Cytochromes P450 2B1 and P450 2B2 Demethylate N-Nitrosodimethylamine and N-Nitrosomethylaniline *in vitro*

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Abstract. The demethylation of carcinogenic N-nitrosodimethylamine (NDMA) and N-nitrosomethylaniline (NMA) is catalyzed by purified rat liver cytochromes P450 2B1 and 2B2 reconstituted with NADPH-P450 reductase and dilauroylphosphatidylcholine. A molar P450 to reductase ratio of about 1.0 is the most appropriate for the catalysis. NMA is a better substrate for both P450 enzymes than NDMA, with K_m values of 0.34 and 0.43 mmol/l for P450 2B1 and P450 2B2, respectively. For NDMA as the substrate, the K_m values were approx. ten times higher than those for NMA. With each isoenzyme only one K_m for NDMA or NMA was observed, whereas with liver microsomes of PB-pretreated rats, multiple K_m values were obtained. The results strongly suggest that both P450 isoenzymes can be involved in the metabolism of nitrosamines.

Key words: Cytochrome P450 isoenzymes — N-Nitrosamines — Catalysis — Demethylation

Introduction

N-Nitrosamines are unique group of chemicals with a broad organ and species specificity as to the toxic and/or carcinogenic effects. The metabolism of these compounds has, hence, been subject of extensive investigations. There is evidence indicating that N-nitrosamines are metabolized by a cytochrome P450-dependent monooxygenase system (Tu and Yang 1983; Yang et al. 1985, 1990; Dipple et al. 1987). Other specific enzymatic systems for N-nitrosamines (such as peroxidases) are also involved in their metabolic activation in tissues (or cells), which are not rich in cytochrome P450 (P450) (Preussmann and Wiessler 1987; Schuller et al. 1990; Stiborová et al. 1992). Some of the target tissues for N-nitrosamines-induced tumorigenesis are abundant in special P450 isoenzymes (e.g. liver, nasal cavity, esophagus) which are supposed to be responsible for the metabolic initiation of

nitrosamine-induced carcinogenesis in these organs (Dipple et al. 1987; Preussmann and Wiessler 1987; Schuller et al. 1990). The specificity of these isoenzymes in reactions converting carcinogenic nitrosamines in crude microsomes (Lorr et al. 1982; Gold et al. 1987) or in the reconstituted NADPH-dependent system has, therefore, been extensively investigated (Yang et al. 1985, 1990). Studies using liver isoenzymes of rabbits treated with several special P450 inducers showed that P450 induced by ethanol (P450 2E1) is mainly responsible for the metabolism of NDMA and/or other nitrosamines, but the role of other P450 isoenzymes cannot be excluded (Yang et al. 1985, 1990).

In the present work, we have studied the *in vitro* metabolism of two N-nitrosamines, namely NDMA and NMA with a carcinogenic potential (Fig. 1), by P450 2B1 and P450 2B2 isoenzymes purified from liver microsomes of phenobarbital (PB)-pretreated rats.

Abbreviations: ΔA_{SAT}, differences between the actual minimum and maximum absorbance; DMSO, dimethylsulfoxide; K_m, Michaelis constant; K_S, spectral dissociation constant; NDMA, N-nitrosodimethylamine; NMA, N-nitrosomethylaniline; PB, phenobarbital; P450, cytochrome P450; P450 2B1, P450 2B2 and P450 2E1, cytochromes P450 isolated from livers of rats pretreated with PB and ethanol, respectively; P450 LM_2, cytochrome P450 isolated from livers of rabbits pretreated with PB; V_{max}, maximal velocity.

Materials and Methods

Chemicals

Chemicals were obtained from the following sources: cytochrome c, NADPH and dilauroylphosphatidylcholine from Fluka, Switzerland; dithiothreitol from Koch-Light, UK; Emulgen 911 from Atlas Co., Japan; glucose-6-phosphate dehydrogenase from Calbiochem, Switzerland; adamantane from Organopharma, Czech Republic; NADH from Boehringer, Germany; and all other chemicals were reagent grade or better. NDMA and NMA were synthesized as described previously (Druckrey et al. 1967). Diamantane and triamantane were prepared according to the published procedure (Curtney et al. 1972; Kafka and Náhůnek 1986).

