplet at about 850 cm^{-1}. This couplet may be a minor impurity of the commercial 4-nitrocatechol, which had been used without purification.

A sample of the ether-free extract of the MNP reaction mixture was partially purified by vacuum sublimation. The ether was removed in a flowing stream of air. When a TMS derivative of the partially purified product was examined by gas chromatography, it was found that the area beneath the peak for MNP was less than 10% of the area of the peak for the unknown. This preparation and authentic nitrohydroquinone were then examined by mass spectrometry. The principal ions of the product of MNP metabolism and of authentic nitrohydroquinone are depicted in Fig. 3 in the form of bar graphs. Both samples produced parent (P) ions of mass 155. The presence of the P + 1 ion as 7.1% of the P ion abundance, the presence of the P + 2 ion (not shown in the figure) as 1.0% of the P ion abundance, and the parent ion are consistent with an empirical formula for the unknown of $C_6H_5NO_4$. The presence of the same principal ions provides additional evidence that the product formed from MNP is nitrohydroquinone. The differences in the relative abundance of the principal ions are

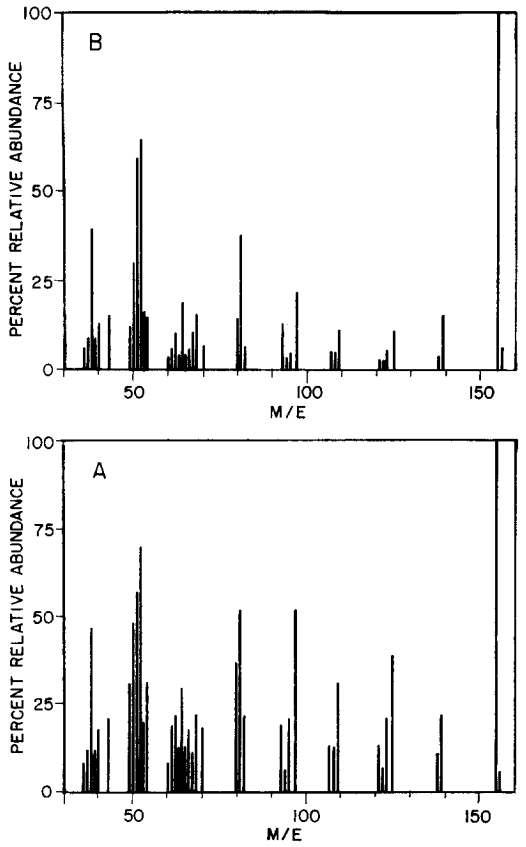

FIG. 3. *Mass spectra of authentic nitrohydroquinone (A) and of the product formed microbiologically from m-nitrophenol (B).*

probably the result of the differences in the amounts of the chemicals used.

To determine whether O_2 was involved in the conversion of MNP to nitrohydroquinone, manometric techniques were employed. Each Warburg flask contained resting cells and 1.0 μmole substrate. After 3 hr at 30°, 0.56 μmole O_2 was consumed with MNP as substrate; that is, about 0.5 μmole O_2/μmole substrate. The MNP had been completely metabolized and converted to nitrohydroquinone, as revealed by spectrophotometric assay. Under identical conditions with PNP as substrate, 6.49 μmole O_2 was consumed and 1.0 μmole nitrite was formed per μmole of PNP provided. Phenol, too, was completely metabolized, as shown by spectrophotometric tests, and 6.4 μmole O_2 was consumed/μmole substrate.

DISCUSSION

The ultraviolet, visible, and infrared spectra and the data from thin-layer and gas chromatography confirm that the substance produced from PNP is indeed 4-nitrocatechol. Similarly, the ultraviolet and visible spectra, the data from thin-layer and gas chromatography, and mass spectrometry confirm that the metabolite generated from MNP by the bacterium is nitrohydroquinone. Although nitrohydroquinone is generated in the detoxication of MNP and *o*-nitrophenol by rabbits (8), this is the first report of its formation by microorganisms.

Nitrohydroquinone is apparently a product of the cometabolism of MNP, inasmuch as the organism carrying out the reaction is unable to use MNP as a source of carbon or energy. The enzyme catalyzing this oxidation presumably has a low substrate specificity and usually is acting on some other substrate, possibly PNP. However, the enzymes concerned with PNP metabolism proceed to degrade further the products formed from PNP, as witnessed by the extensive oxidation observed manometrically and the growth of the bacterium on this compound. MNP, however, is not further degraded, presumably because of a narrower substrate specificity of the catechol-metabolizing enzymes.

The growth of this bacterium on PNP and its release of nitrite from the substrate is akin to the ability of an *Arthrobacter* to grow on and release nitrite from DNOC, as reported by Jensen and Lautrup-Larsen (3), but these investigators did not isolate the organic product of the nitro cleavage reaction. Possibly an initial step in the reaction with DNOC is its hydroxylation, as shown in the present report. On the other hand, the isolate studied herein behaves quite differently from the pseu-

domonad studied by Tewfik and Evans (2); the latter bacterium reduced the nitro group and only then introduced a hydroxyl onto the ring to replace the nitro group that was cleaved. Further work is necessary to clarify the routes by which such pesticides are transformed both in laboratory culture and in nature.

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