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Identification of a Hydride-Meisenheimer Complex as a Metabolite of 2,4,6-Trinitrotoluene by a *Mycobacterium* Strain

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A bacterial strain, *Mycobacterium* sp. strain HL 4-NT-1, enriched with 4-nitrotoluene as its sole source of nitrogen, was able to metabolize 2,4,6-trinitrotoluene under aerobic conditions. The dark red-brown metabolite, which accumulated in the culture fluid, was identified as a hydride-Meisenheimer complex by comparison with an authentic synthetic sample.

In the course of large-scale manufacturing and handling of explosives, both soil and groundwater were contaminated extensively with polynitroaromatic compounds such as 2,4,6-trinitrotoluene (TNT) (5). Large amounts of TNT were synthesized during World War II. The high concentrations still found in the environment indicate the resistance of TNT to microbial degradation. Nitro groups, just like chloro substituents, reduce the electron density of the aromatic π system, and thus hamper electrophilic attack by oxygenases. Initial reactions in the course of a degradative process of polynitroarenes thus must be reductive rather than oxidative. Several reports in the literature (1, 4, 15) deal with the reductive metabolism of TNT under anaerobic conditions. Little is known, however, about aerobic catabolism of TNT; most reports deal with cometabolic reactions by bacterial species leading to aminodinitrotoluenes (2, 6, 12, 14, 16). The hydroxylamino- or nitrosodinitrotoluenes, intermediates of such coreductions, usually couple with each other, generating biologically inert azoxy compounds (2, 6, 12, 14, 16).

Recently, a novel reductive degradation mechanism by an aerobic organism has been described for the utilization of picric acid by *Rhodococcus erythropolis* HL PM-1 (9). An orange-red metabolite, accumulating transiently in the culture fluid, was characterized as the hydride-Meisenheimer complex formed by the nucleophilic addition of a hydride ion at C-3 of picric acid. Subsequently, this Meisenheimer complex was converted to 2,4-dinitrophenol and nitrite. From these data, though without any direct evidence, Duque et al. (3) proposed a corresponding mechanism for the metabolism of TNT yielding nitrite, dinitrotoluenes, nitrotoluenes, toluene, and major amounts of dead-end metabolites.

We now present unequivocal evidence that the hydride-Meisenheimer complex of TNT (H^--TNT complex) is the product of TNT bioconversion by *Mycobacterium* sp. strain HL 4-NT-1. 4-Nitrotoluene (4-NT)-grown cells of this organism produced sufficient amounts of the H^--TNT complex to allow a spectroscopic characterization, in contrast to another newly isolated strain, CV TNT-8, which utilized TNT as a nitrogen source but accumulated only minor amounts of this rather unstable metabolite (15a).

Bacterial strain and media. *Mycobacterium* sp. strain HL 4-NT-1 was isolated from a mixed soil sample from the Stuttgart (Germany) area with 4-NT as the sole source of nitrogen (8a). This strain was characterized by its biochemical reactions and on the basis of the mycolic acid, menaquinone, and fatty acid compositions of the cell envelope (8). The strain was grown in batch culture with the mineral medium described by Lenke et al. (10) containing 0.5 mM 4-NT and 10 mM succinate. The cultures were incubated at 30°C in fluted Erlenmeyer flasks on a rotary shaker (at 120 rpm). Growth was determined by measuring the optical density at 546 nm. The organism was cultivated on agar plates (containing 1.5% [wt/vol] agar no. 1; Oxoid Ltd., London, United Kingdom) with succinate (10 mM) in mineral medium. 4-NT was supplied through the gas phase in a desiccator containing crystals of 4-NT.

