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Metabolism of ^{14}C-labelled 5-nitro-1,2,4-triazol-3-one (NTO): comparison between rat liver microsomes and bacterial metabolic pathways

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Abstract

In the present study, we have investigated the metabolism of the explosive 5-nitro-1,2,4-triazol-3-one (NTO) **1**. $^{14}C_5$- and $^{14}C_3$-labelled NTO were synthesized to facilitate the elucidation of its bacterial and mammalian metabolism. The metabolites formed were characterised, and the degradative pathways compared. The *Bacillus licheniformis* strain was isolated from industrial waste containing high concentrations of the explosive (15 g l^{-1}). Microbial metabolism of NTO **1** proceeded through an oxygen-insensitive nitroreduction leading to the primary amine ATO (5-amino-1,2,4,-triazol-3-one) **2**, followed by cleavage of the triazolone ring. The maximum microbial nitroreduction occurred at pH 6 in the presence of sucrose, while the ring cleavage occurred at pH 8. A permanent control and adjustment of the pH was required to achieve the complete degradation of NTO by *B. licheniformis*. The triazolone ring resulted from the hydrolysis of the 'pseudo guanido' group ($R-NH-C(NH_2)=N-R'$). Mammalian degradation of NTO was catalysed by dexamethasone-induced murine hepatic microsomes, and influenced by the presence of oxygen. Thus, under a nitrogen atmosphere, NTO **1** is exclusively reduced to the amine **2**. In the presence of oxygen, besides a low amount of **2**, 5-hydroxy-1,2,4-triazol-3-one urazole **3** is the major metabolite formed. This compound is obtained through an oxidative denitrification of NTO accompanied by simultaneous formation of nitrite. Both nitroreduction and oxidative-denitrification of NTO were NADPH-dependent and totally inhibited by carbon monoxide. These observations suggest a possible implication of cytochrome *P*-450, which was confirmed by inhibition and induction experiments. No ring cleavage was observed in the microsomal metabolism of NTO.

Keywords: NTO; Cytochrome *P*-450; Nitroreductase; Denitrification; Biodegradation; Triazolones; *Bacillus licheniformis*

1. Introduction

Aromatic and heterocyclic nitro-compounds are common industrial chemicals. They are associated with many industrial processes and particularly, the ammunitions industry. There is a great deal of evidence that these recalcitrant compounds are toxic and hazardous [1–3]. The risk that classical explosives such as TNT, RDX, and HMX pose to health and the environment make them less attractive for industrial use, and justifies the need for powerful, stable and poten-

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tially less toxic explosives. The 5-nitro-1,2,4-triazol-3-one (NTO) **1** is a promising candidate [4,5]. This compound is thermally stable and impact-insensitive [6]. Its high performance and stability [4,7] make it of considerable interest to both the defense and civilian sectors. The impact of NTO on the environment has not been investigated yet, and any potential risk can only be accurately predicted when knowledge of its metabolism will be available. Summary of toxicological studies on NTO indicates that the molecule is not toxic to mice or rats when given orally ($LD_{50} > 5$ g kg^{-1}), and is a mild irritant when applied to rabbit skin [5]. However, these investigations did not account for the risk that NTO metabolites may represent. Such intermediates produced during the nitroreduction of nitro-compounds have been shown to cause local damage in the liver [8]. They are responsible for the cytotoxicity, neurotoxicity and chemosensitization of 2-nitroimidazole derivatives [9,10] as well as for the mutagenicity, genotoxicity and carcinogenicity of nitro aromatic [8,11] and nitro aliphatic compounds [12]. Intermediates, including hydroxylamines, ring-opening products and activated oxygen species, are probably responsible for the biological activity of nitro-compounds [13].

We have therefore synthesized ^{14}C-labelled NTO and investigated its microbial and mammalian metabolism. The aim of our study is to characterize the intermediates formed in each case, and to compare both metabolic pathways.

