Initial Hydrogenation and Extensive Reduction of Substituted 2,4-Dinitrophenols

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***Rhodococcus erythropolis* HL 24-1 isolated as a 2,4-dinitrophenol-degrading organism can utilize 2-chloro-4,6-dinitrophenol as the sole nitrogen, carbon, and energy source under aerobic conditions. This compound is metabolized with liberation of stoichiometric amounts of chloride and nitrite. Under anaerobic conditions, 2,4-dinitrophenol was transiently accumulated in the culture fluid, indicating a reductive elimination of chloride. During aerobic bioconversion of 2-amino-4,6-dinitrophenol by *R. erythropolis* HL 24-1, a reductive elimination of nitrite leading to 2-amino-6-nitrophenol was observed. Elimination of chloride or nitrite by the initial formation of a hydride Meisenheimer complex is discussed. A methyl group in the *ortho* position of the 2,4-dinitrophenol gives rise to an extensive reduction of the aromatic ring under aerobic conditions. Thus, 2-methyl-4,6-dinitrophenol was shown to be converted to the two diastereomers of 4,6-dinitro-2-methylhexanoate as dead-end metabolites which were identified by spectroscopic methods.**

Nitrophenolic compounds are widely used in industry as intermediates in the production of pesticides, dyes, and explosives. Therefore, nitrophenolic compounds occur as contaminants of industrial effluents, soil, groundwater, and the atmosphere. The dinitrophenol derivatives 2-methyl-4,6-dinitrophenol (DNOC) and 2-*sec*-butyl-4,6-dinitrophenol (Dinoseb) were used in agriculture, mainly as herbicides. DNOC is rapidly inactivated in soil (14, 15), but Dinoseb is more persistent (17, 27).

Oxidative as well as reductive reactions are known to initiate degradation of nitrophenolic compounds. The removal of the nitro group from 2- or 4-nitrophenol may be catalyzed by monooxygenases (23, 24, 34, 35). Thus, 2-nitrophenol is converted to catechol and 4-nitrophenol is converted to hydroquinone. Recently, an alternative pathway for the degradation of 4-nitrophenol by a *Arthrobacter* sp. has been described (13). The catabolism of 4-nitrophenol is initiated by hydroxylation of the aromatic ring followed by a monooxygenase-catalyzed removal of the nitro group leading to 1,2,4-trihydroxybenzene (1,2,4-benzenetriol). In the case of 2,6-dinitrophenol, a dioxygenase-catalyzed elimination of nitrite has been reported as the initial reaction of the degradative pathway (8). Tewfik and Evans (28) reported very briefly on a pseudomonad that metabolized DNOC by a reductive pathway. 4-Amino-2-methyl-6-nitrophenol, 5-amino-3-methylcatechol, and 2,4,5-trihydroxytoluene were postulated as metabolites of DNOC. In contrast to reduction of the nitro groups of DNOC, several authors describe that microbial degradation of DNOC is indicated by accumulation of nitrite and decoloration of the culture fluid (9, 10, 14, 15).

Recent analysis of degradative pathways of polynitroaromatic compounds demonstrated the initial reduction of the aromatic ring by a hydride ion, which gives rise to a colored Meisenheimer complex (20). This ring hydrogenation plays an important role in the aerobic metabolism of 2,4,6-trinitrophenol (picric acid [18]). A nucleophilic attack of the aromatic ring

by a hydride ion can be rationalized by the highly electrophilic character of the nitro substituents, which makes the aromatic ring electron deficient and thus less susceptible to electrophilic reactions by oxygenases. The addition of nucleophilic ions like hydroxide, methoxide, or hydride, resulting in the formation of Meisenheimer complexes (σ -complexes), is a well-known chemical reaction of polynitroaromatic compounds (4, 5, 20). A hydride Meisenheimer complex was identified as the initial metabolite of picric acid by a *Rhodococcus erythropolis* HL PM-1 (18, 22) and of 2,4,6-trinitrotoluene by *Mycobacterium* sp. strain HL 4-NT-1 (30). In the case of picric acid, the hydride Meisenheimer complex regenerates aromaticity by the elimination of nitrite and formation of 2,4-dinitrophenol (2,4-DNP). The present communication describes the reductive metabolism of *ortho*-substituted ($-Cl$, $-CH_3$, and $-NH_2$) 2,4-dinitrophenols as structural analogs of picric acid and gives further support to its hydrogenation as the crucial initial reaction.

MATERIALS AND METHODS

Culture conditions and measurements of growth. *R. erythropolis* HL 24-1 was grown in nitrogen-free mineral medium, as described by Lenke et al. (19), with 2,4-DNP, 2-amino-4,6-dinitrophenol (2-A-4,6-DNP), 2-chloro-4,6-dinitrophenol (2-Cl-4,6-DNP) or DNOC (0.5 mM each) and succinate (10 mM), or with only 2-Cl-4,6-DNP (0.5 mM). Fluted Erlenmeyer flasks were incubated at 30°C on a rotary shaker at 150 rpm, and growth was determined by measuring the optical density at 546 nm. For the protein content, the value reported by Lenke et al. (19) was used. The organism was cultivated on agar plates (containing 1.5% [wt/vol] agar, no. 1; Oxoid Ltd., London, United Kingdom) with 2,4-DNP, 2-A-4,6-DNP, 2-Cl-4,6-DNP, or DNOC (0.5 mM each) and succinate (10 mM).

