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NITRITE FORMATION FROM 2-NITROPROPANE BY MICROSOMAL MONOOXYGENASES

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Abstract—Rat liver microsomes in the presence of NADPH and dioxygen catalyze the oxidative denitrification of 2-nitropropane to actone and nitrite. Pretreatment of the animals with phenobarbital and 3-methylcholanthrene increases the specific activity. Inhibitors of microsomal monooxygenases inhibit the formation of nitrite and the photochemical action spectrum of the CO-inhibited reaction indicates the involvement of cytochrome P450. The K_m value and the spectral dissociation constant, K_s, of the substrate binding spectrum are both around 10^{-2} M. 1-nitropropane also undergoes oxidative denitrification. It is suggested that aliphatic nitro compounds may exhibit mutagenic or carcinogenic effects as recently demonstrated for 2-nitropropane in rats.

INTRODUCTION

The microsomal drug monooxygenase system of liver and other organs consists of multiple forms of cytochrome P450 species which exhibit different, but overlapping, substrate specificities [1–4]. This enables an organism to attack a broad spectrum of lipophilic organic compounds by introduction of an oxygen atom leading to alcohols, phenols or epoxides as the main products. Usually these metabolites are stable and can be excreted from the body, but from some compounds unstable intermediates are formed. They can stabilize either by reaction with nucleophiles or by internal rearrangement. In the latter case dealkylations, desulfurations or deaminations may be observed [5].

With this respect it seemed interesting to study the metabolism of aliphatic nitro compounds by the microsomal cytochrome P450-dependent monooxygenase system. Unlike aromatic nitro derivatives the carbon atom of secondary or primary aliphatic nitro compounds contains one or two hydrogens, respectively, which could undergo a hydroxylation reaction. The resulting hypothetical geminal hydroxy nitro intermediate would be unstable and could release nitrite under formation of the corresponding ketone or aldehyde.

After *in vivo* application of aliphatic nitro derivatives nitrite has been found in the blood of experimental animals [6], but its route of formation has not yet been elucidated. This was the aim of the present study.

MATERIALS AND METHODS

Male Sprague–Dawley rats (about 120 g b.w.) were used either as controls or pretreated with phenobarbital (Pb) (80 mg/kg b.w. for three days) or 3-methylcholanthrene (3-MC) (20 mg/kg b.w. for two days). The animals were starved 24 hr before decapitation and the microsomal fraction was prepared as described previously [7]. All chemicals were commercially available and were used in highest purity grade. Since a 2-nitropropane–water mixture released nitrite from the substrate, additions to the incubation medium were made from 5 M ethanolic solutions. The standard incubation mixture contained the following concentrations in a total volume of 10 ml: 0.1 M Tris-HCl buffer pH 7.6, 5×10^{-2} M 2- (or 1-) nitropropane, 2 mg of microsomal protein/ml, 1.5 mM 5′-AMP (disodium salt), 5 mM Na_3-isocitrate, 0.1 mM NADPH and 5 U isocitrate dehydrogenase.

After starting the reaction with NADPH 0.5 ml samples were taken at 0, 2, 4, 6 and 8 min and pipetted into an icecold 0.5 M zinc acetate solution in 50% ethanol. 0.5 ml of a 0.5 M Na-carbonate solution was added under shaking. The precipitate adsorbed the microsomal membranes as well as NADPH and was removed by centrifugation. One ml of the clear supernatant was diluted with 1.5 ml of water and 0.5 ml of the diazo reagent (obtained by mixing equal volumes of 10^{-3} M β-naphthyl ethylenediamin in 3 N HCl and 0.1 M sulfanilamide in 3 N HCl before use) were added. The azo dye was measured photometrically at 546 nm after 15 min [8].

Acetone was determined by GLC using a Hewlett–Packard 5700 A gaschromatograph and a column containing Chromosorb W-AW coated with 5% FFAP (2-nitroterephtalic acid).

Substrate binding difference spectra were recorded on an Aminco-DW-2 spectrophotometer. Six ml of a microsomal suspension containing 2 mg of protein/ml were equally divided between two cuvettes and the baseline was established. Microliter additions of methanolic 2-nitropropane were made to the same cuvette and corresponding amounts of methanol to the reference cell. After each addition the spectrum was recorded in the range from 350 to 500 nm.

