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THE INTERACTION OF ALIPHATIC NITRO COMPOUNDS WITH THE LIVER MICROSOMAL MONOOXYGENASE SYSTEM

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Abstract—It was previously shown that liver microsomes from phenobarbital pretreated rats can convert 2-nitropropane to acetone and nitrate in the presence of NADPH and dioxygen. The investigation was now extended to the compounds phenylnitromethane, ω-nitrostyrene, nitrocyclohexane and nitromethane, which were also denitrificated but with weaker specific activities than 2-nitropropane. Untreated rats and 3-methylcholanthrene induced animals yielded microsomes with even lower activities. The observation that addition of nitromethane to an oxidized rat liver microsomal suspension did not result in a substrate binding difference spectrum like the other nitro compounds, but gave rise to a peak at 437 nm, was unexpected. With the aid of e.p.r.-spectroscopy, this was interpreted as the formation of a cytochrome P450-NO complex. Parallel to this complex formation, oxidized rat liver microsomes catalyzed the production formaldehyde from nitromethane in an NADPH-independent reaction. In contrast, liver microsomes from phenobarbital pretreated pigs produced a normal substrate binding spectrum with nitromethane and were unable to release formaldehyde from nitromethane. Reduced liver microsomes from all sources yielded a cytochrome P450-ligand spectrum with peaks around 453 nm, which probably reflects the formation of a ferrous cytochrome P450–nitrosoalkane complex.

Aliphatic nitro compounds are commonly used organic chemicals and solvents with more or less toxic effects in organisms. Some of their molecular interactions with cell constituents have been described only recently. Schnabel and Ullrich [1] reported that 2-nitropropane forms a ligand complex with microsomal cytochrome P450 in the presence of sodium dithionite, which is very likely a nitroso complex of the ferrous hemoprotein [2]. The same compound was found to undergo a denitrification when it was incubated aerobically with liver microsomes and NADPH [3]. Nitrite and acetone were found as metabolites. Similarly, phenyl-acetone was a product of a microsomal incubation of 1-phenyl-2-nitropropane, but the formation of nitrite was not reported [4]. In view of the toxicity of aliphatic nitroalkanes and the reactivity of nitrite in the potential formation of nitrosamines, we were interested in the microsomal metabolism of other aliphatic nitro compounds.

MATERIALS AND METHODS

Male Sprague–Dawley rats (about 120 g body wt) were pretreated with either phenobarbital (Pb) (80 mg/kg body wt, for 3 days) or 3-methylcholanthrene (MC) (20 mg/kg body wt, for 2 days). The animals were starved 24 hr before decapitation and the microsomal fraction was prepared as described previously [5].

The methods and procedures for both nitrite determination and difference spectroscopy were published in the preceding paper [3], with the following additions. As substrates, phenylnitromethane, ω-nitrostyrene (EGA-Chemie), nitrocyclohexane (EGA-Chemie), nitromethane (Fluka AG) and tetranitromethane (Fluka AG) were used, together with 2-nitropropane (Fluka AG). These compounds were added to the incubation mixtures as 1 M methanolic solutions. When formaldehyde from nitromethane was determined, a suspension of the substrate in buffer was used.

Formaldehyde was determined by the Nash reagent according to Cochim *et al.* [6]. Electron paramagnetic resonance spectra were obtained with a Varian E-9 spectrometer connected to a digital computer (Data General, Nova 820) for base line subtraction and spectra integration. *g*-Values were calculated from magnetic field values obtained from the 'Fieldial' with diphenylpicrylhydrazine as a *g*-marker. Spin concentrations were determined from the double integrals by means of a Cu (II)–EDTA standard [7].

RESULTS AND DISCUSSION

When the commercially available compounds phenylnitromethane, ω-nitrostyrene, nitrocyclohexane and nitromethane were incubated aerobically with liver microsomes from phenobarbital pretreated rats in the presence of NADPH, a linear formation of nitrite was observed. Relative to 2-nitropropane, the denitrification of these compounds was lower, as can be seen from Table 1.

Besides nitrite the products of nitrocyclohexane and nitromethane were identified as cyclohexanone and formaldehyde, respectively. The stoichiometry of nitrite to formaldehyde formation in the case of nitromethan was exactly 1:1, as shown in Fig. 1.