Analytical methods. Concentrations of substrates and metabolites were monitored by high-pressure liquid chromatography (HPLC) with Hyperchrome SC columns (length, 125 mm; diameter, 4.6 mm) filled with 5- μ m particles of Li-chrospher 100 RP8 (Bischoff, Leonberg, Germany) and photometric detection at 210 nm. To quantify DNOC, nitrite, and 4,6-dinitro-2-methylhexanoate, the mobile phase was methanol-water- H_3PO_4 (250:750:2.6 [vol/vol/vol]). To analyze the methyl ester of 4,6-dinitro-2-methylhexanoate, the mobile phase was methanol-water- H_3PO_4 (400:600:2.6 [vol/vol/vol]). Concentrations of 2,4-DNP, 2-Cl-4,6-DNP, 2-A-4,6-DNP, 2-amino-6-nitrophenol, and nitrite were estimated by HPLC with a mobile phase of acetonitrile-water- H_3PO_4 (300:700:2.6 [vol/vol/vol]).

Nitrite and ammonium ion concentrations were measured by photometric methods as described by Lenke et al. (18, 19). The release of chloride was determined with an ion-selective combination electrode (model 96/17; Orion Research, Cambridge, Mass.).

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Analytical thin-layer chromatography was carried out with thin-layer chromatography plates, silica gel 60 PF₂₅₄ (Merck, Darmstadt, Germany), and a solvent system of chloroform-acetone (4:1 [vol/vol]).

Resting-cell experiments. Cells of *R. erythropolis* HL 24-1 were grown in mineral medium as described above with 2,4-DNP or 2-Cl-4,6-DNP (0.5 mM each) as the nitrogen source and succinate (10 mM) as the carbon source. Induced cells were obtained by addition of 0.5 mM 2,4-DNP or 2-Cl-4,6-DNP during exponential growth 3 h before they were harvested by centrifugation. Cells were resuspended in 50 mM phosphate buffer (pH 7.4) and incubated with 2-Cl-4,6-DNP (0.5 mM), DNOC (0.5 mM), or 2-A-4,6-DNP (0.25 mM) on a water bath shaker at 30°C. The concentrations of the substrates and metabolites were determined by HPLC analysis. Nitrite was estimated photometrically.

For resting-cell experiments under anaerobic conditions, air was replaced by nitrogen and the cells in 50 mM phosphate buffer (pH 7.4) were incubated with 2-Cl-4,6-DNP in Hungate tubes by standard procedures (12).

Isolation and identification of 2-amino-6-nitrophenol. Resting-cell experiments were carried out to isolate a metabolite of 2-A-4,6-DNP. Cells of *R. erythropolis* HL 24-1 pregrown with 2,4-DNP and succinate (1,000 ml of mineral medium in a 3-liter fluted Erlenmeyer flask) were harvested, resuspended in 50 mM phosphate buffer (pH 7.4; optical density, about 10), and incubated with 2-A-4,6-DNP (0.5 mM) at 30°C on a rotary shaker. The transformation of 2-A-4,6-DNP was analyzed by HPLC. After total conversion of the substrate, the cells were removed by centrifugation and the fluid was extracted three times with equal volumes of ethyl acetate. The ethyl acetate extracts were combined, dried over MgSO₄, and evaporated to dryness. The isolated metabolite was purified on preparative silica gel layers (20 by 20 cm; 0.1-cm silica gel 60 PF₂₅₄; Merck, Darmstadt, Germany) with a solvent system of chloroform-acetone (4:1 [vol/vol]). The band which corresponded to the metabolite 2-amino-6-nitrophenol was located under UV light and eluted with ethyl acetate. After evaporation of the solvent, 2-amino-6-nitrophenol was recrystallized from ethanol-water (1:1 [vol/vol]). The melting point (corrected) of 2-amino-6-nitrophenol was determined with a heatable microscope (Reichert Thermovar, Nussloch, Germany). An MAT 711 mass spectrometer (Varian, Palo Alto, Calif.) was used for recording the mass spectrum at a source temperature of 25°C and an electron energy of 70 eV. The ¹H nuclear magnetic resonance (NMR) spectrum was recorded on a CXP 300 spectrometer (7,046 T; Bruker, Rheinstetten, Germany). The high-resolution spectrum was obtained at 300.13 MHz in CDCl₃ with tetramethylsilane as an internal standard.