Microsomes and microsomal enzymes

Microsomes from rat livers were prepared as described by Kimura et al. (1982), from male Wistar rats pretreated with PB as described by Stiborová et al. (1988), and stored at −70 °C.

Cytochromes P450 2B1 and P450 2B2 were isolated from liver microsomes of PB-pretreated rats by a procedure described previously (Anzenbacher et al. 1984;

Hodek et al. 1988). The P450 preparations were electrophoretically homogeneous (Anzenbacher et al. 1984; Hodek et al. 1988), and specific contents, expressed as nmol P450 per mg protein, were 17.3 and 19.8 for P450 2B1 and P450 2B2, respectively. Electrophoretically homogeneous NADPH-P450 reductase was isolated from rat liver microsomes as described previously (Yasukochi et al. 1979). The specific activity of the reductase was 60 μmol reduced cytochrome c per min per mg protein. The enzymes were stored at −70 °C.

Analytical methods

Quantitative determination of P450 was carried out according to Omura and Sato (1964). The concentration of NADPH-P450 reductase was estimated as described earlier (Vermilion and Coon 1978). The activity of NADPH-P450 reductase was measured according to Sottocasa et al. (1967) using cytochrome c as the substrate (i.e. NADPH-cytochrome c reductase).

Protein concentrations were estimated according to Bradford (1976) or Lowry et al. (1951) with bovine serum albumin as a standard.

In vitro binding of substrates of P450 was monitored by difference spectroscopy (Jefcoate 1978). The substrates were directly added to the sample cuvette containing oxidized P450 isoenzymes, an identical volume of solvent being added to the reference cuvette. Absorption spectra were recorded between 370 and 500 nm 30 s after mixing. Results were expressed as ΔA (380–425 nm).ml^{-1} obtained after the addition of the substrate. When shifts were observed in absorbance minima or maxima, ΔA between actual minima and maxima were estimated (ΔA_{SAT}). The substrate spectra of P450 isoenzymes (2 μmol/l) were measured at 20 °C in 20 mmol/l potassium phosphate pH 7.5. The concentrations of the tested compounds were 0.1–400 μmol/l.

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

One ml of the assay mixture for the demethylation reactions of NDMA an NMA contained 0.3 nmol P450 2B1 or P450 2B2, 0.3 nmol rat liver NADPH-P450 reductase, 50 mmol/l potassium phosphate (pH 7.4), 7.5 mmol/l $MgCl_2$, 8 mmol/l semicarbazide, 4 mmol/l glucose-6-phosphate, 0.4 units of glucose-6-phosphate dehydrogenase, 100 μg of D,L-dilauroylphosphatidylcholine, and 0.06-75 mmol/l NMA or 0.5-25 mmol/l NDMA dissolved in dimethylsulfoxide (DMSO). After 1 min of preincubation, the reaction was started by addition of NADPH (final concentration 0.5 mmol/l). The mixture was 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).

To estimate the optimal ratio in the reconstitution experiments, 0.15–1.2 nmol of the NADPH-P450 reductase and a constant 0.3 nmol of P450 2B1 or P450 2B2 in 1 ml were used, or 0.15–1.2 nmol of both P450 isoenzymes were tested with

a constant 0.3 nmol of the reductase. The NDMA or NMA concentration was 3 mmol/l.

One ml of the assay mixture for demethylation of NDMA or NMA catalyzed by rat liver microsomes contained 50 mmol/l potassium phosphate (pH 7.4), 2 nmol P450 (measured in microsomal preparations containing 1.5 mg/ml protein), 0.4 units of glucose-6-phosphate dehydrogenase, 7.5 mmol/l $MgCl_2$, 8 mmol/l semicarbazide, and 0.05–12 mmol/l NMA or 0.12–90 mmol/l NDMA. The procedure was the same as that described for the reconstitution experiments (see above).

Inhibition of demethylation by adamantane and diamantane was performed by the addition of 1.5–600 μmol/l and 0.3–240 μmol/l of adamantane and diamantane, respectively, dissolved in DMSO.