The photochemical action spectrum was obtained by measuring the reactivation by light of a carbon monoxide inhibited incubation mixture [9]. The light of a 2500 W xenon lamp was passed first through a copper sulfate solution and then through an interference filter (Schott, Mainz) and

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focussed on a square 5 mm cell of 2 ml total volume. Before starting the reaction with NADPH the assay mixture was gassed with a carbon monoxide–dioxygen mixture (4:1 v/v) through two stainless steel tubings inserted at both sides of the cuvette. The light intensities of the various wavelengths were measured with a thermopile (CAI-713002, Kipp & Zonen, Holland) in order to correct the reactivation data for equal light quantities.

RESULTS AND DISCUSSION

When 2-nitropropane was incubated aerobically with rat liver microsomes and NADPH a linear formation of nitrite with time could be established (Fig. 1).

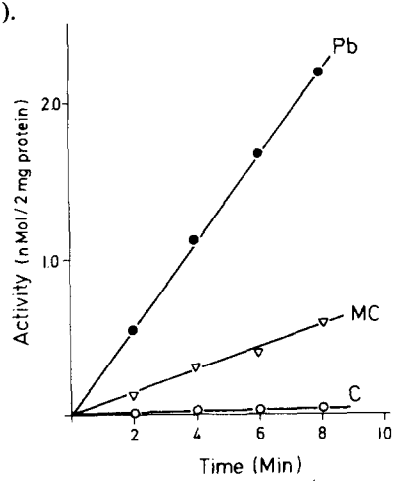


Fig. 1. Time course of nitrite formation from 2-nitropropane in rat liver microsomes. The incubation mixture (10 ml) contained 2 mg of protein/ml with the following cytochrome P450 concentrations: Pb = 2.4 nmole/mg, 3-MC = 1.5 nmole/mg, control (c) = 0.8 nmole/mg protein.

The activity in untreated controls varied but generally was very low. Pretreatment with phenobarbital increased the specific activity to about 10 nmole/min mg of protein. Induction with 3-methylcholanthrene had a much lower, but still stimulating, effect. In these preparations the specific activity was around 0.5 nmole/min · mg of protein.

In addition to nitrite the product of the reaction was acetone, which was determined by GLC in the protein-free supernatant of a 20 min incubation mixture containing 6 mg of microsomal protein per ml from phenobarbital pretreated rats. In order to avoid interference by organic solvents in the gas chromatogram a corresponding amount of pure 2-nitropropane was added to this assay and the ethanolic zinc acetate solution was replaced by an aqueous solution. The concentration of acetone in the supernatant corresponded to the concentration of nitrite determined in an aliquot of the incubation mixture. It can be assumed therefore, that the monooxygenation of 2-nitropropane proceeded by the following equation:

The pH-optimum of the reaction was found at 7.6 with a broad shoulder around 8.0 in microsomes from Pb-pretreated rats. Microsomes from MC-pretreated rats exhibited a very broad optimum around 8.4. The affinity of the monooxygenase system for 2-nitropropane was rather poor as judged from the K_m-value of 2×10^{-2} M found for Pb- as well as for MC-pretreated rats (Fig. 2).

This low affinity was not unexpected, since in general the affinity towards the microsomal monooxygenase system decreases with decreasing chain length and increasing polarity of the organic compound.

It was also expected that typical inhibitors of cytochrome P450 catalyzed monooxygenations, like carbon monoxide, metyrapone (2-methyl-1.2 bis-(3-pyridyl-1-propanone), and 7.8-benzoflavone affected the oxidative denitrification reaction (Table 1).

Metyrapone proved to be most effective in microsomes from Pb-pretreated rats, whereas 7.8-benzoflavone showed a higher per cent of inhibition in microsomes from MC-induced animals. This is similar to findings with other substrates [10] and indicates that different populations of cytochromes P450 participate in both systems. Clear evidence for the involvement of cytochrome P450 was presented by the photochemical action spectrum of the CO-inhibited reaction. A maximal reactivation was achieved with light of 450 nm (Fig. 3).