Isolation and identification of 4,6-dinitro-2-methylhexanoate, diastereomers 1 and 2. Resting cells of *R. erythropolis* HL 24-1 were used to accumulate 4,6-dinitro-2-methylhexanoate (diastereomers 1 and 2). These cells were obtained by growth in mineral medium (8 × 1,000 ml in 3-liter fluted Erlenmeyer flasks) with 2,4-DNP and succinate (see above). Cells were harvested, resuspended in 500 ml of 50 mM phosphate buffer (pH 7.4; optical density at 546 nm, 7 to 8), and incubated at 30°C with DNOC (0.5 mM). The disappearance of DNOC and the formation of 4,6-dinitro-2-methylhexanoate (diastereomers 1 and 2) were monitored by HPLC. To obtain larger amounts of both metabolites, additional quantities of DNOC (portions with 0.5 mM each) were supplied after total consumption of the substrate. After complete conversion of DNOC, the cells were removed by centrifugation and the cell-free culture fluid was acidified to pH 3 with H₃PO₄ and extracted three times with equal volumes of ethyl acetate. The ethyl acetate extracts were combined, dried over MgSO₄, and evaporated to dryness. Both diastereomers of 4,6-dinitro-2-methylhexanoate were purified by semipreparative reversed-phase HPLC on a Hyperchrome SC column (length, 250 mm; diameter, 20 mm) filled with 5-μm-diameter particles of Lichrosorb 100 RP8 (Bischoff), using an aqueous solvent system containing 25% methanol (vol/vol) and 0.1% trifluoroacetic acid (vol/vol). The combined solvents from the eluates of the two metabolites were removed by flush evaporation. Both diastereomers of 4,6-dinitro-2-methylhexanoate were obtained as nearly colorless oils.

Parts of the extracted metabolites were derivatized with diazomethane (6). The HPLC analysis showed that two products (methyl 4,6-dinitro-2-methylhexanoate, diastereomers 1 and 2) were formed. The methyl esters were purified by preparative reversed-phase HPLC on a Hyperchrome SC column (length, 250 mm; diameter, 40 mm) filled with Lichrosorb 100 RP8 (Bischoff), using an aqueous solvent system containing 30% methanol (vol/vol) and 0.1% trifluoroacetic acid (vol/vol). The methyl esters were detected photometrically at 210 nm. The combined eluates of the two methyl esters were evaporated to dryness. Both diastereomers of methyl 4,6-dinitro-2-methylhexanoate were obtained as nearly colorless oils. ¹H and ¹³C NMR spectra were recorded on a Bruker CXP 300 spectrometer. High-resolution ¹H NMR spectra were obtained at 300.13 MHz, and decoupled ¹³C NMR spectra were obtained at 75.47 MHz in CDCl₃ with tetramethylsilane as an internal standard. Positive-ion chemical ionization spectra were recorded on a mass spectrometer (70-250 S; VG Analytical Ltd., Manchester, United Kingdom).

RESULTS

Growth of *R. erythropolis* HL 24-1 with different ortho-substituted 2,4-DNPs. The 2,4-DNP-degrading *R. erythropolis* HL 24-1 (19) could also utilize 2-Cl-4,6-DNP (0.5 mM) as the sole nitrogen source in the presence of succinate (10 mM) as the

FIG. 1. Growth of *R. erythropolis* HL 24-1 with 2-Cl-4,6-DNP as the sole source of nitrogen, carbon, and energy. 2-Cl-4,6-DNP was added in portions such that its concentration never exceeded 0.6 mM in the culture fluid. The culture was incubated in a 500-ml fluted Erlenmeyer flask at 30°C on a rotary shaker. The increase in cell density was determined at 546 nm (○). Disappearance of 2-Cl-4,6-DNP (■) was monitored by HPLC, and nitrite (▲) was detected photometrically.

carbon and energy source. In contrast, the structural analogs 2-A-4,6-DNP and DNOC were transformed but not utilized as the sole nitrogen source.

R. erythropolis HL 24-1 was shown to utilize 2,4-DNP not only as a source of nitrogen but also as the sole carbon and energy source if 2,4-DNP was added to the culture medium in amounts such that its concentration never exceeded 0.6 mM (19). To demonstrate the complete mineralization of 2-Cl-4,6-DNP by *R. erythropolis* HL 24-1, the strain was inoculated into a mineral medium containing 2-Cl-4,6-DNP (0.5 mM) without succinate. After depletion of the substrate, three additional portions of 2-Cl-4,6-DNP (0.4 to 0.5 mM) were added (Fig. 1). The consumption of 1.9 mM 2-Cl-4,6-DNP correlated with an increase in the optical density of 0.46 (Fig. 1, $t_d = 7.6$ h). Nitrite (3.23 mM) was the only metabolite detectable by HPLC.