For the other two nitro compounds the corre-

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Table 1. Denitrification activity of rat liver microsomal monooxygenases*

Substrates	Specific activity (± S.E.M.) nmol/mg protein · min
2-Nitropropane	1.9 ± 0.5
Phenylnitromethane	1.8 ± 0.5
Phenylnitromethane	1.0 ± 0.3†
ω-Nitrostyrene	0.7 ± 0.2
Nitrocyclohexane	0.6 ± 0.2
Nitrocyclohexane	0.15 ± 0.1†
Nitromethane	0.2 ± 0.1

* Specific activities were calculated from the values obtained after an incubation time of 8 min for each substrate. Four experiments with different microsomal preparation were run in duplicates.

† From rats pretreated with 3-methylcholanthrene (20 mg/b.w. for three days.)

sponding formation of a keto group is also very likely, so that a complete analogy to the results obtained with 2-nitropropane is suggested. This also applies for the induction with 3-methylcholanthrene, which as with 2-nitropropane resulted in a decrease of the specific activities for phenylnitromethane and nitrocyclohexane. A similar low activity was observed in untreated control rats. One therefore can conclude that only the forms of cytochrome P450 induced by phenobarbital exhibit appreciable activities towards these substrates. To what extend other CH-bonds of these molecules were hydroxylated was not determined. This might be of interest to answer the question whether these nitro compounds are weak substrates in general, or whether only the sterical shielding of the CH-bond next to the nitro group causes the low denitrification rates.

The denitrification of 2-nitropropane was completely inhibited by carbon monoxide, indicating the exclusive dependence on cytochrome P450. This is important with respect to the previously reported participation of O_2^- and OH-radicals in the oxidation of 2-nitropropane to nitrite and acetone [8]. In further support for the cytochrome P450 dependence of the microsomal denitrification, we have incubated microsomes with NADPH, dioxygen, nitropropane and inhibitors for O_2^-, H_2O_2 and OH radicals (Table 2).

None of the inhibitors showed a significant effect in agreement with the postulated role of cytochrome P450 in the reaction [3].

Addition of nitrocyclohexane, ω-nitrostyrene and phenylnitromethane to liver microsomes causes the formation of a substrate binding spectrum with cytochrome P450. This indicates that, like 2-nitropropane, these compounds are typical substrates for cytochrome P450 and could be hydroxylated and denitrificated in the presence of NADPH and dioxygen (Table 3). In contrast to 2-nitropropane, the affinities as measured by the spectral dissociation constants (K_s) are higher for the more lipid soluble compounds nitrocyclohexane, ω-nitrostyrene and phenylnitromethane.

Under reducing conditions achieved by addition

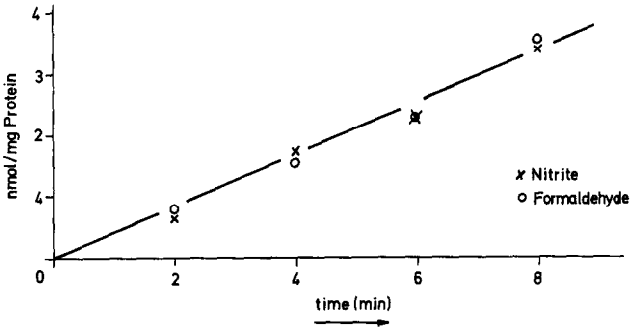


Fig. 1. Stoichiometry of formaldehyde and nitrite formation from nitromethane by rat liver microsomes in the presence of NADPH and dioxygen. A final volume of 10 ml of a microsomal suspension from phenobarbital-pretreated rats (3.5 mg protein/ml) was incubated aerobically with 50 mM nitromethane, 10^{-4}M NADPH and an NADPH-regenerating system (1.5×10^{-5}M 5'AMP, 5×10^{-3}M isocitrate, 1.25 U isocitrate-dehydrogenase) in 0.1 M Tris–HCl buffer pH 7.6. At the times indicated, 0.5 and 1.0 ml of the mixture were used for the nitrite and formaldehyde determination, respectively.