2. Materials and methods

2.1. Chemicals

NTO **1** was a gift from the Commissariat à l'Energie Atomique (Monts, France). Urazole **3** was supplied by Aldrich, 3-methylcholanthrene, dexamethasone, phenobarbital, methimazole and *N*-octylamine were by Sigma, clofibrate by Fluka and miconazole by Janssen. EDTA, ascorbate and cumene hydroperoxide were supplied by Merck. Glc6P–NADP and Glc6PDH were from Boehringer. 5-Amino-1,2,4,-triazol-3-one (ATO) **2** and 1,2,4-triazol-3-one **4** were prepared according to a published procedure [14,6]. ^{14}C-labelled NTO and ^{14}C-labelled ATO were prepared as described earlier [15].

2.2. Microorganism

Bacillus licheniformis was isolated from an aqueous industrial waste containing NTO. The microorganism was grown in a liquid medium containing (per litre): 0.5 g KH_2PO_4, 1 g K_2HPO_4, 30 g D-glucose, 10 g corn steep, 0.5 g $MgSO_4$, 2 g $NaNO_3$, 0.5 g KCl, 0.02 g $FeSO_4$ at 27°C on a rotary shaker (200 rpm). Biomass from 3-day old cultures was recovered by centrifugation and introduced into the aqueous waste containing 15 g l^{-1} NTO. Samples of the medium were centrifuged and the supernatant analyzed by HPLC. When the nitroreduction was complete, the mixture was centrifuged at $10\,000 \times g$ for 15 min. The supernatant was passed through a Sephadex G-10 gel column (Pharmacia Biotech) then eluted with distilled water to give pure amine **2**. M.p.: 240–245°C. ^{13}C-NMR (DMSO, 300 MHz) δ: 155.1 (C=O), 147.8 (C=N). IR: 3684, 3620, 3023, 2980, 2889, 2403, 1525, 1469, 1216, 1040, 920. EIMS: 100 (M_+, 83), 57 (35), 43 (100). CIMS: m/z 101 ($M + H_+$). Anal.: $C_2H_4N_4O$. Calc.: C, 24.0; H, 4.0; N, 55.0; 0, 16.0. Found: C, 24.6; H, 3.8; N, 54.1; 0, 17.4.

2.3. Microsomes

Male Sprague–Dawley rats (175–200 g) were treated with: phenobarbital (0.1% in drinking water for 5 days), 3-methylcholanthrene (20 mg kg^{-1} intraperitoneal in corn oil for 3 days), dexamethasone (100 mg kg^{-1} intraperitoneal in corn oil for 3 days) or clofibrate (500 mg kg^{-1} intraperitoneal in corn oil for 3 days). Animals were then killed and liver microsomes and 9000 $\times g$ supernatant (S9) fractions were prepared.

Protein and cytochrome *P*-450 contents were measured using standard techniques [16]. Experiments with human liver cytochrome *P*-450 produced by yeast were conducted according to the published procedure [17]. Anaerobic incubations were done in phosphate buffer (0.1 M, pH 7.4) deaerated by bubbling argon for over 1 h. Microsomal suspensions were then added to give 4 nmol cytochrome *P*-450 ml^{-1}, and NTO (111 kBq) was added to give a final concentration of 100 μM. The mixture was further deaerated with argon for 3 min, mixed with the NADPH-generating system (NADP, Glc6P and Glc6PDH), and incubated with the desired inhibitors or cofactors at 37°C. Incubations in the presence of oxygen were performed under the same conditions, except that the solutions and buffers were not deaerated. Incubations were stopped by adding CH_3CN (1 ml) and freezing at -20°C. Samples (500 μl) were evaporated to dryness on a Speed-Vac Concentrator (Savant), the residue was taken up in water (500 μl) and the mixture analyzed by HPLC.

2.4. Analytical methods

HPLC apparatus consists of a Wisp 712 injector, a 484 UV detector set at 220 nm, a 745 integrator, a 600 pump (flow rate 1 ml min^{-1}) (Waters). HPLC analysis were performed on a porous graphite column (Hypercarb 7 μm), 100 $\times$ 4.6 mm^2 inter diameter (Shandon HPLC, FRANCE). The elution gradient was: $t_{0-25\ min}$: 100% A (0.05% trifluoroacetic acid in water), $t_{30-45\ min}$: 100% B (15% acetonitrile:0.05% trifluoroacetic acid in water), $t_{50\ min}$: 100% A. Radioactivity was measured by a HPLC radioactivity monitor LB506 C-1 (Berthold).