Carbon monoxide inhibition of demethylation was performed by the addition of a 50 μl aliquot of 50 mmol/l phosphate buffer saturated with CO to the incubation medium, either in darkness or under white light, from a 15 cm distant 150 W heat-filtered quartz lamp.

Values given in Figures 2–5 are averages of three parallel experiments.

Results

Metabolism of NDMA and NMA by liver microsomes

NMA and NDMA are demethylated by liver microsomes of PB-pretreated rats. The demethylation had an absolute requirement for NADPH. The reaction was negligible when NADPH regenerating system was omitted (Table 1). NADH is a less efficient cofactor than NADPH.

Demethylation of both nitrosamines was significantly inhibited by carbon monoxide, and this effect was partly reversed upon irradiation of the incubation mixture (Table 1).

Table 1. Demethylation of NDMA and NMA by microsomes from rats pretreated with PB

Incubation conditions	Relative activity of demethylation of NDMA	NMA
Complete	100.0 ± 8.1[a]	100.0 ± 5.6[b]
NADPH regenerating system	2.1 ± 0.3	3.6 ± 0.8
+ NADH (0.5 mmol/l) instead of NADPH	22.3 ± 1.3	15.4 ± 1.2
+ CO-buffer (50 μl) darkness	41.8 ± 4.3	43.5 ± 4.6
+ CO-buffer (50 μl) light	88.9 ± 7.5	91.9 ± 8.2

The demethylation was measured as described in Materials and Methods. 100% activity was 0.31[a] and 1.32 nmol HCHO/min/nmol P450[b]. Means and standard deviations of three parallel experiments are shown.

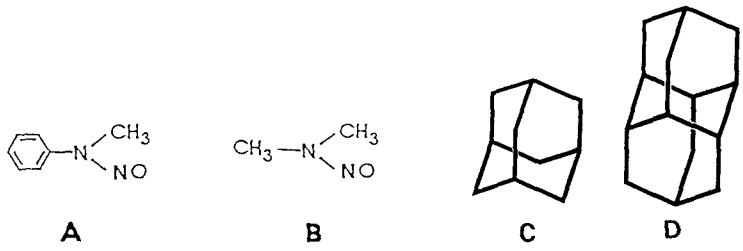

Figure 1. Chemical structures of NMA (*A*), NDMA (*B*), adamantane (*C*) and diamantane (*D*).

