An Investigation of the Metabolism of N-Nitrosodimethylamine and N-Nitrosomethylaniline by Horseradish Peroxidase *in vitro*

M Stiborová[1], H Hansíková[1], H H Schmeiser[2] and E Frei[2]

1 *Department of Biochemistry, Faculty of Natural Sciences, Charles University Albertov 2030, 128 40 Prague Czech Republic*
2 *Department of Molecular Toxicology, German Cancer Research Center, Im Neuenheimer Feld 280 691 20 Heidelberg Germany*

Abstract. The demethylation of carcinogenic N-nitrosodimethylamine (NDMA) and N-nitrosomethylaniline (NMA) is catalyzed by horseradish peroxidase in the presence of hydrogen peroxide NMA is a better substrate for peroxidase than NDMA The K_m values are 0 74 and 3 12 mmol/l for NMA and NDMA, respectively The oxidation of NDMA and NMA is inhibited by radical trapping agents (nitrosobenzene, glutathione, ascorbate, NADH) This indicates the radical mechanism for the peroxidase-mediated oxidation of both N-nitrosamines The *in vitro* metabolism of NMA using peroxidase was investigated in detail Beside formaldehyde, the metabolites formed from NMA by peroxidase include aniline, *p*-aminophenol and phenol Phenol formation presumably arose from N-demethylation of NMA via a benzenediazonium ion (BDI) intermediate while aniline and *p*-aminophenol from denitrosation of this carcinogen The results are discussed from the point of view of the role of peroxidases in the metabolism of N-nitrosamines

Key words: Carcinogens — N-nitrosamines — Peroxidase — Catalysis — Demethylation — Radical mechanism

Abbreviations: BDI, benzenediazonium ion, K_m, Michaelis constant, NDMA, N-nitrosodimethylamine NMA, N-nitrosomethylaniline, P450, cytochrome P450, tRNA, transfer RNA, V_{max}, maximal velocity

Correspondence to M Stiborová, Dept of Biochemistry, Faculty of Natural Sciences, Charles University, Albertov 2030 12840 Prague, Czech Republic, Fax 420-02-21952331 E-mail hudecek@prfdec natur cuni cz

Introduction

N-Nitrosamines are a unique group of chemicals with a broad organ and species specificity of the toxic and/or carcinogenic effects Tumors can be produced in nearly all organs, depending on the route of administration and the structure of the nitrosamine (Preussmann and Stewards 1984, Preussmann and Wiessler 1987) The metabolism of these chemicals has, hence, been subject of extensive investigations Microsomal cytochromes P450 (P450) are supposed to be the main enzyme systems participating in N-nitrosamine metabolism (Tu and Yang 1983, Dipple et al 1987, Preussmann and Wiessler 1987, Yang et al 1990, Shu and Hollenberg 1996, Stiborová et al 1996 a,b) Nevertheless, the involvement of other specific enzyme systems for nitrosamine metabolism (such as peroxidases) cannot be excluded in the tissues (or cells), which are not rich in P450s (Sivarajah et al 1982, Dipple et al 1987, Preussmann and Wiessler 1987, Schuller et al 1990, Stiborová et al 1992 a,b)

Peroxidases (such as prostaglandin H synthase and/or non-specific peroxidases) are present at significant concentrations in several tissues (i e urinary bladder, kidney, lung) (Dipple et al 1987, Preussmann and Wiessler 1987, O'Brien 1988, Schuller et al 1990) The involvement of these enzymes in the activation of some N-nitrosamines has been suggested (Schuller et al 1990), based on indirect *in vivo* studies with inhibitors of one peroxidase, prostaglandin H synthase Aspirin and indomethacin inhibited efficiently the metabolism of N-nitrosodiethylamine in alveolar type II lung cells which are rich in prostaglandin H synthase (Falzon et al 1986) Furthermore, in hamsters injected with these inhibitors prior to administration of radiolabelled N-nitrosodiethylamine a pronounced decrease in alkylation of macromolecules of pulmonary cells was demonstrated, while inhibitors of P450 did not affect the level of alkylation (Hegedus et al 1987, Schuller et al 1990)