Table 2. Effect of inhibitors for OH-radicals, O_2^- radicals and hydrogen peroxide on the denitrification of 2-nitropropane

	Specific activity nmol/mg protein	percent of control
Control	0.92	100
+ Mannitol (10^{-2}M)	1.00	109
+SOD (25 U/ml)	0.80	87
+ Catalase	0.90	98

* The normal incubation assay was supplemented with mannitol, superoxide dismutase (SOD) and catalase, respectively. Average values from two experiments.

Table 3. Difference spectra of rat liver microsomes from phenobarbital-pretreated rats with various nitro compounds*

Compound	State	Absorbance max (nm)	Absorbance min (nm)	ΔE (Max–min) x nmol Cyt. P450	K_s(M)
Nitrocyclohexane	ox.	388	423	0.0067	$3{,}4 \times 10^{-4}$
	red.	422,461	439		
Phenylnitromethane	ox.	387	424	0.0043	$1{,}0 \times 10^{-3}$
	red.	423,451	435		
2-Nitropropane	ox.	385	422	0.0053	$1{,}0 \times 10^{-2}$
	red.	425,455	433		
Nitromethane	ox.	437	396,421	0.0043	$8{,}3 \times 10^{-2}$
	red.	424,453	432		
Tetranitromethane	ox.	354,435	419	0.0038	
	red.	424,454	432		

* The nitro compounds were dissolved in methanol and added in μl amounts. ΔE-values were taken from maxima to minima and used for K_s-determinations in Lineweaver-Burk plots.

of dithionite, all compounds showed peaks between 450 and 460 nm in the difference spectrum indicating a ligand binding under formation of a hyperporphyrin spectrum [9, 10]. It has been suggested recently that under these conditions aliphatic nitro compounds can be reduced to nitroso derivatives which show strong complexing properties with hemoproteins [2, 11].

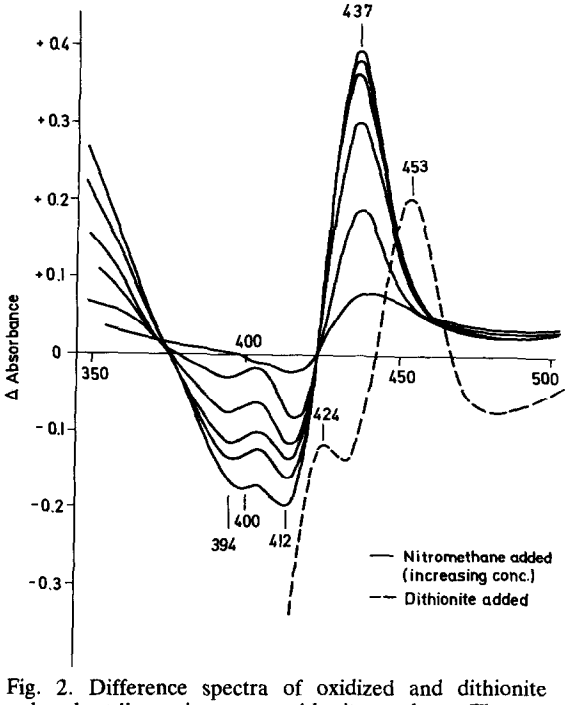


Fig. 2. Difference spectra of oxidized and dithionite reduced rat liver microsomes with nitromethane. The two cuvettes contained 6.0 mg of protein (2.2 nmol cytochrome P450/mg protein) from phenobarbital pretreated rats in 3 ml of 0.1 M Tris–HCl buffer, pH 7.6. Solid lines: 1,2,3,4,5,7 μl of 10 M nitromethane (in methanol) were added to the sample cuvette and corresponding amounts of methanol to the reference cuvette. Dashed line: Further addition of 2 mg of sodium dithionite to both cuvettes.

Another type difference spectrum appeared in the presence of nitromethane (Fig. 2).

A peak at 437 nm developed within seconds accompanied by the formation of weaker bands at 540, 572 and 602 nm which must be attributed to a derivative of oxidized cytochrome P450 since no reductant was present and conversion of cytochrome P450 to cytochrome P420 abolished the spectrum. Addition of sodium dithionite to both cuvettes resulted in the usual difference spectrum of cytochrome P450 with other aliphatic nitro compounds exhibiting peaks at 453, 525 and 580 nm.