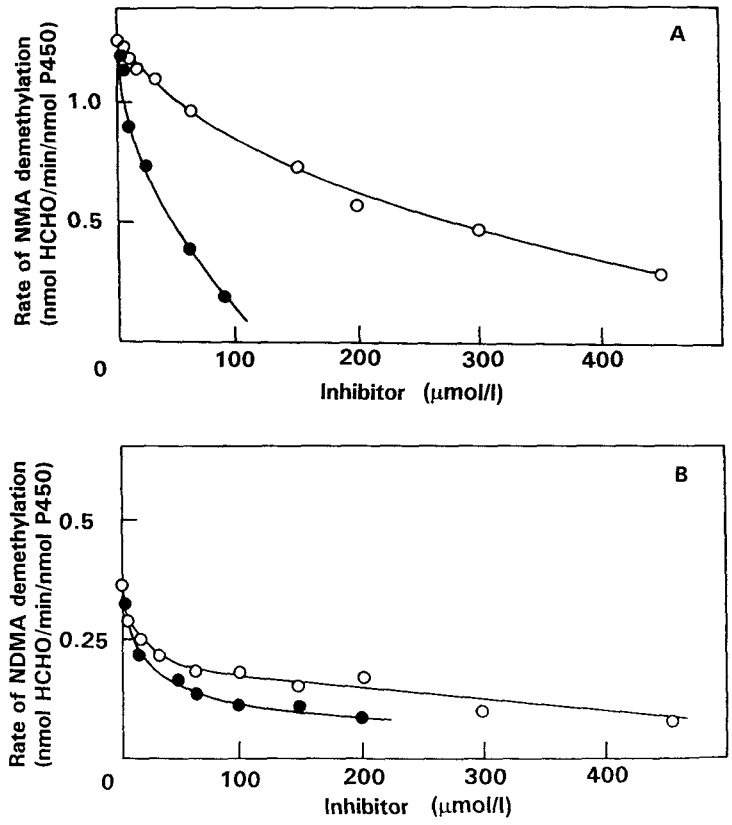

Figure 2. Inhibition of NMA (*A*) and NDMA (*B*) demethylation by adamantane (○) and diamantane (●) in PB-induced microsomes (1.5 mg/ml). The NDMA or NMA concentration was 3 mmol/l.

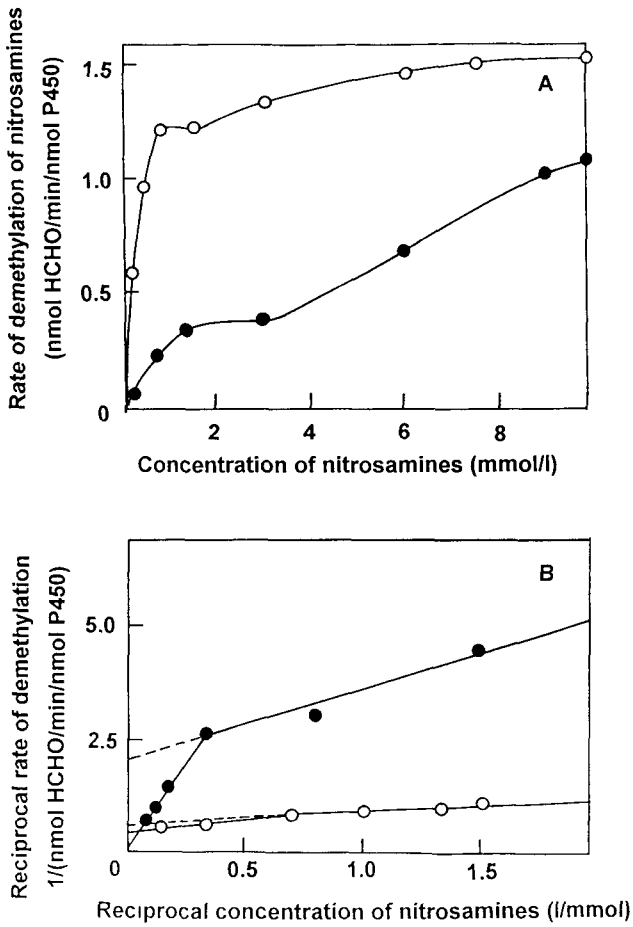

Figure 3. Kinetic curves for NDMA (●) and NMA (○) demethylation by PB-induced microsomes (*A*). (*B*) Reciprocal plots of the data from panel *A*. 50 mmol/l potassium phosphate (pH 7.4), 1.5 mg/ml microsomal protein. For other conditions, see Materials and Methods section.

Adamantane and diamantane (Fig. 1) known as substrates of cytochromes P450 having a high affinity to the enzymes (Anzenbacher et al. 1984; Hodek et al. 1988, 1995) inhibited the demethylation of N-nitrosamines in a concentration-dependent manner (Fig. 2), presumably because these agents compete effectively with N-nitrosamines for the occupancy of the active site. Diamantane had more significant effect than did adamantane.

Non-Michaelian saturation curves were seen when the initial velocity was plotted as a function of NDMA or NMA concentrations for liver microsomal enzymes

Table 2. Spectral characteristics resulting from the binding of substrates to P450 2B1 and P450 2B2

Compound	K_S (μmol/l)	ΔA_{SAT}	Values of extremes (nm)	
			maxima	minima
P450 2B1				
Adamantane	12.40 ± 2.40	0.010 ± 0.002	387	420
Diamantane	1.87 ± 0.07	0.035 ± 0.002	385	421
Triamantane	14.73 ± 3.40	0.069 ± 0.008	387	421
NMA	12.90 ± 2.10	0.002 ± 0.0001	387	420
NDMA	192.00 ± 32.00	0.008 ± 0.001	425	396
P450 2B2				
Adamantane	92.00 ± 4.10	0.038 ± 0.001	380	421
Diamantane	13.30 ± 3.20	0.013 ± 0.007	380	425
Triamantane	83.00 ± 9.90	0.040 ± 0.005	384	421
NMA	2.61 ± 0.20	0.003 ± 0.0005	378	425
NDMA	15.30 ± 0.70	0.004 ± 0.001	418	385