We have found that carcinogenic N-nitrosomethylaniline (NMA) is oxidized *in vitro* by horseradish peroxidase to ultimate carcinogens which bind to DNA and transfer RNA (tRNA) (Stiborova et al 1992 a,b) However, a detailed study of the oxidation of this compound and/or other N-nitrosamines by peroxidase is still missing

Here, we report the results of an *in vitro* study of the metabolism of two carcinogenic N nitrosamines, namely, symmetric N-nitrosodimethylamine (NDMA) and asymmetric N-nitrosomethylaniline (NMA) by horseradish peroxidase The kinetics and the mechanism of the peroxidase-mediated oxidation of both nitrosamines were studied in details

Materials and Methods

Chemicals

Chemicals were obtained from the following sources NADH, horseradish peroxidase, glutathione and nitrosobenzene from Boehringer, Germany, benzenediazonium hexafluorophosphate from Aldrich, USA, NDMA and NMA were synthesized as described previously (Druckrey et al 1967), all other chemicals were reagent grade or higher

Analytical methods

Kinetic analyses were carried out using the non-linear least-square method described previously (Cleland 1983)

The assay mixture for the demethylation of NDMA and NMA contained (per 1 ml) 50 mmol/l potassium phosphate buffer (pH 8 0), 200 μg horseradish peroxidase (EC 1 11 1 7), 0 03–3 mmol/l NMA dissolved in dimethylsulfoxide or 0 03–6 mmol/l NDMA dissolved in distilled water The mixtures were incubated for 10 min at 37 °C The reaction was terminated by addition of 500 μl 20% trichloroacetic acid The amount of formaldehyde formed was measured as described by Nash (1953) Alternatively, after 10 min incubation (37 °C) 0 5 ml aliquots of the mixtures were reacted with 2,4-dinitrophenylhydrazine in orthophosphoric acid The 2,4-dinitrophenylhydrazone formed from formaldehyde raised from NMA and NDMA was determined by HPLC as described by Janzowski et al (1982)

The incubation mixtures containing one of the nitrosamines studied (NMA) were applied to a C18 SepPak cartridge to quantify the products formed from NMA in the peroxidase system The cartridge was washed with 0 1 mmol/l NaOH and the products were eluted with 50% methanol in water The products were separated by HPLC on a Separon SGX C18 column with a linear gradient of methanol in water (20–70% (v/v) methanol from 0 to 12 min, 70–100% methanol from 12 to 16 min) Absorbance of the eluent was monitored at 250 nm Reaction products were identified by comparison of their retention times with those of authentic standards (aniline, phenol, *p*-aminophenol) The quantities were calculated by peak areas relating to external standards Recoveries of products were around 80% after 10 min incubation in the presence of peroxidase without hydrogen peroxide, except the recovery of phenol (it was around 60%) The recovery of phenol when benzenediazonium hexafluorophosphate was added to the incubation mixture containing peroxidase but without hydrogen peroxide was about 20% after 10 min incubation

NADH oxidation was measured in a final volume of 2 ml containing 50 mmol/l potassium phosphate buffer pH 8 0, 0 1 mmol/l NADH, 0–114 μmol/l hydrogen peroxide, 0–5 μg horseradish peroxidase, and 0–1 5 mmol/l NDMA or NMA Reactions were started by addition of the respective nitrosamine, and the disappearance of

NADH with time was followed at 340 nm using a Beckmann D-62 spectrophotometer.

Values given in Figures 1–3 are averages of three parallel experiments.

Results

Peroxidase in the presence of hydrogen peroxide oxidizes carcinogenic N-nitrosamines NDMA and NMA. The formation of formaldehyde illustrating N-demethylation of these carcinogens was proven (Table 1). No activity was observed when any of the component was omitted from the reaction mixture (Table 1).

Table 1. Demethylation of NDMA and NMA by peroxidase

Incubation conditions	Rate[a] of demethylation of			
	NDMA		NMA	
	0.3 mmol/l	3.0 mmol/l	0.3 mmol/l	3.0 mmol/l
Complete system	0.010 ± 0.001	0.206 ± 0.030	0.195 ± 0.020	0.630 ± 0.070
H_2O_2	0	0	0	0
peroxidase	0	0	0	0

Experimental conditions are described in Materials and Methods. Values given are averages and standard deviations of three parallel experiments.
[a] nmol HCHO/min per nmol peroxidase

The rate of demethylation is dependent on pH. N-demethylation of NMA and NDMA showed an optimum in slightly alkaline pH (8.0–8.5) (Fig. 1).