This reduced spectrum very probably originated from a ligand reaction of nitrosomethane formed by reduction of nitromethane [2, 11].

The oxidized spectrum was more difficult to explain, since it resembled a ligand type spectrum, but nitromethane has no liganding properties unless it would undergo a chemical reaction under these conditions. This assumption was supported by the observation that during the incubation of nitromethane a continuous and linear formation of formaldehyde was measured by the Nash-reaction (Fig. 3) at a rate of 0.2 nmol/mg microsomal protein × min.

In contrast to the monooxygenase reaction performed with nitromethane in the presence of NADPH and dioxygen, the release of formaldehyde from this substrate was not paralleled by a release of nitrite as described in Fig. 1. Only a small amount was detected at the start of the reaction, but no further increase with time was observed.

Obviously the C–N bond of the nitromethane molecule was cleaved and, besides formaldehyde, nitric oxide could have been formed. Indeed, the oxidized difference spectrum closely resembles that obtained with nitric oxide and cytochrome P450 [12]. Such a complex would not be detectable by its e.p.r.-spectrum because of the tight spin coupling between the ferric iron and the unpaired electron of nitric oxide. However, after addition of dithionite and immediate cooling down to the temperature of liquid nitrogen, a spectrum appeared with g-values at 2.089

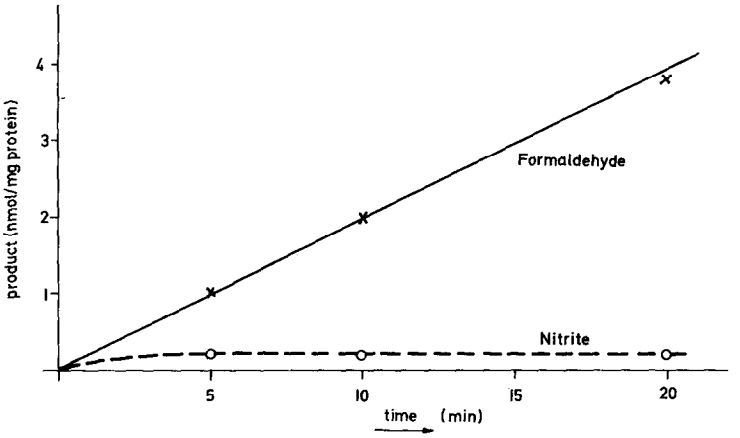


Fig. 3. Time dependence of formaldehyde and nitrite formation in oxidized rat liver microsomes under nitrogen. Microsomal suspension from phenobarbital-pretreated rats (10 ml; 10 mg protein/ml) was incubated anaerobically under nitrogen with 5×10^{-3}M nitromethane (added without solvent). At the times indicated, 0.5 and 1.0 ml of the mixture were used for the nitrite and formaldehyde determination, respectively.

and 2.011 which are almost identical to the two g-values reported for the bacterial cytochrome P420–NO complex [13].

Also the splitting constant of the g_z-absorption was consistent with that of the cytochrome $P420_{CAM}$-NO complex. Integration of the spectrum and standardization with a Cu-EDTA sample gave a value of 10% of microsomal cytochrome P450 being present as the NO complex.

It is known that the reduced cytochrome P450–NO complex is highly unstable at room temperature. This would explain why the reduced difference spectrum of microsomes shows the absorption bands of the nitrosomethane complex at 453 nm and not that of the nitric oxide complex which would have shown peaks at 443 and 586 nm. The additional band at 425 nm could correspond to a cytochrome P420–NO complex, which is formed from the reduced cytochrome P450–NO complex at room temperature. The calculated value of about 10% complex formation would be in rough agreement with the height of this peak.