Spectral dissociation constants (K_S) and differences between the actual minimum and maximum absorbance (ΔA_{SAT}) were estimated as described in Material and Methods. The numbers in the Table (K_S and ΔA_{SAT}) are averages and standard deviations of three parallel experiments.

of PB-pretreated rats (Fig. 3*A*). Double reciprocal plots of the same data were not linear (Fig. 3*B*). These plots consisted of at least two linear portions. The apparent Michaelis constant (K_m) values were 0.71 and 25.0 mmol/l with the corresponding maximal velocity (V_{max}) values of 0.49 and 5.0 nmol/min/nmol P5450 for NDMA. The apparent K_m values were 0.52 and 1.02 mmol/l, and V_{max} values were 1.47 and 1.67 nmol/min/nmol P450 for NMA (Fig. 3*B*).

The results strongly suggest that demethylation of the studied nitrosamines is catalyzed by cytochrome P450 enzymes present in rat liver microsomes of PB-pretreated rats. This is consistent with the results of Lorr et al. (1982) and Scheper et al. (1991) who postulated that P450 isoenzymes induced in liver microsomes by PB are able to metabolize N-nitrosamines, NMA being the best substrate.

Metabolism of NDMA and NMA by P450 isoenzymes in the reconstituted system

In order to examine whether demethylation reactions are catalyzed solely by the P450-dependent monooxygenase system, a reconstituted system composed of purified P450 isoenzymes isolated from microsomes of PB-pretreated rats (P450 2B1 or P450 2B2), NADPH-P450 reductase, and dilauroylphosphatidylcholine was used.

Also, we have studied the interactions of P450 2B1 and P450 2B2 isoenzymes with NMA and NDMA as well as with saturated polycyclic carbohydrates adaman-

Table 3. Conditions for the reconstitution of P450 isoenzymes catalyzing NDMA and NMA demethylation

Conditions	Reaction rate (nmol HCHO/min per nmol P450)			
	P450 2B1		P450 2B2	
	NDMA	NMA	NDMA	NMA
Complete system	1.7 ± 0.2	3.1 ± 0.3	0.9 ± 0.1	1.3 ± 0.2
– P450 isoenzymes	0	0	0	0
– NADPH-P450 reductase	0	0	0	0
NADPH regenerating system	0	0	0	0
– dilauroylphosphatidylcholine	0.05 ± 0.001	0.11 ± 0.1	0.02 ± 0.003	0.05 ± 0.001

One ml of the complete reaction system contained 0.3 nmol P450 2B1 or P450 2B2, 0.3 nmol NADPH-P450 reductase, 100 μg dilauroylphosphatidylcholine and 8 mmol/l NDMA or NMA. Means and standard deviations of three parallel experiments are shown.

tane, diamantane and triamantane. Difference spectroscopy was used for these studies.

NMA, adamantane, diamantane and triamantane gave type I binding spectra with P450 2B1 and P450 2B2 isoenzymes with a maximum at around 390 nm and a minimum at around 420 nm (Table 2). NDMA produced a reverse type of spectrum with both isoenzymes (Table 2). Table 2 shows the values of spectral dissociation constants (K_S) and the absorbance differences (ΔA_{SAT}) for these compounds. Among the compounds tested, the highest affinity of P450 2B1 was found for diamantane, followed by NMA and adamantane (Table 2). P450 2B2 was shown to have the highest affinity for NMA, with the dissociation constant value of 2.61 μmol/l.

Upon mixing P450 2B1 or P450 2B2 with NADPH-P450 reductase and dilauroylphosphatidylcholine, the reconstituted monooxygenase systems efficiently catalyzed the demethylation of NDMA and NMA. Very low, if any activity was observed when any of the component was omited from the reconstituted system (Table 3).