Conversion of 2-Cl-4,6-DNP by resting cells of *R. erythropolis* HL 24-1. To quantify the elimination of nitrite and chloride from 2-Cl-4,6-DNP, resting cells of *R. erythropolis* HL 24-1 (optical density at 546 nm, 0.8) pregrown with 2,4-DNP and succinate were incubated with 2-Cl-4,6-DNP (0.51 mM) under aerobic conditions. After complete consumption of the substrate (specific activity, 33 μmol/min/g of protein), nearly stoichiometric amounts of chloride (0.5 mM) and nitrite (1.01 mM) were liberated into the culture fluid (Fig. 2). During aerobic conversion of 2-Cl-4,6-DNP, no other metabolites were detected by HPLC analysis. Resting cells of *R. erythropolis* HL 24-1 pregrown with 2-Cl-4,6-DNP and succinate showed the same specific activity against 2-Cl-4,6-DNP as the resting cells pregrown with 2,4-DNP and succinate.

Under anaerobic conditions, 2,4-DNP was completely reduced to 4,6-dinitrohexanoate by *R. erythropolis* HL 24-1 (19). Under the same conditions, cells of *R. erythropolis* HL 24-1 (optical density at 546 nm, 7.0) pregrown with 2,4-DNP and succinate converted 2-Cl-4,6-DNP to 2,4-DNP, which appeared transiently in the culture medium. It was further transformed to 4,6-dinitrohexanoate in almost quantitative amounts (Fig. 3). 2,4-DNP as an intermediate was identified by comparison with authentic 2,4-DNP. 4,6-Dinitrohexanoate as the final metabolite was identified by the authentic metabolite of

FIG. 2. Conversion of 2-Cl-4,6-DNP by resting cells of *R. erythropolis* HL 24-1. Resting cells were obtained by growth in mineral medium containing 2,4-DNP (0.5 mM) and succinate (10 mM). The cells were harvested, resuspended in phosphate buffer (optical density at 546 nm, 0.8), and incubated at 30°C with 0.51 mM 2-Cl-4,6-DNP on a rotary shaker. Conversion of 2-Cl-4,6-DNP (■) was measured by HPLC, nitrite release (▲) was detected photometrically, and the liberation of chloride (●) was determined with a chloride electrode.

2,4-DNP metabolism (19) by comparing chromatographic properties and the UV-visible absorption spectra (2,4-DNP, retention volume of 8.16 ml and $\lambda_{\max}$ of 260 nm with a shoulder at 295 nm; 4,6-dinitrohexanoate, retention volume of 2.94 ml and $\lambda_{\max}$ of 210 nm with a broad band of low intensity at 255 nm). The rate of biotransformation of 2-Cl-4,6-DNP under anaerobic conditions (specific activity, 3 $\mu\text{mol}/\text{min}/\text{g}$ of protein) was lower than that observed for the anaerobic bioconversion of 2,4-DNP (specific activity, 6 $\mu\text{mol}/\text{min}/\text{g}$ of protein).

Isolation and identification of 2-amino-6-nitrophenol as a metabolite of 2-A-4,6-DNP. Growth experiments of *R. erythropolis* HL 24-1 with 2-A-4,6-DNP showed that the substrate did not serve as the sole source of nitrogen although nitrite was

FIG. 3. Conversion of 2-Cl-4,6-DNP under anaerobic conditions by resting cells of *R. erythropolis* HL 24-1. Resting cells were obtained by growth in mineral medium with 2,4-DNP (0.5 mM) and succinate (10 mM). The cells were harvested, resuspended in phosphate buffer (optical density at 546 nm, 7), and incubated at 30°C with 0.4 mM 2-Cl-4,6-DNP on a rotary shaker. Conversion of 2-Cl-4,6-DNP (■) and formation of 2,4-DNP (◆) and 4,6-dinitrohexanoate (●) were determined by HPLC.

TABLE 1. Characteristic signals from the electron impact mass spectrum of 2-amino-6-nitrophenol

Mass (<i>m/z</i>)	Intensity (% of basic peak)	Fragment ion
154	100	M^+
137	40.5	$\text{M} - \text{NH}_3^{+}$
109	7.7	$\text{M} - \text{NH}_3$ and CO^{+}
108	16.4	$\text{M} - \text{NO}_2^{+}$
91	21.7	$\text{M} - \text{NO}_2$ and NH_3^{+}
90	6.4	$\text{M} - \text{H}_2\text{O}$ and NO_2^{+}
80	7.8	$\text{C}_5\text{H}_4\text{NH}_2^{+}$
79	23.9	$\text{M} - \text{CHO}$ and NO_2^{+}
63	5.0	$\text{M} - \text{CHO}$, NO_2 and NH_2^{+}
52	26.3	C_4H_4^{+}
30	3.6	$\text{CH}_2\text{NH}_2^{+}$

liberated into the culture fluid during conversion of 2-A-4,6-DNP by resting cells of *R. erythropolis* HL 24-1. Besides nitrite, a colored metabolite was formed from 2-A-4,6-DNP. After complete transformation of 2-A-4,6-DNP, the metabolite was extracted from the culture fluid with ethyl acetate. The red-brown oil obtained after evaporation of the ethyl acetate was purified by thin-layer chromatography. Recrystallization gave dark-red needles with a melting point of 109 to 110°C (corrected; melting point of 2-amino-6-nitrophenol, 111 to 112°C uncorrected [3]). The UV-visible absorption spectrum (in ethanol) showed absorption maxima at $\lambda_{\max}$ of 236 nm (ϵ 13,600), 303 nm (ϵ 5,800), and 418 nm (ϵ 1,100).