Synthesis and identification of the H^--TNT complex. The H^--TNT complex was prepared from TNT, with tetramethylammonium octahydrotriborate (Alfa; Johnson Matthey, Karlsruhe, Germany) as the hydride donor, at −10°C as described by Kaplan and Siedle (7). In contrast to the results described by Kaplan and Siedle, the product precipitated as dark lustrous needles only after 6 to 8 h. After 24 h, the needles were filtered off, washed with cold acetonitrile, and dried in vacuo. The isolated compound displayed more or less the absorption maxima (in acetonitrile) described by Kaplan and Siedle (data from reference 7 are in parentheses): 255 nm, ε = 9,380 (256 nm, ε = 11,000); 477 nm, ε = 24,870 (478 nm, ε = 25,000); and 578 nm, ε = 14,050 (580 nm, ε = 34,000). For a more definite characterization, ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra of the H^--TNT complex were determined in *d*-6-dimethyl sulfoxide with tetramethylsilan as an internal standard on a Bruker (Rheinstetten, Germany) AC 250 (^1H nominal frequency, 250.134 MHz; ^{13}C nominal frequency, 62.896 MHz).

The ^1H NMR data are comparable to those reported by Kaplan and Siedle (7). They clearly demonstrated that the hydride ion was added exclusively in the 3 position of TNT (Fig. 1; Table 1). Kaplan and Siedle had rationalized the absence of any hydride attack at C-1 in terms of the inability of the methyl group to coordinate with the developing B_3H_7 moiety in the transition state.

The ^{13}C NMR data of the Meisenheimer complex are also consistent with the presence of a C-3 hydride adduct. One signal corresponds to the CH_2 group (30.21 ppm) and one signal corresponds to the methyl group (18.72 ppm). There are five signals in the sp^2 region corresponding to C-1, C-2, and C-4 to C-6.

Stability of the H^--TNT complex under physiological conditions. For the identification of the hydride-Meisenheimer complex as a metabolite of TNT, it was necessary to determine

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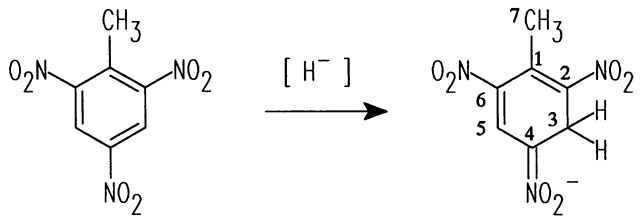

FIG. 1. Formation of the C-3 H^--TNT complex.

the stability of the complex under physiological conditions. The H^--TNT complex, prepared independently by chemical synthesis, was incubated in phosphate buffer (pH 7.4) at 30°C. The concentration of the H^--TNT complex was monitored by ion-pair high-pressure liquid chromatography (HPLC) (Sykam, Gilching, Germany) with a solution of tetrabutylammonium phosphate (PicA; Waters, Milford, Conn.) in acetonitrile-water (7:13 [vol/vol]) as the mobile phase and with a reversed-phase Lichrocart column (length, 125 mm; diameter, 4.6 mm) filled with 5-μm-diameter particles of Lichrospher 100 RP8 (Merck, Darmstadt, Germany). Detection was carried out at 240 nm. During incubation the concentration of the H^--TNT complex decreased to half its initial value within 5 h (Fig. 2). In the course of this spontaneous chemical decomposition nonstoichiometric amounts of TNT were formed (30% of H^--TNT; Fig. 2). No other major products of this chemical transformation were observed.

Conversion of TNT by resting cells. In order to investigate the metabolism of TNT, resting-cell experiments were carried out. 4-NT-grown cells of *Mycobacterium* sp. strain HL 4-NT-1 were cultivated in mineral medium with 4-NT (0.5 mM) as the sole nitrogen source and succinate (10 mM) as the carbon source. The cells were harvested by centrifugation during exponential growth, washed with phosphate buffer (50 mM) (pH 7.4), suspended in phosphate buffer (optical density of 10 at 546 nm), and incubated with TNT (0.5 mM) and NH_4SO_4 (1 mM) at 30°C on a water bath shaker. Concentrations of TNT and metabolites were monitored by ion-pair chromatography as described above. Nitrite formation was determined simultaneously by reversed-phase HPLC with a Lichrocart column (length, 125 mm; diameter, 4.6 mm) filled with 5-μm particles of Lichrospher 100 RP8 (Merck). Photometric detection was at 210 nm, and the mobile phase was acetonitrile-water-H_3PO_4 (400:600:2.6, vol/vol/vol).