The rate of demethylation was dependent on the P450/NADPH-P450 reductase molar ratio. The optimal molar P450 isoenzymes to reductase ratios were close to the value of 1.0 for both N-nitrosamines (Fig. 4). This molar ratio was used to determine the kinetic parameters of NMA and NDMA demethylation catalyzed by the reconstituted cytochrome P450 system.

Kinetics of NDMA and NMA demethylation in the reconstituted system

Formaldehyde formation was measured in the reaction medium, which contained P450 reconstituted enzymic system and various NDMA concentrations. The reac-

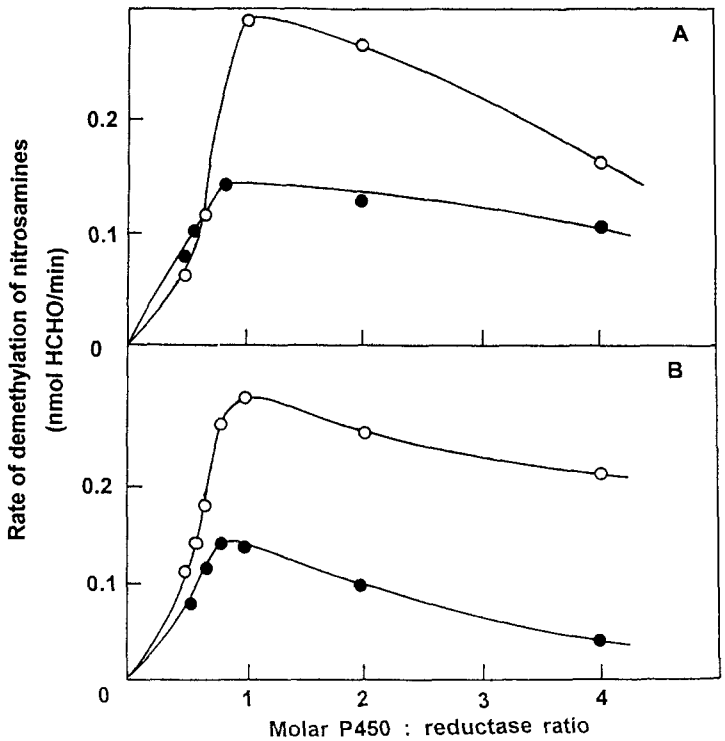

Figure 4. Dependence of the rate of NDMA (●) and NMA (○) demethylation on the molar ratio of cytochrome P450 2B1 (A) and P450 2B2 (B) to NADPH-P450 reductase in the reconstituted enzyme system. For experimental conditions, see Materials and Methods.

Table 4. Kinetics of NDMA and NMA metabolism by the reconstituted cytochrome P450 2B1 and P450 2B2 system

Isoenzyme	NDMA		NMA	
	K_m (mmol/l)	V_{max} (nmol/min per nmol P450)	K_m (mmol/l)	V_{max} (nmol/min per nmol P450)
P450 2B1	2.3 ± 0.2	3.2 ± 0.2	0.34 ± 0.03	3.3 ± 0.3
P450 2B2	3.5 ± 0.3	1.4 ± 0.1	0.43 ± 0.03	1.6 ± 0.1

Experimental conditions are described in Materials and Methods. Values given in the Table are averages and standard deviations of three parallel experiments.

tion rate was higher for P450 2B1 than for P450 2B2 (Fig. 5A). A single K_m and V_{max} were observed in the reconstituted systems with both isoenzymes. The ap-