The metabolite was further characterized by mass-spectroscopic and NMR analysis. The compound had an apparent molecular ion (M^+) of 154 when analyzed by electron impact mass spectroscopy. High resolution of the molecular ion (mass of 154.0378) revealed the following formula $\text{C}_6\text{H}_6\text{N}_2\text{O}_3$ (calculated mass, 154.0378) corresponding to an aminonitrophenol. The significant fragment ion at *m/z* 137 is due to the elimination of ammonium after proton transfer from the phenolic hydroxy group. The fragment ion at 108 can be explained by the loss of an NO_2 radical (11). The fragmentation pattern of the aminonitrophenol is shown in Table 1. For the final identification of the positions of substituents at the aromatic ring, a ^1H NMR spectrum was recorded. One of the five signals (Table 2; Fig. 4), which appears at 4.01 ppm (two protons), is due to the amino group. The other singlet, highly shifted to low field (10.73 ppm, one proton), corresponds to a phenolic hydroxy group. The remaining three protons appeared as a doublet of doublets and resonated at 6.96, 6.79, and 7.48 ppm. The low-field shift of one of the signals indicates that it is bound to a nitro-substituted carbon atom (5-H [11]). One of the protons (4-H) showed two *ortho* couplings, and the other two protons

TABLE 2. ^1H NMR data for 2-amino-6-nitrophenol

Nucleus ^a	Chemical shift (ppm)	Splitting of signals ^b	Coupling constant (Hz)
1-H	10.73	s	
2-H	4.01	s	
3-H	6.96	dd	3J (3-H, 4-H) = 7.8
			4J (3-H, 5-H) = 1.6
4-H	6.79	dd	3J (4-H, 3-H) = 7.8
			3J (4-H, 5-H) = 8.5
5-H	7.48	dd	3J (5-H, 4-H) = 8.4
			4J (5-H, 3-H) = 1.4

^a The chemical structure is given in Fig. 4.

^b s, singlet; dd, double of doublets.

FIG. 4. Chemical structure for the data in Table 2.

(3-H and 5-H) both showed an *ortho* and a *meta* coupling, which is compatible with the structure of 2-amino-6-nitrophenol (Table 2; Fig. 4).

Conversion of 2-A-4,6-DNP by resting cells of *R. erythropolis* HL 24-1. Resting cells (optical density at 546 nm, 7) pregrown with 2,4-DNP and succinate turned over 2-A-4,6-DNP without a lag phase (Fig. 5). The conversion of 2-A-4,6-DNP (specific activity, 4 $\mu\text{mol}/\text{min}/\text{g}$ of protein), however, was much slower than that of 2,4-DNP (specific activity, 59 $\mu\text{mol}/\text{min}/\text{g}$ of protein [11]) or 2-Cl-4,6-DNP (specific activity, 33 $\mu\text{mol}/\text{min}/\text{g}$ of protein [see above]). Besides 2-amino-6-nitrophenol (0.18 mM) stoichiometric amounts of nitrite (0.25 mM) were liberated from 2-A-4,6-DNP (0.23 mM). Ammonia was not found in the culture fluid. The reductive elimination of nitrite leading to 2-amino-6-nitrophenol could also be induced by 2-Cl-4,6-DNP as the growth substrate.

Isolation and identification of the two diastereomers of 4,6-dinitro-2-methylhexanoate as metabolites of DNOC. For the isolation and identification of the two unknown metabolites which were observed during conversion of DNOC, this compound was metabolized by resting cells of *R. erythropolis* HL 24-1 grown with 2,4-DNP and succinate. After complete conversion of DNOC, the metabolites were extracted from the acidified culture fluid with ethyl acetate. After evaporation of the solvent, a slightly brown oil was obtained. The metabolites were separated and purified by semipreparative HPLC. Thus, two nearly colorless fractions which did not solidify on evaporation of the solvents were obtained. For further purification, the oily material was derivatized with diazomethane and the products were purified by preparative HPLC. The methyl de-

FIG. 5. Conversion of 2-A-4,6-DNP by resting cells of *R. erythropolis* HL 24-1. Resting cells were obtained by growth in mineral medium containing 2,4-DNP (0.5 mM) and succinate (10 mM). The cells were harvested, resuspended in phosphate buffer (optical density at 546 nm, was 7), and incubated at 30°C with 0.23 mM 2-A-4,6-DNP on a rotary shaker. The concentrations of 2-A-4,6-DNP (■) and 2-amino-6-nitrophenol (●) were determined by HPLC. The amount of nitrite (▲) was estimated photometrically.