It was essential for the postulated mechanism to establish the involvement of cytochrome P450 since recently a bacterial dioxygenase was isolated which could denitrificate 2-nitropropane with an overall stoichiometry of one molecule of molecular oxygen for two substrate molecules [11]. This reaction proceeds by dehydrogenation and subsequent oxygenation and hence follows a different pathway compared to cytochrome P450-dependent monooxygenations.

Typical for substrates of cytochrome P450 is their ability to induce a blue-shift of the Soret band in the ferric state [1]. This was also found for

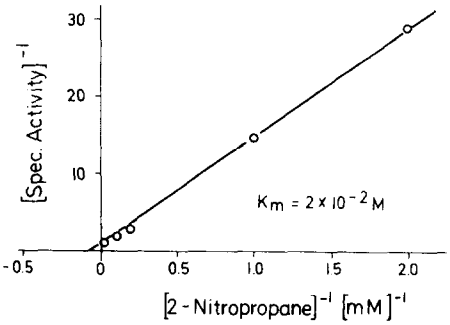
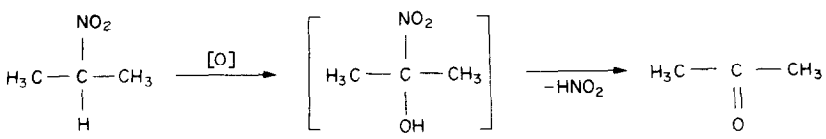


Fig. 2. Lineweaver–Burk plot for the K_m-determination for 2-nitropropane in liver microsomes from phenobarbital pretreated rats. 2-nitropropane was added as a 5 M methanolic solution.



Scheme 1.

Table 1. Inhibitors for the denitrification of 2-nitropropane in rat liver microsomes

Inhibitor	% Inhibition MC-induced	Pb-induced
Metyrapone 3×10^{-5} M	11	82
7.8-Benzoflavone 3×10^{-5} M	37	2
Tetrahydrofuran 1×10^{-2} M	0	30
CO/O_2 (4:1, v/v)	72	88

MC – methylcholanthrene, 20 mg/kg b.w. 2 d.
Pb = phenobarbital, 80 mg/kg b.w. 3 d.

Metyrapone and tetrahydrofuran were added in aqueous solutions. 7.8-benzoflavone was first dissolved in acetone (0.1 M) and then diluted to 3×10^{-3} M in water containing 20 mg/ml of bovine serum albumin. Carbon monoxide and dioxygen were mixed in a 4:1 (v/v) ratio in a calibrated container from which the incubation flask was gassed at a flow rate of 200 ml/min. The preincubation time was 5 min.

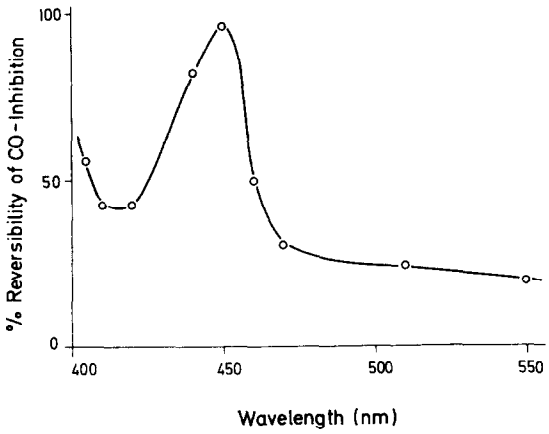


Fig. 3. Photochemical action spectrum of the CO-inhibited denitrification of 2-nitropropane in Pb-microsomes. The activities were determined as described under "Methods". Interference filters of 10 nm band width were used.

2-nitropropane as indicated from the difference spectrum of Pb-microsomes (Fig. 4a).

The magnitude of the difference spectrum compared to other aliphatic hydrocarbon substrates [12] was rather small and explains in part the much lower specific activities for the denitrification reaction. When the difference spectra were related to the concentration of 2-nitropropane in a Lineweaver–Burk plot, a spectral dissociation constant of 1×10^{-2} M was obtained (Fig. 4b).