In the course of TNT metabolism four metabolites were released into the culture fluid. One of these metabolites was identified as 4-amino-2,6-dinitrotoluene by comparing the chromatographic properties (metabolite retention time [RT], 9.7 min; reference RT, 9.7 min) and UV–visible-light absorption spectra (metabolite absorption maxima [λ_{max}], 233 and 342 nm; reference λ_{max}, 233 and 342 nm), recorded under stop flow conditions during HPLC, with those of an authentic synthetic sample (11, 13). During turnover of TNT, a dark red-brown metabolite transiently accumulated. HPLC analysis and UV–visible-light absorption spectra, recorded under stop flow conditions, proved the identity of this metabolite (RT, 15.72 min; λ_{max}, 255, 477, and 578 nm) (Fig. 3) with the authentic synthetic H^--TNT complex (RT, 15.97 min; absorption maxima, 255, 477, and 578 nm) (Fig. 3).

Remarkably, the metabolism of TNT by *Mycobacterium* sp. strain HL 4-NT-1 involves two different initial reductive enzyme systems: one enzyme system is responsible for the reduction of one nitro group to an amino group, and the other one is responsible for the nucleophilic attack of a hydride ion,

TABLE 1. ^1H and ^{13}C NMR data for the H^--TNT complex[a]

Position	δ (ppm)	
	^{13}C	^1H (relative intensity)[b]
C-3	30.21	3.90 (3.90), 2 H[c]
C-5	129.67	8.37 (8.38), 1 H
1-CH_3	18.72	2.57, 3 H[c]
$N(CH_3)_4$	54.27	3.10 (3.12), 12 H[d]
C-1, C-2, C-4, C-6	141.76, 133.75, 129.47, 123.79	

[a] The complex was prepared by the method of Kaplan and Siedle (7).
[b] Relative intensities are from reference 7.
[c] ^5J (3-H, 1-CH_3) 1.1 Hz.
[d] Signal of the cation.

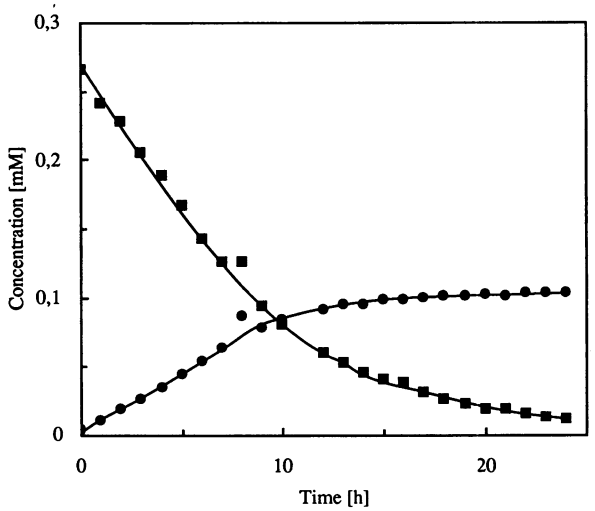

FIG. 2. Spontaneous chemical decomposition of the hydride-Meisenheimer adduct under physiological conditions. Concentrations of the complex (■) and TNT (●) were monitored by HPLC.

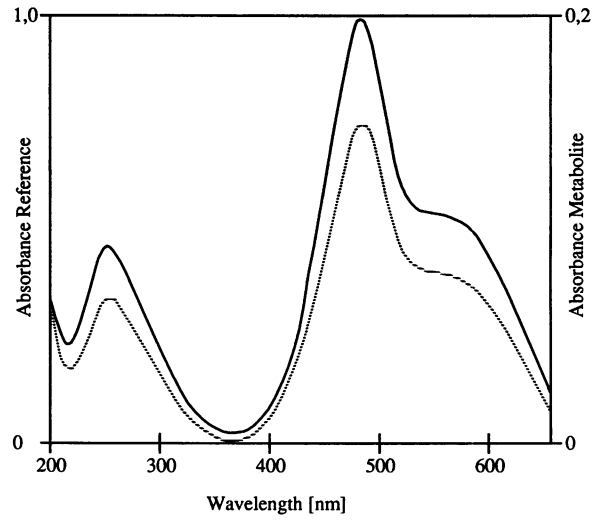

FIG. 3. UV–visible-light spectra of the H^--TNT complex obtained chemically (——) and biologically (·····).