TABLE 3. Characteristic signals from the chemical ionization mass spectrum of methyl 4,6-dinitro-2-methylhexanoate (diastereomer 1)

Mass (<i>m/z</i>)	Intensity (% of basic peak)	Fragment ion
252	21.4	MH + NH ₃ ⁺
219	9.6	(MH + NH ₃) ⁺ - CH ₃ OH ₂ ⁺
203	7.5	MH - CH ₃ OH ⁺
188	28.1	MH - HNO ₂ ⁺
172	20.0	MH - HNO and CH ₃ OH ⁺
156	27.8	MH - HNO ₂ and CH ₃ OH ⁺
141	23.12	MH ⁺ - CH ₃ NO ₂ and CH ₃ OH ₂ ⁺
125	100	MH - CH ₃ OH, HNO ₂ , and HNO ⁺

rivatives, which were obtained as almost colorless oils, were analyzed by NMR and mass spectroscopy.

Chemical ionization (ammonia) mass spectroscopy revealed the same ion mass of *m/z* 252 for both metabolites because of the ammonium cluster of the molecular ion. According to the identification of 4,6-dinitrohexanoate as a metabolite of 2,4-DNP (19), mass-spectrometric data indicated that DNOC is also subject to a complete reduction of the aromatic ring, yielding 4,6-dinitro-2-methylhexanoate. Because 4,6-dinitro-2-methylhexanoate or its methyl derivative contains two asymmetric carbon atoms, two diastereometric structures of this compound must exist. This is supported by the similar fragmentation pattern of both methyl derivatives of the metabolites (Table 3 and 4) which correspond to the formula of C₈H₁₅N₂O₆. The fragmentation patterns of both metabolites are strictly analogous to that of 4,6-dinitrohexanoate (19) as a homologous structure. For final identification, ¹H and ¹³C NMR spectra of both diastereomers were recorded. In both cases, the integrals of the resonance lines added up to the expected 15 protons. In both ¹H NMR spectra (Table 5; Fig. 6), one singlet at 3.71 ppm (diastereomer 1) or 3.72 ppm (diastereomer 2) corresponds to a methyl ester and one doublet at 1.26 ppm (diastereomer 1) or 1.23 ppm (diastereomer 2) corresponds to the methyl group. The doublet can easily be explained by a ³J coupling between the methyl group and the proton at the tertiary C-2 carbon atom (³J = 6.9 Hz, diastereomer 1; ³J = 10.0 Hz, diastereomer 2). The remaining eight protons appeared in both spectra as multiplets. In both cases, two multiplets corresponding to one and two protons, respectively, resonated at 4.70 to 4.77 ppm (diastereomer 1) or 4.66 to 4.75 ppm (diastereomer 2) and at 4.37 to 4.54 ppm (diastereomer 1) or 4.38 to 4.51 ppm (diastereomer 2). The low-field shift of these protons demonstrates that they are bound to nitro-substituted carbon atoms which have a high anisotropic effect (11). The assignment of the remaining two multiplets which differ in each spectrum occurred in accordance to the ¹H

TABLE 4. Characteristic signals from the chemical ionization mass spectrum of methyl 4,6-dinitro-2-methylhexanoate (diastereomer 2)

Mass (<i>m/z</i>)	Intensity (% of basic peak)	Fragment ion
252	74.7	MH + NH ₃ ⁺
219	29.2	(MH + NH ₃) ⁺ - CH ₃ OH ₂ ⁺
203	14.6	MH - CH ₃ OH ⁺
188	100	MH - HNO ₂ ⁺
172	39.1	MH - HNO and CH ₃ OH ⁺
156	42.7	MH - HNO ₂ and CH ₃ OH ⁺
141	24.6	MH ⁺ - CH ₃ NO ₂ and CH ₃ OH ₂ ⁺
124	41.3	MH ⁺ - CH ₃ OH ₂ ⁺ , HNO ₂ , and HNO

TABLE 5. ^1H and ^{13}C NMR decoupled data for methyl 4,6-dinitro-2-methylhexanoate (diastereomers 1 and 2)

Diastereomer 1		Diastereomer 2	
Nucleus	Chemical shifts (ppm)	Nucleus	Chemical shifts (ppm)
2-H ^a	2.45–2.67 (multiplet)	2-H ^b	2.46–2.64 (multiplet)
2-H ₍₃₎ ^a	1.26 (doublet)	2-H ₍₃₎ ^b	1.23 (doublet)
3-H _A	2.07–2.19 (multiplet)	3-H _A ,H _B	1.84–1.94 (multiplet)
5-H _A ,H _B , 3-H _B	2.45–2.67 (multiplet)	5-H _A ,H _B	2.46–2.64 (multiplet)
4-H	4.70–4.77 (multiplet)	4-H	4.66–4.75 (multiplet)
6-H _A ,H _B	4.37–4.54 (multiplet)	6-H _A ,H _B	4.38–4.51 (multiplet)
7-H ₍₃₎	3.71 (singlet)	7-H ₍₃₎	3.72 (singlet)
C-1	174.91	C-1	175.20
C-2	36.62 ^c	C-2	37.05 ^c
C-3	36.04 ^c	C-3	36.17 ^c
C-4	82.71	C-4	83.49
C-5	30.43	C-5	30.84
C-6	70.98	C-6	70.93
C-7	52.16	C-7	52.17
C-8	16.73	C-8	18.01

^a 3J (2-H, 2-CH₃) = 6.9 Hz.^b 3J (2-H, 2-CH₃) = 10.0 Hz.^c Assignments of C-2 and C-3 are tentative.