Formaldehyde formation was measured in the reaction medium which contained peroxidase, hydrogen peroxide and various NDMA or NMA concentrations. The reactions followed the Michaelis-Menten kinetics (Fig. 2*A*). NMA is a better substrate of peroxidase than NDMA. Under the conditions used, the maximal velocity (V_{max}) of the reaction with NMA is approx. two times higher than that with NDMA (Fig. 2*B*, Table 2). The value of the apparent Michaelis constant (K_m) for the formaldehyde formation from NMA is smaller than that for the formaldehyde formation from NDMA (Table 2).

Because N-demethylation of NDMA to formaldehyde was detected in the peroxidase system, this enzyme catalyzes the oxidation of NDMA similarly to P450, producing formaldehyde and methyldiazonium ion (Kroeger-Koepke et al. 1981, Yang et al. 1990, Stiborova et al. 1996b).

More complicated reactions of NMA oxidation were generated by the P450-dependent system (Scheper et al. 1991, Stiborova et al. 1996a). Therefore, special

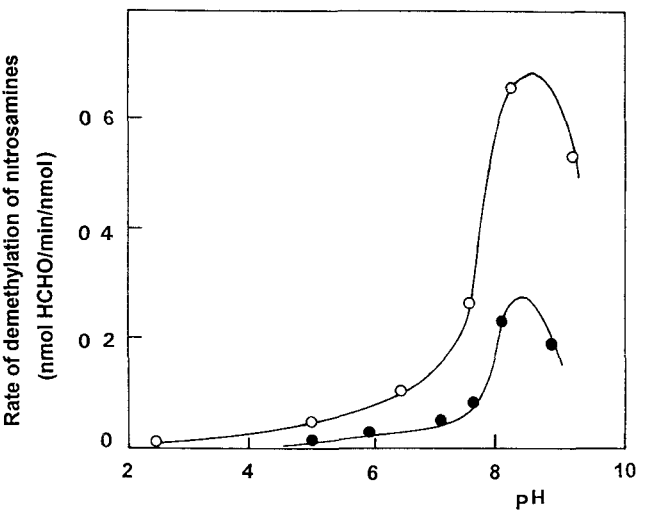

Figure 1. The effect of pH on peroxidase mediated N-demethylation of NDMA (●) and NMA (○) The experimental conditions are described in Materials and Methods except that 50 mmol/l potassium phosphate buffers pH 2 3 9 0 were used

emphasis was laid on the identification and quantitation of not only formaldehyde but also of other NMA metabolites formed by peroxidase

The products of NMA conversion generated by the peroxidase system were separated by HPLC The metabolites were identified by comparison of their retention times with those of authentic standards The unchanged NMA is not detected here as it remains bound to the SepPak cartridge Beside formaldehyde the metabolites formed from NMA by peroxidase included aniline and *p*-aminophenol (Fig 3) Phenol which is the representative metabolite for the oxidative bioactivation pathway via the intermediary BDI formation, was also determined and the yields were corrected for the about 20% recovery of phenol from BDI in the presence of peroxidase This compound represents the main metabolite with about 20% in relation to the NMA concentration (3 mmol/l) within 10 min under the conditions used (Table 3) Aniline which is formed by the denitrosation reactions and *p*-aminophenol (its hydroxylation metabolite) are minor products (Table 3) Based on the products formed we can postulate that peroxidase catalyzes both N-demethylation reaction (leading to the formation of formaldehyde and BDI) and denitrosation (Fig 4)

The conversion of NDMA and NMA is strongly inhibited by NADH, ascorbate glutathione and nitrosobenzene, which are known to be radical scavengers (O'Brien 1988) (Table 4) These findings indicate that radical mechanisms are involved in the reactions