Nitrite concentrations were determined using a procedure based on the Griess reaction. Aliquots (50 μl) of solution were added to an equal volume of Griess reagent (5% sulfanilamide in 2 M HCl plus 0.5% *N*-1-naphthylethylenediamine in 2 M HCl; by vol.) in a microplate at room temperature for 20 min. The absorbance at 540 nm was measured in a microplate reader (Dynatech).

Urea formation was assayed by two methods: (1) commercial test based on the formation of a coloured complex between urea and diacetyl monoxime (Sigma), and (2) TLC method coupled to radioactivity detection. Spot-associated radioactivity was measured using a linear TLC analyzer Tracemaster 20 (Berthold). Cellulose F plates (Merck) were eluted by butanol–acetic acid–water mixture (50%, 25%, and 25%, respectively). Silica gel plates 60F (Merck) were used to separate NTO and ATO from polar derivatives. The plates were eluted by methanol–chloroform (1:1) saturated with ammonia; under such conditions ATO and NTO are not completely separated.

Radioactivity was determined by mixing 1 ml of the incubation medium with 10 ml of aqueous scintillation cocktail (Aquasafe 300 plus from Zinsser Analytic). The mixture is counted in a LKB 1214 Rackbeta counter. Values correspond to the mean of three samples $\pm$ standard deviation.

3. Results

The HPLC separation of three hypothetical metabolites of NTO on a porous graphite carbon column was described earlier [18].

3.1. Microbial degradation of NTO by B. licheniformis

The use of microorganisms to catalyse the transformation of NTO offers safe experimental conditions, and may contribute to our knowledge of the biodegradation of this compound. Among various microorganisms screened, *B. licheniformis* gave the best results. This strain was isolated from an industrial waste containing NTO, and corresponds to a widespread strain in the environment. The microorganism was grown in a rich medium, recovered by centrifugation

and incubated in the aqueous waste containing NTO. The pH of the solution was adjusted to 6 by adding NaOH pellets to avoid dilution. The physical and chemical factors that influence nitroreduction were investigated; temperature, pH, aeration and sucrose were optimized. The appropriate conditions were pH 6, 27°C, 1.5% sucrose and stirring at 150 rpm. There was no significant difference between aerated and partially deaerated incubations, suggesting that the nitroreduction is not inhibited by oxygen. The reaction was scaled-up to 1 l of industrial waste containing 15 g NTO, 90 g wet *B. licheniformis* cells and 15 g sucrose. Nitroreduction was finished in 24 h, and the amine was easily recovered at this stage.

The second step in NTO degradation is the ring cleavage of ATO, catalysed by *B. licheniformis*. This reaction is inhibited at pH 6 and maximal at pH 8; it did not involve any cyclic intermediate and follows the total nitroreduction of NTO (Fig. 1).

To elucidate the mechanism involved in the triazolone ring cleavage, ^{14}C-labelled NTO and ATO were synthesised. The $^{14}C_3$ (C=O) or $^{14}C_5$ (C–NO_2 or C–NH_2) labelled compounds were used to follow the outcome of each region of the triazolone ring. Various results confirmed that the urea region of ATO was not hydrolysed. According to the literature, such hydrolysis provokes spontaneous liberation of carbon dioxide from the carbonyl group of ATO. During the incubation of $^{14}C_3$-ATO (^{14}C=O) with *B. licheniformis*, formation of $^{14}CO_2$ was not

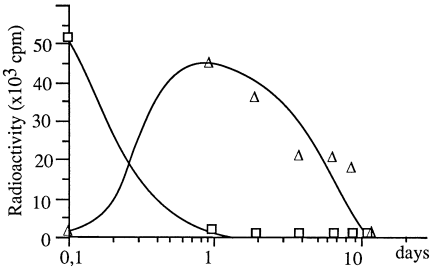

Fig. 1. Degradation of ^{14}C-NTO (□) by *B. licheniformis* and formation of ^{14}C-ATO (△). Samples of the incubation medium were centrifuged and analysed by HPLC.