NMR data of 4,6-dinitrohexanoate and 1,3,5-trinitropentane (18, 19). For diastereomer 1, one proton (3-H_A) resonated at 2.07 to 2.19 ppm (multiplet) and the other three protons (3-H_B, 5-H_A, and 5-H_B) appeared as a multiplet at 2.45 to 2.67 ppm. In contrast, in the spectrum of diastereomer 2, two multiplets, which correspond to 3-H_A and 3-H_B at 1.84 to 1.94 ppm and to 5-H_A and 5-H_B at 2.46 to 2.64, appeared.

The decoupled ^{13}C NMR data of the two methyl esters are similar (Table 5; Fig. 6) and give further evidence for the postulated structures of the 4,6-dinitro-2-methylhexanoate diastereomers which are formed from DNOC. In both cases, two signals correspond to the methyl ester function. The other five signals are due to the aliphatic structure of five sp^3 carbon atoms. Two of these signals were shifted to low field (C-4; 82.71 ppm, and C-6, 70.98 ppm, for diastereomer 1; C-4, 83.49 ppm, and C-6, 70.93 ppm, for diastereomer 2) because of the high anisotropic effect of the nitro groups (16). Besides these seven signals, which are in accordance with the decoupled ^{13}C NMR data of 4,6-dinitrohexanoate (19), another signal resonated at 16.73 ppm (diastereomer 1) or 18.01 ppm (diastereomer 2), corresponding to the additional methyl group.

Conversion of DNOC by resting cells of *R. erythropolis* HL 24-1. To quantify the formation of the two diastereomers of 4,6-dinitro-2-methylhexanoate, resting cells of *R. erythropolis* HL 24-1 (optical density at 546 nm, 3.8) pregrown with 2,4-DNP and succinate were incubated with DNOC (0.48 mM). After complete transformation of DNOC (Fig. 7; specific activity, 10 $\mu\text{mol}/\text{min}/\text{g}$ of protein), 69% ($\pm 1\%$) of the organic nitrogen was recovered as 4,6-dinitro-2-methylhexanoate (19% $\pm 1\%$ of diastereomer 1 and 50% $\pm 1\%$ of diastereomer 2) in the culture fluid. In addition, some nitrite was eliminated during conversion of DNOC (8% $\pm 1\%$ of the total organic

FIG. 6. Chemical structure for the data in Table 5.

FIG. 7. Conversion of DNOC by resting cells of *R. erythropolis* HL 24-1. Resting cells were obtained by growth in mineral medium containing 2,4-dinitrophenol (0.5 mM) and succinate (10 mM). The cells were harvested, resuspended in phosphate buffer (optical density at 546 nm, 3.8), and incubated at 30°C with 0.48 mM DNOC on a rotary shaker. Concentrations of DNOC (■) and 4,6-dinitro-2-methylhexanoate (diastereomer 1, ○; diastereomer 2, ◆) were measured by HPLC, and nitrite (▲) release was detected photometrically.

nitrogen). Because the analogous compound 4,6-dinitrohexanoate decomposes with liberation of nonstoichiometric amounts of nitrite, a corresponding decomposition of the diastereomers of 4,6-dinitro-2-methylhexanoate was assumed. Actually, when each of the diastereomers of 4,6-dinitro-2-methylhexanoate was incubated in phosphate buffer (pH = 7.4) at 30°C, small amounts of nitrite were released (approximately 15% of the total organic nitrogen), indicating that the liberation of nitrite is due to an abiotic reaction. Although both diastereomers are subject to chemical decomposition, a nearly quantitative nitrogen balance was obtained, indicating that no other nitrogen-substituted metabolite was formed from DNOC in significant amounts.

DISCUSSION

Studies on the catabolism of nitroaromatic compounds with two nitro substituents, such as 2,4-dinitrotoluene (25), 1,3-dinitrobenzene (7, 21), and 2,6-dinitrophenol (8), have demonstrated an oxidative initial removal of the nitro groups by dioxygenase-catalyzed reactions. In contrast, reductive initial reactions were postulated for the catabolism of 2,4-DNP and picric acid by *R. erythropolis* HL 24-1, HL 24-2, or HL PM-1

FIG. 8. Proposed mechanism for the complete reduction of DNOC by *R. erythropolis* HL 24-1.

FIG. 9. Proposed initial reactions in the catabolism of 2-A-4,6-DNP (a) and 2-Cl-4,6-DNP (b) by *R. erythropolis* HL 24-1.

(18, 19). The present study supports this mechanism by feeding *ortho*-substituted analogs of 2,4-DNP such as DNOC, 2-A-4,6-DNP, and 2-Cl-4,6-DNP.