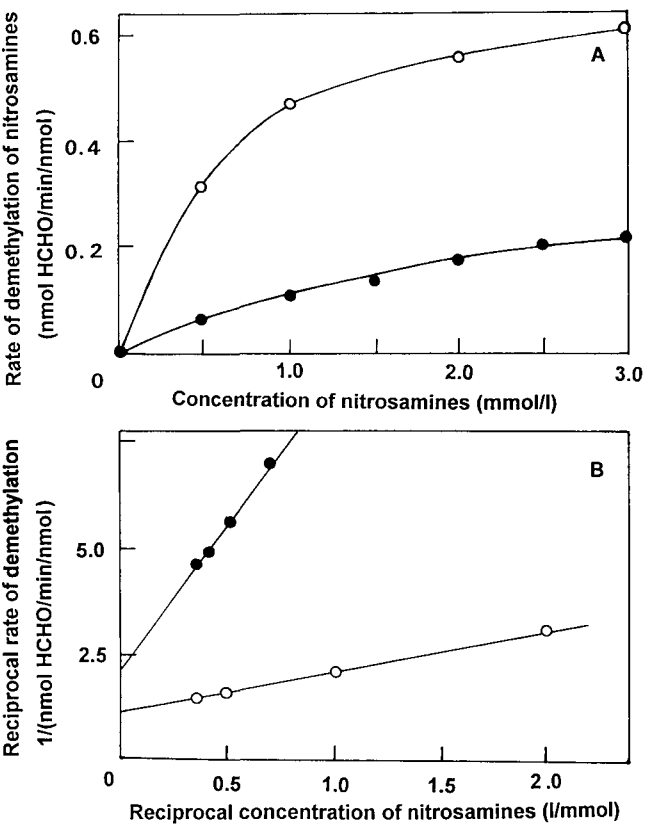

Figure 2. Kinetic curves for NDMA (●) and NMA (○) demethylation by horseradish peroxidase (*A*) (*B*) Reciprocal plots of the data from panel *A* For experimental conditions, see Materials and Methods

Table 2. Kinetics of NDMA and NMA oxidation by peroxidase

Substrate	K_m (mmol/l)	V_{max} (nmol/min per nmol peroxidase)
NDMA	3 12 ± 0 030	0 45 ± 0 040
NMA	0 74 ± 0 008	0 8 ± 0 080

Experimental conditions are described in Materials and Methods Values shown are averages and standard deviations of three parallel experiments

We studied the mechanism of inhibition of NMA oxidation by ascorbate and NADH

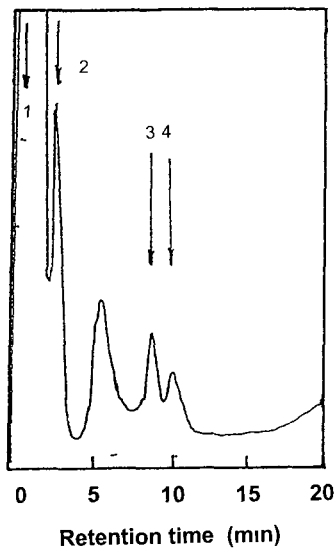

Figure 3. HPLC separation of products formed by horseradish peroxidase Experimental conditions are described in the text The peak numbers refer to the following metabolites (1) solvent front, (2) *p*-aminophenol, (3) phenol, (4) aniline The dotted line indicates the HPLC chromatogram of the control sample (without hydrogen peroxide)

Table 3. *In vitro* metabolism of NMA by the peroxidase system

Metabolite	Yields (%)[a]
p-Aminophenol	2 90 ± 0 20
Phenol	20 00[b]± 1 75
Aniline	1 47 ± 0 13

Experimental conditions are described in Materials and Methods Values represent means and standard deviations of three experiments
[a]Based on added NMA (3 mmol/l)
[b]Corrected for 20% recovery of phenol from peroxidase

Low formaldehyde production was detected when 1 0 mmol/l ascorbate was present in the incubation mixture Two possibilities of the mechanism of ascorbate inhibition of NMA conversion could be suggested Ascorbate may be the substrate of peroxidase, and it may compete with NMA, or it may react with the reactive metabolites of NMA Ascorbate is practically not converted by peroxidase as its substrate (measured at 265 nm) under the conditions used in the experiments with the peroxidase system alone (ΔA/min = 0 05), but a rapid oxidation occurred in the presence of 50 μmol/l NMA (ΔA/min = 0 96) Ascorbate is therefore not a peroxidase substrate but reduces the radicals formed from NMA back to NMA

Similarly, free radical(s) from NMA act as catalyst in the oxidation of NADH by H_2O_2 and peroxidase In the absence of NMA in the reaction mixture, the oxida-