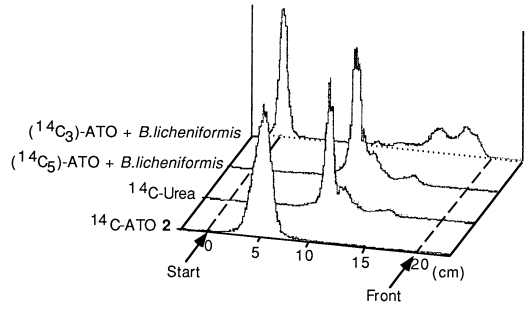

Fig. 2. Degradation of ATO by *B. licheniformis*. $^{14}C_3$-ATO (C=O) or $^{14}C_5$-ATO (C–NH_2) were incubated with the microorganism for 9 days. Supernatants were analyzed by TLC on Cellulose F coupled to radioactivity detection as described in Section 2. Authentic ATO and urea were used as standards.

observed. The carbonyl associated radioactivity was totally recovered in highly polar compounds as shown by TLC analysis (Fig. 3); radioactive urea was not formed in this case. $^{14}C_5$-ATO (^{14}C–NH_2) was totally transformed to ^{14}C-urea, suggesting that carbon 5 is the site of hydrolysis (Fig. 2).

The use of *B. licheniformis* allowed us to achieve the total degradation of NTO in 2 weeks. Starting from $^{14}C_5$-NTO (^{14}C–NO_2) we obtained 40% mineralization arising probably from urea and hydrolysis by urease-like activity. At

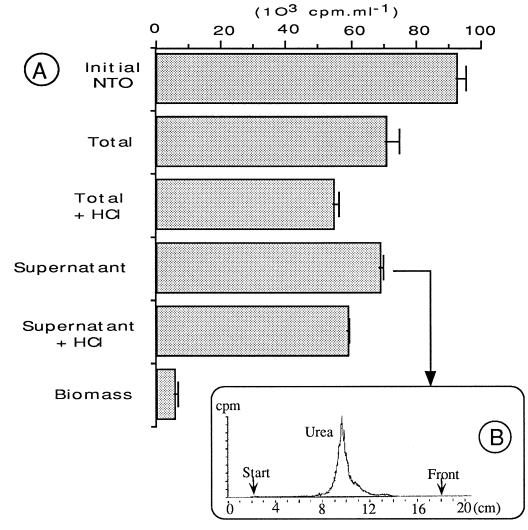

Fig. 3. Bioremediation of NTO. (A) Distribution of radioactivity after two weeks of incubation. (B) Analysis of the supernatant by TLC on Cellulose F.

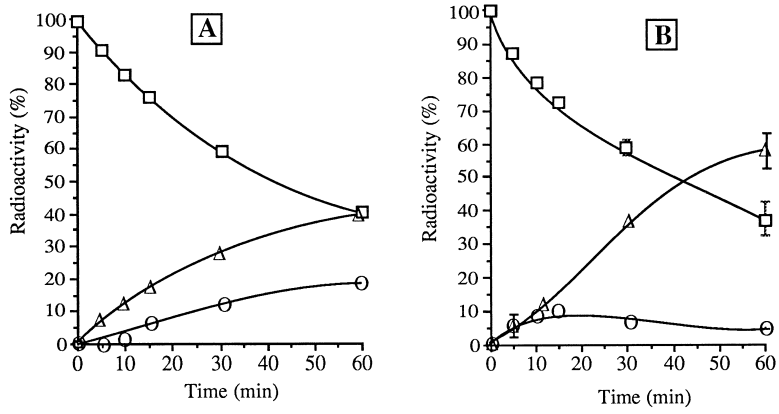

Fig. 4. Microsomal transformation of NTO (□) under aerobic (A) and anaerobic (B) conditions. The amounts of metabolites (ATO, △ and urazole, ○) were determined by monitoring the radioactivity of the HPLC effluent, and are reported as a percentage of the initial ^{14}C-NTO radioactivity.

the end of the incubation, the residual radioactivity was recovered in the supernatant as urea (Fig. 3).