Obviously, the homolog DNOC follows the initial catabolic sequence of 2,4-DNP. Its methyl group in the *ortho* position, however, interrupts the metabolic pathway by generating diastereometric 4,6-dinitro-2-methylhexanoate as dead-end metabolites. According to the reaction sequence in Fig. 8, two hydride ions must be added to DNOC. Subsequent protonation may give rise to 4,6-dinitrohexanone, which, by hydrolysis, yields the 4,6-dinitro-2-methylhexanoates. They are chemically unstable and slowly release some nitrite and thus allow negligible growth. In contrast, the structural analog 2,4-DNP can be mineralized by *R. erythropolis* HL 24-1 (19).

If picric acid is used as a structural analog, initial addition of a hydride ion gives rise to a stable hydride Meisenheimer complex. This can accept another hydride, generating 2,4,6-trinitrocyclohexanone as a dead-metabolite. The crucial step for the productive catabolism of picric acid is the elimination of nitrite from the hydride Meisenheimer complex. Thus, rearomatization generates 2,4-DNP, which is assimilated (18).

During transformation of 2-A-4,6-DNP by *R. erythropolis* HL 24-1, a reductive removal of one nitro group was observed, leading to 2-amino-6-nitrophenol and nitrite. The formation of a Meisenheimer complex as the initial enzymatic reaction would explain the formation of 2-amino-6-nitrophenol and the elimination of nitrite by rearomatization (Fig. 9a). To our knowledge, this is the first description of a reductive removal of a nitro group from a dinitroaromatic compound.

Although nitrite was released from 2-A-4,6-DNP, it does not serve as a nitrogen source for *R. erythropolis* HL 24-1 or HL 24-2. Similar observations were made with both 2,4-DNP-degrading strains HL 24-1 and HL 24-2 when picric acid was supplied as the sole source of nitrogen. Picric acid was metabolized only when the cells were induced by 2,4-DNP (18). In contrast to the wild-type strain, the mutant strain HL PM-1 acquired the ability to induce the catabolic pathway of 2,4-DNP by picric acid. 2-A-4,6-DNP, however, does not induce any catabolic activity toward nitrophenols.

In contrast to 2-A-4,6-DNP, 2-Cl-4,6-DNP serves as the sole source of nitrogen in the presence of succinate. The compound was also used as the only source of carbon and energy if nontoxic amounts of 2-Cl-4,6-DNP were supplied. Remarkably, the growth yield with 2-Cl-4,6-DNP (change in optical density for 1 mM 2-Cl-4,6-DNP = 0.24) was higher than with 2,4-DNP (change in optical density for 1 mM 2,4-DNP = 0.14,

19). This can be rationalized because approximately 20% of 2,4-DNP was metabolized to 4,6-dinitrohexanoate and accumulated in the culture fluid without further bioconversion (19).

With 2,4-DNP- or 2-Cl-4,6-DNP-grown resting cells of *R. erythropolis* HL 24-1, stoichiometric amounts of chloride and nitrite were eliminated from 2-Cl-4,6-DNP. Under anaerobic conditions, 2,4-DNP-grown cells of *R. erythropolis* HL 24-1 converted 2-Cl-4,6-DNP to 4,6-dinitrohexanoate with transient accumulation of 2,4-DNP. Compared with the transformation rate under aerobic conditions, the rate of 2-Cl-4,6-DNP turnover was rather low and nitrite was not liberated. The formation of 2,4-DNP from 2-Cl-4,6-DNP can be explained by the hydride addition mechanism, leading to a hydride Meisenheimer complex. Aromaticity is regenerated by elimination of chloride and formation of 2,4-DNP (Fig. 9b). The reductive removal of chloride from 2-Cl-4,6-DNP was also noticed in cell extracts of *R. erythropolis* HL PM-1 by Rieger et al. (22). In contrast to the observation made with picric acid, the elimination of chlorine instead of nitrite seemed to be the crucial initial enzymatic reaction in the aerobic catabolism of 2-Cl-4,6-DNP. The replacement of chlorine against hydrogen is an unusual enzymatic reaction in the catabolism of halogenated aromatic compounds under aerobic conditions. It has been described only in a few cases, e.g., 2,4-dichlorobenzoate, 2,4-D and pentachlorophenol (1, 2, 26, 29, 33). In the catabolism of pentachlorophenol, a glutathione transferase which accomplished the reductive dehalogenation was characterized (31, 32). In the case of 2-Cl-4,6-DNP the two nitro groups reduce the electron density of the aromatic π system to such an extent that an enzyme-mediated addition of a H^- from a hydride donor such as NADH is favored. Future work must show the nature of the actual H^- donor and whether initial ring hydrogenation is of general importance for the catabolism of aromatic compounds that are highly electron deficient by a multitude of electron-withdrawing substituents like halogen or nitro groups. Whether 4,6-dinitrohexanoate is actually an intermediate of the aerobic catabolism of 2-Cl-4,6-DNP and 2,4-DNP is the subject of current investigations.

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