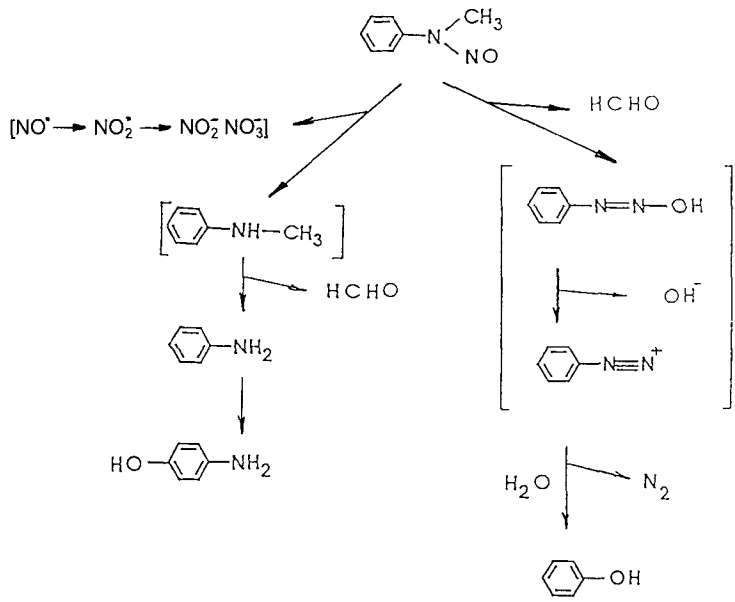

Figure 4. Proposed metabolic scheme of NMA in the peroxidase system The metabolites in the parentheses were not detected

tion of NADH by the peroxidase system was negligible (0 002 nmol NADH/min per nmol peroxidase) the same was true for the system with this compound but without H_2O_2 (0 003 nmol NADH/min per nmol peroxidase) However, in the complete system (peroxidase H_2O_2, NADH, NMA) 7 6 nmol NADH/min per nmol peroxidase was oxidized Hence the inhibition of the peroxidase-mediated oxidation of NMA is caused by reduction of the reactive intermediates by NADH the latter getting simultaneously oxidized

We suggest that a similar mechanism is also operating for the glutathione inhibition Neither glutathione is a substrate for peroxidase under the conditions used (Subrahmanyam et al 1987 O'Brien 1988) but it reduces the radicals formed from NMA back to the parent compound as it has been described earlier for several other substrates of peroxidase (O Brien 1988 Eling et al 1990)

Discussion

In the present work we have shown the kinetic characteristics of oxidation of two carcinogenic nitrosamines by peroxidase NDMA as a carcinogen causing tumors of the liver and NMA as an agent with cancerogenic potential for esophageal

Table 4. The effect of radical scavengers on the demethylation of NDMA and NMA

Compound	Rate of N-demethylation of	
	NDMA	NMA
	(nmol HCHO/min per nmol peroxidase)	
None	0 206 ± 0 030	0 630 ± 0 07
Ascorbate		
0 5 mmol/l	0 100 ± 0 012 (48 5)	0 310 ± 0 033 (49 2)
1 0 mmol/l	0 021 ± 0 002 (10 2)	0 060 ± 0 007 (9 5)
Glutathione		
0 5 mmol/l	0 089 ± 0 010 (43 2)	0 350 ± 0 038 (55 5)
1 0 mmol/l	0 019 ± 0 002 (9 2)	0 068 ± 0 007 (10 8)
NADH		
0 5 mmol/l	0 123 ± 0 015 (59 7)	0 410 ± 0 040 (65 1)
1 0 mmol/l	0 024 ± 0 002 (11 6)	0 072 ± 0 007 (11 1)
Nitrosobenzene		
1 0 mmol/l	0 141 ± 0 012 (68 1)	0 420 ± 0 038 (66 7)