3.2. Mammalian transformation of NTO by rat liver microsomes

Nitroreduction of NTO was principally catalysed by the microsomal fraction of rat liver cells. This reaction required a NADPH-generating system and led to the formation of two metabolites, 5-amino-1,2,4-triazol-3-one **2** and 5-hydroxy-1,2,4-triazol-3-one **3**. The presence of oxygen did not affect the overall conversion rate of NTO, but considerably altered the proportions of the metabolites (Fig. 4). Amine **2** was the major derivative formed under anaerobic conditions, while urazole **3** accounted for less than 5% of the mixture. Urazole **3** represented 40% of the metabolites formed under aerobic conditions, while nitroreduction decreased by 75%. There was no further degradation of NTO or its metabolites during the experiments, as the initial radioactivity was recovered in compounds **1–3**.

^{14}C-labelled ATO was incubated with dexamethasone-induced microsomes to determine whether urazole **3** derived directly from NTO or from the corresponding amine. ^{14}C-ATO was not transformed by rat liver microsomes into either urazole **3** or any other derivative, even when unlabelled NTO was added to the incuba-

Table 1
Relationship between urazole **3** and nitrite formation in aerobic incubations

Experiment no.	Amine **2** (μM)	Urazole **3** (μM)	Nitrite (μM)
1	0	0	0
2	38.2	0	1.3
3	3.2	14.8	15.4
4	5.7	17.4	23

Urazole and amine concentrations were calculated from radioactive peaks, related to the initial ^{14}C-NTO concentration. Nitrite concentration was determined using Griess reagent, as described in Section 2.

Table 2
Influence of the gas phase and cofactor requirement

Conditions	Urazole **3** (%)	Amine **2** (%)	NTO **1** (%)
Complete system (N_2)	3.1	38.2	58.7
+ Air	17.6	9.6	72.8
− NADPH	0	0	100
+ CO	0	0	100
+ Cumene hydroperoxide	0	0	100
+ Iron, ascorbate	0	0	100
Boiled preparation	0	0.9	99.1
Heating 5 min at 42°C	17.6	9.6	72.8

The amounts of metabolites were determined by monitoring the radioactivity of the HPLC effluent, as a percentage of the initial ^{14}C-NTO radioactivity.

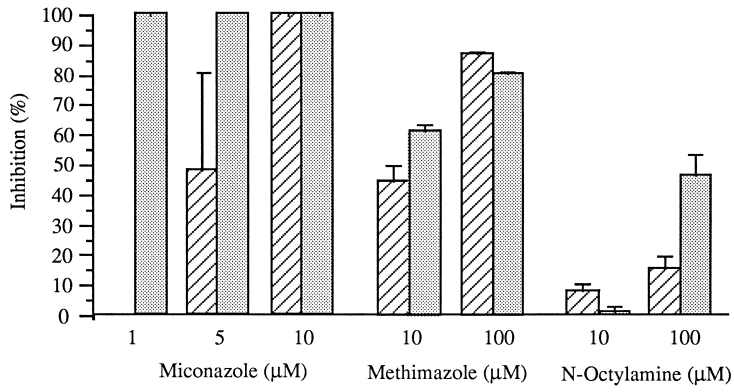

Fig. 5. Effect of cytochrome *P*-450 inhibitors on the aerobic microsomal formation of ^{14}C-ATO (square with diagonal lines inside) and ^{14}C-urazole (dotted square). Microsomes and substrate were incubated for 30 min with the indicated concentrations of inhibitors.

tion. This finding suggests that urazole is formed directly from NTO. Nitrite measurement tends to support an oxidative denitrification of NTO rather than an amine oxidase catalysed reaction. Thus, as shown in Table 1, nitrite was detected only in the experiments where urazole was formed, the concentrations of both compounds being relatively similar.

Table 2 summarises the cofactor requirements and the influence of various gas phases on the metabolism of NTO by rat liver microsomes. These results indicate that cytochrome *P*-450 type enzymes are involved in the formation of both **2** and **3**.