This is in accordance with the observed K_m-value and again proves the low affinity of this substrate to the monooxygenase system. Furthermore, it adds to the hypothesis that the spectral change from 420 to 390 nm in the difference spectrum reflects the formation of an enzyme–substrate complex at cytochrome P450 [13, 14].

In the ferrous state the cytochrome is a strong reductant and at low concentrations of oxygen or especially in the anaerobic state may transfer electrons to the nitro group, as it was observed for aromatic nitro [15] or polyhalogenated aliphatic compounds [16, 17]. This competition for electrons could diminish the rate of oxygen activation and consequently the oxidative denitrification rate. However, an experiment conducted under reduced oxygen pressure of 4% oxygen in the gas phase gave the same specific activities as under 20%. Consequently, the reduction of the nitro group is unlikely to occur under our incubation conditions.

It can be expected from the unspecifity of the microsomal monooxygenase system that lipophilic aliphatic nitro compounds in general are subject to the oxidative denitrification. Very recently the conversion of 2-nitro-1-phenylpropane to phenylactone was reported and the incorporation of molecular oxygen demonstrated [18].

The oxidative denitrification is not restricted to secondary nitro groups but 1-nitropropane as a representative of primary nitro compounds is also denitrificated with a specific activity of 0.6 nmole/min × mg of microsomal protein from Pb-induced rats. The specific activity and hence the extent of denitrification of a given compound will be influenced by the quality of the microsomal

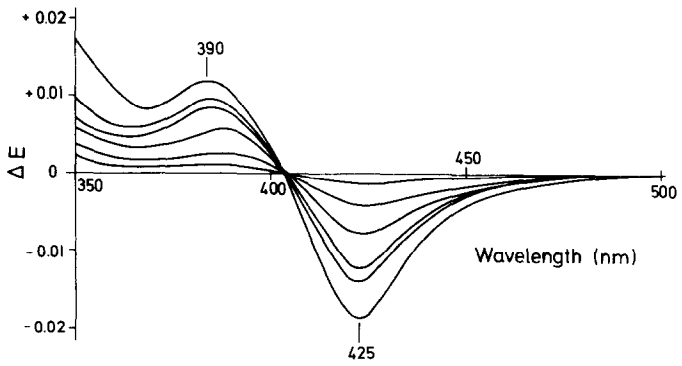


Fig. 4a. Optical difference spectra of Pb-microsomes with increasing concentrations of 2-nitropropane. Additions of 2-nitropropane (5 M in methanol) were made to the sample cuvette with corresponding amounts of methanol to the reference cell. Further details are described under "Methods".

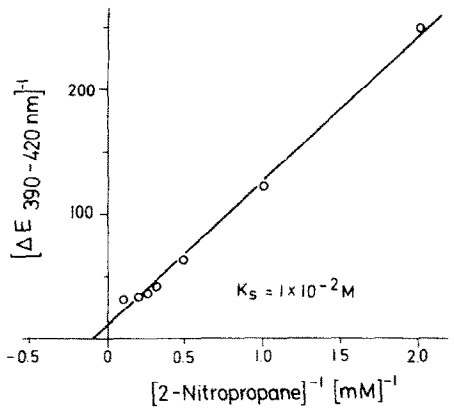


Fig. 4b. Spectral dissociation constant, K_s, for 2-nitropropane and Pb-microsomes. The experimental conditions of Fig. 4a were used.

monooxygenase system in relation to the various pretreatments that the cytochrome P450 species present in microsomes from control rats exhibit almost no activity, whereas pretreatment by phenobarbital or methylcholanthrene causes the induction of isoenzymes with higher activities. These findings were similar to those reported for other drugs [10].

Although the extent of denitrification for the two nitropropanes is low the potential toxicity of nitrite should not be underestimated. When applied externally small amounts of nitrite are not known to cause deleterious effects [19], but this may change when it is liberated within the cell. Diazo and *N*-nitroso compounds are typical reaction products of nitrite with primary or secondary amines and could lead to chemical modifications of biologically important macromolecules [20]. Recent warning about 2-nitropropane as a solvent with carcinogenic properties in rats [21] may have their molecular basis in the oxidative denitrification reaction. This aspect is under further study.

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