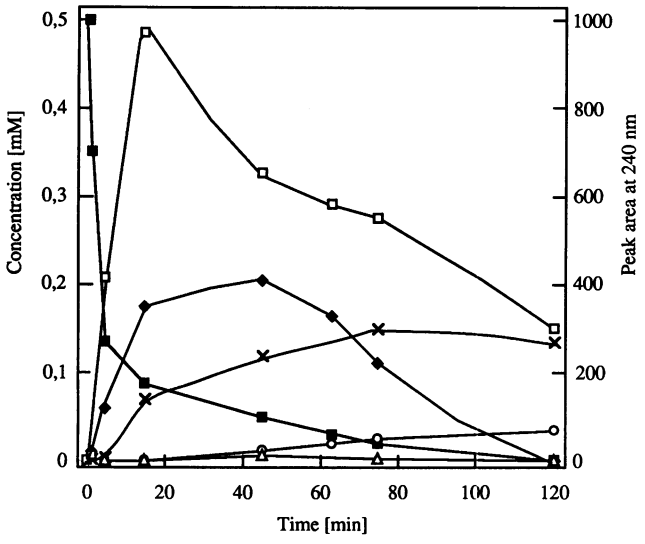

FIG. 4. Conversion of TNT by resting cells of *Mycobacterium* sp. strain HL 4-NT-1. Concentrations of TNT (●), the Meisenheimer complex (◆), 4-amino-2,6-dinitrotoluene (○), not-yet-identified metabolites with RTs of 6.7 (□) and 3.3 (△; determined by ion-pair HPLC), and nitrite (×; determined by reversed-phase HPLC) are shown.

leading to a Meisenheimer complex, which was first demonstrated for picric acid (9). With TNT as the substrate (Fig. 4) only 5% of the compound was reduced to 4-amino-2,6-dinitrotoluene, whereas 40% were accumulated transiently as the H^--TNT complex. This clearly indicates the importance of the hydride addition mechanism for the metabolism of TNT. Reversed-phase HPLC analysis demonstrated that nitrite was released into the culture fluid. After 75 min of the resting-cell experiment (Fig. 4) 0.15 mM nitrite was liberated per 0.48 mM TNT. TNT was not utilized, however, as the sole nitrogen source by this organism. Besides the H^--TNT complex and 4-amino-2,6-dinitrotoluene, two additional metabolites with respective RTs of 3.3 and 6.7 min transiently accumulated in the culture fluid (Fig. 4). They were not identified, although it was shown that they were neither 2,4- nor 2,6-dinitrotoluene. On the other hand, these two dinitrotoluenes were reported but not quantified in concentrated culture supernatants during growth of *Pseudomonas* sp. strain C1S1 (3). The authors postulated, without presenting straightforward evidence, that one nitro group had been removed via a Meisenheimer complex. Whether the H^--TNT complex is actually formed and plays a key role during metabolism of TNT by *Pseudomonas* sp. strain C1S1 remains an open question, particularly because the organism is not available for comparative studies. The organism was reported (3) to grow with TNT as the sole source of nitrogen, although aminodinitrotoluenes as well as azoxy dimers were the most abundant products. Accumulation of such dead-end products limits complete degradation, although partial mineralization was observed for the constructed strain *Pseudomonas* sp. clone A(pWWO-km).

To our knowledge the present report demonstrates, for the first time, that a Meisenheimer complex (the H^--TNT complex) is the initial metabolite of TNT under aerobic conditions. Whether the H^--TNT complex serves as a nitrogen source for bacteria, as described for the hydride-Meisenheimer complex of picric acid metabolized by *Rhodococcus erythropolis* HL PM-1, is the subject of further investigations. The unusual hitherto-unknown biochemical reaction of hydride addition to picric acid proceeds without detectable reduction of nitro groups (9). Formation of hydride-Meisenheimer complexes may be a general reaction which makes highly electron-deficient polynitroarenes acceptable for aerobic catabolism.

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