Miconazole, an imidazole-containing inhibitor of cytochrome *P*-450, was a very potent inhibitor of NTO transformation. Formation of urazole **3** was totally inhibited by 1 μM miconazole while the level of amine **2** remained unchanged. Nitroreduction was inhibited by 50% with 5 μM miconazole. NTO transformation was completely inhibited by 10 μM miconazole (Fig. 5). Methimazole decreased both urazole and amine formation with an IC_{50} of about 10 μM, and *N*-octylamine inhibited the oxidative denitrification of NTO to urazole (46% at 100 μM) (Fig. 5).

The influence of various cytochrome *P*-450 inducers was evaluated under aerobic conditions. The liver microsomes from untreated, clofibrate or 3-methylcholanthrene-induced rats transformed NTO to the corresponding amine, but no urazole was detected. Clofibrate induced the highest nitroreductase activity (Table 3). Microsomes from dexamethasone and phenobarbital treated rats catalysed both nitroreduc-

Table 3
Metabolism of NTO by liver microsomes from inducer treated rats

Inducer	Activity (nmol min^{-1} mg protein^{-1})		Cytochrome *P*-450 content (nmol mg protein^{-1})
	Amine **2**	Urazole **3**	
Untreated	0.265 ± 0.025	0	1.01
Dexamethasone	0.17	0.29 ± 0.025	2.13
Phenobarbital	0.35 ± 0.04	0.07	2.41
Clofibrate	1.225 ± 0.015	0	1.83
3-Methylcholanthrene	0.32	0	1.73

The microsomal activity was evaluated under aerobic conditions. Protein and cytochrome *P*-450 contents were measured by standard techniques.

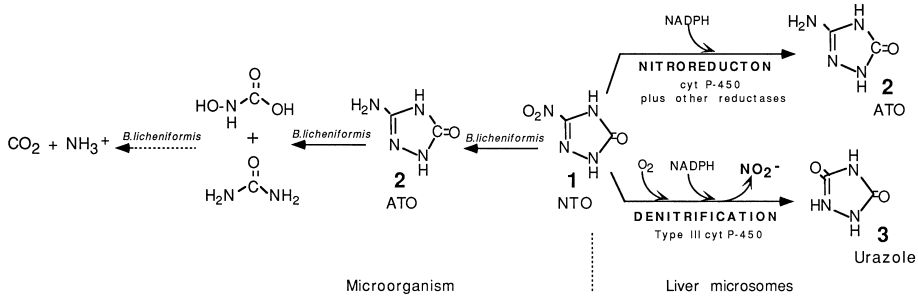

Fig. 6. Proposed metabolic pathways for the microsomal and microbial transformations of NTO.

tion and oxidative denitrification of NTO, but urazole formation by microsomes from phenobarbital-treated rats was relatively slow.

4. Discussion

The potential carcinogenicity of aromatic and heterocyclic nitro-compounds had led to considerable attention being focused on their metabolism in mammals [8–10]. The general pathway of their biodegradation is nitroreduction which may generate potent cytotoxic intermediates, such as primary amines and hydroxylamines [8]. Less extensively, the oxidative denitrification of nitroaliphatic compounds [12], and polycyclic nitroaromatics [19,20] have been reported.

Our results have shown major differences between the mammalian and the microbial metabolism of NTO (Fig. 6). Among the reactions observed, nitroreduction of NTO is common to both systems and led to the primary amine ATO. Hepatic microsomal enzymes involved in this reaction are extensively inhibited by oxygen compared to the microbial enzymes.

Nitroreduction is the sole microbial reaction that affects NTO, but the derived amine ATO is subsequently degraded through triazolone ring cleavage. During this process, *B. licheniformis* cells are not affected by the high concentrations of NTO and ATO (between 10 and 15 g l^{-1}), attesting for a reduced cytotoxicity of these compounds against bacteria. The degradation of NTO with *B. licheniformis* is relatively efficient, inexpensive and attractive for the bioremediation of aqueous NTO. The mechanism involved in the cleavage of the triazolone ring was not established.

In the mammalian system, besides nitroreduction, NTO undergoes an oxidative denitrification, providing urazole and nitrite. This type of reaction has been reported for nitropropane, and suspected to generate genotoxic NO_x species [12]. Inhibition and enzymatic induction experiments suggest that both nitroreduction and oxidative denitrification are catalysed by cytochrome *P*-450 type enzymes.

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