Tetranitromethane addition to microsomes resulted in the same difference spectra as with nitromethane, but no formaldehyde was found. If the 437 nm peak is indicative for the release of NO from

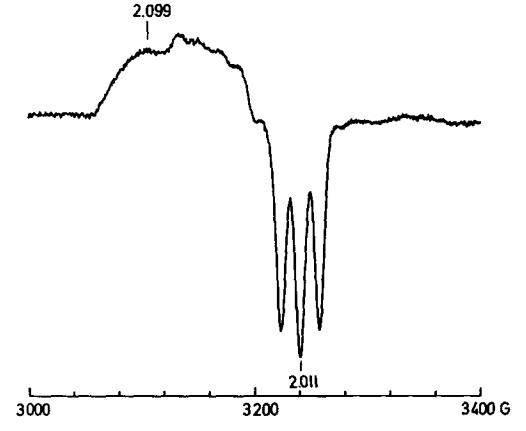


Fig. 4. EPR spectrum of rat liver microsomes after incubation with nitromethane and subsequent reduction by dithionite. Fifty-one milligrams microsomal protein/per ml in Tris–HCl buffer, pH 7.6, were supplemented with 10 μl of 10 M nitromethane (in methanol) and 1 min later with 1 mg of sodium dithionite at room temperature. Then the spectrum was recorded at −180° with 10 mW microwave power, 9.15 GHz frequency, 10 G modulation amplitude and gain 400.

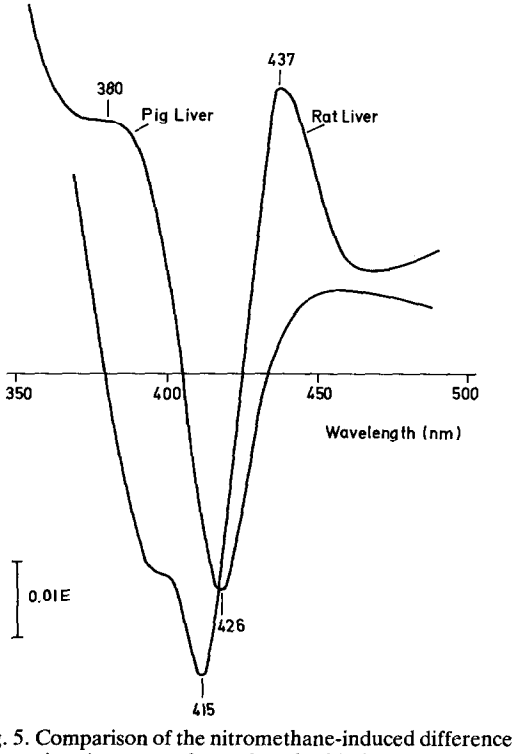
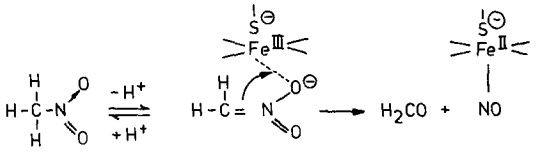


Fig. 5. Comparison of the nitromethane-induced difference spectra in microsomes from phenobarbital-pretreated rats and pigs. Ten microlitres of 10 M nitromethane (in methanol) were added to the sample cuvettes and 10 μl methanol to the reference cells.

the nitro group, then nitromethane and tetranitromethane are unique in their reaction with cytochrome P450, since the other aliphatic nitro compound listed in Table 3 gave normal substrate binding spectra. The splitting of NO from the nitromethanes seemed to be even a characteristic feature of rat liver cytochrome P450, since no corresponding difference spectrum was seen in liver microsomes from phenobarbital-pretreated pigs.

Interestingly, these microsomes were also inactive in catalyzing the formaldehyde release from nitromethane, which suggested that a specific interaction of the substrate molecule to a special form of cytochrome P450 was necessary to initiate the cleavage of the C–N bond. The simplest mechanism according to which this cleavage could have occurred would have been a migration of an oxygen atom from the nitrogen to the carbon atom. In view of the known properties of cytochrome P450 to catalyze the transfer of oxygen atoms, e.g. from iodosylbenzene to other acceptors [14], such an intramolecular rearrangement in the following transition state is likely to occur.

In the subsequent steps, the nitric oxide complex must be formed and a one-electron oxidation must take place, which also could be mediated by the



ferric ion. Model studies are now carried out to verify this proposed mechanism.

The interaction of cytochrome P450 with aliphatic nitro compounds and especially the nitromethanes represents a further interesting facet in the variety of catalytic reaction of heme–sulfur proteins.

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