Experimental conditions are described in Materials and Methods Values represent means and standard deviations of three experiments The figures in the parentheses are percentage values relative to controls

and/or lung tissue were chosen for the study Horseradish peroxidase was used as a model, because this peroxidase has been shown to be a suitable system to mimic the metabolism of some carcinogens (i e benzidine, 2-naphthylamine, N N-dimethyl-4-aminoazobenzene, etc) catalyzed by mammalian peroxidases (Meunier 1987, Yamazoe et al 1988 Eling et al 1990, Stiborová et al 1992 c) Moreover, our preliminary results with mammalian peroxidase, lactoperoxidase, have illustrated that NDMA and NMA are oxidized by this peroxidase in a similar way as by horseradish peroxidase (Stiborová et al 1996 c) Likewise, NMA was found to be N-demethylated to formaldehyde by another mammalian peroxidase, prostaglandin H synthase (Sivarajah et al 1982) However, the experiments with horseradish peroxidase as a model cannot be generalized Prostaglandin H synthase and horseradish peroxidase are mechanistically similar yet differences do exist (Eling et al 1990)

NMA activation by peroxidase has already been described in our previous papers (Stiborova et al 1992 a,b) We have found that the reactive intermediates (or products) which are formed during the oxidation of NMA by horseradish peroxidase are bound to nucleic acids (DNA and tRNA) *in vitro* (Stiborová et al 1992 a,b) However, the kinetics of this reaction was not studied and other products formed during the reactions catalyzed by horseradish peroxidase (Stiborová et al 1992 a,b) or by prostaglandin H synthase (Sivarajah et al 1982) were not determined

Formaldehyde was formed from both nitrosamines, N-demethylation is, hence

the reaction catalyzed by peroxidase N-demethylation secondary to α C hydroxylation reaction (catalyzed by P450s) is supposed to be an important activation pathway for many nitrosamines (Dipple et al 1987) It leads to the formation of highly reactive electrophilic intermediates (alkyl or aryldiazonium ions) which are capable of reacting with cellular nucleophiles (Kroeger Koepke et al 1981 Dipple et al 1987 Koepke et al 1990) There is also evidence for the formation of nitrite during the metabolism of nitrosamines (Lori et al 1982 Yang et al 1990 Scheper et al 1991 Stiborova et al 1996a) It may represent a detoxication pathway for nitrosamines

The kinetic data indicate that NMA is a better substrate for peroxidase than NDMA The oxidation of this carcinogen was, therefore, studied in more detail Peroxidase catalyzes both NMA activation (resulting in the formation of the ultimate carcinogenic BDI) and its inactivation by denitrosation (Fig 4)

From a comparison of the kinetic data obtained for NDMA and NMA oxidation by P450 isoenzymes and horseradish peroxidase, we can postulate that both nitrosamines are oxidized by P450 more efficiently than by peroxidase Particularly P450 2B1 and P450 2E1 very effectively activate NMA and NDMA respectively (Yang et al 1985, 1990, Stiborova et al 1996 a,b) The V_{max} values obtained for NDMA and NMA oxidation by peroxidase are approx seven and four times smaller than those obtained for their oxidation by P450, respectively P450 isoenzymes (P450 2E1 and P450 2B1) might hence be responsible for the metabolism of both nitrosamines also *in vivo*

The products of NMA oxidation catalyzed by peroxidase are the same as those catalyzed by P450 2B1 (Stiborova et al 1996 a, present paper) The yields of individual products are, however different Phenol as the product of the N demethylation reaction is formed preferentially to products formed by denitrosation (Table 3, Fig 4)

Although some of the products formed from NMA (and/or NDMA) under the activity of peroxidase are identical with those formed by P450, the mechanisms with respect to the two enzymes are different N-dealkylation of several substrates by peroxidases is known to be different from the N dealkylation mechanism suggested for P450 (Lasker et al 1981, Sivarajah et al 1982, Guengerich 1990) N Demethylation catalyzed by peroxidases results in the formation of free radical cation and iminium cation by sequential one electron oxidations, the latter being hydrolyzed to formaldehyde and demethylated amine (Griffin and Ting 1978 Sivarajah et al 1982, Guengerich 1990) This mechanism has however, not been confirmed for N demethylation of NMA and NDMA as yet Nevertheless, we found that their oxidation by peroxidase is inhibited by compounds which also act as radical scavengers Free radicals are, therefore, formed during the demethylation of both nitrosamines Hence, we suggest that the radical mechanism of N-demethylation catalyzed by peroxidase (postulated for other enzyme substrates) also occurred

for NDMA and NMA. Further experiments are, however, needed to confirm this suggestion.

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