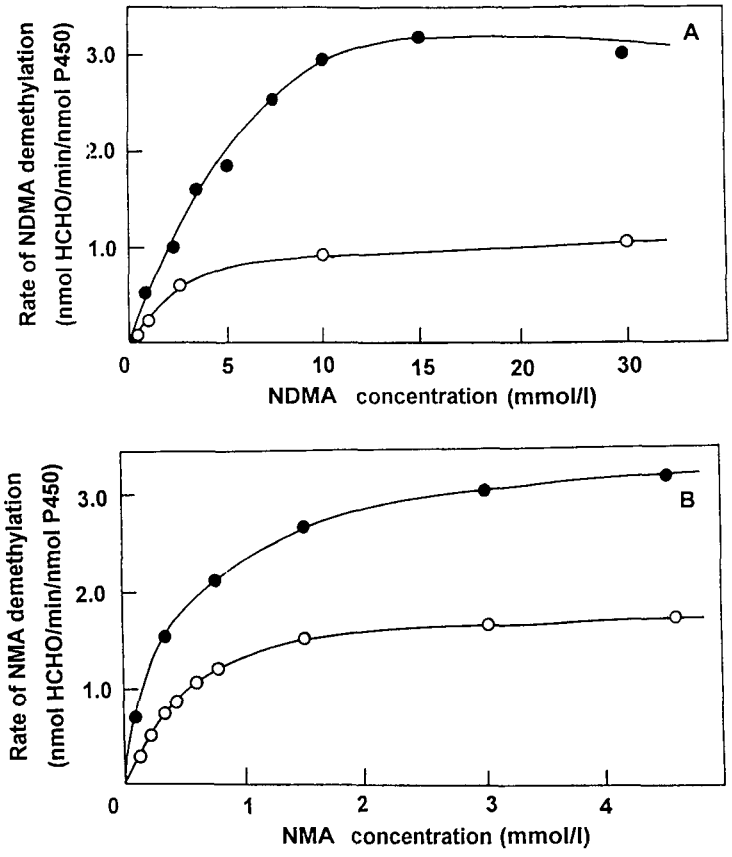

Figure 5. Demethylation of NDMA (*A*) and NMA (*B*). Kinetic curves for NDMA (*A*) and NMA (*B*) concentrations for P450 2B1 (●) and P450 2B2 (○) in the reconstituted system. 50 mmol/l potassium phosphate (pH 7,4), 0.3 nmol P450 2B1 and P450 2B2, 0.3 nmol NADPH-P450 reductase, 100 μg of dilauroylphosphatidylcholine. For other experimental conditions, see the text.

parent K_m values for formaldehyde formation are of the same order of magnitude for both isoenzymes (Table 4).

Also, formaldehyde formation was measured in reaction mixture containing P450 reconstituted system and NMA using various NMA concentrations. The reactions followed the Michaelis-Menten kinetics (Fig. 5*B*). The maximal reaction rate at which formaldehyde is produced when catalyzed by P450 2B1 is more than 2 times higher than when catalyzed by P450 2B2 (Table 4). The apparent K_m values for formaldehyde formation are of the same order of magnitude for both isoenzymes (Table 4).

Discussion

Multiple forms of liver microsomal cytochromes P450 are known to have the ability to bind N-nitrosamines (Lorr et al. 1982; Yang et al. 1985; Scheper et al. 1991). In the present work we have clearly shown another example of this principle: two N-nitrosamines (NDMA and NMA) interact with isoenzymes of rat liver P450 induced by PB. Two N-nitrosamines were chosen as the model compounds (symmetric NDMA and asymmetric NMA). Both are metabolized by the crude microsomal P450 system as well as by the reconstituted P450-dependent system.

Because different P450 isoenzymes present in liver microsomes of PB-pretreated rats have different affinities for NDMA and NMA, it is understandable that multiple K_m values were obtained in our experiments. Our present approach, namely that we pooled the values into two major K_m values without correction for overlap (Fig. 3) is a treatment by approximation. In addition, the kinetic data and the binding affinity constants of NDMA and NMA to major P450 isoenzymes isolated from PB-microsomes (P450 2B1, P450 2B2) were determined. Scheper et al. (1991) postulated that NMA is preferentially metabolized by the PB-induced mouse liver P450-dependent system. In our study, we could confirm that P450 2B1 and P450 2B2 isoenzymes have high affinities to NMA, being able to demethylate this substrate effectively. These P450 isoenzymes thus might be responsible for the metabolism of NMA *in vivo*. The same P450 isoenzymes are also able to demethylate symmetric NDMA. As expected, NMA is a better substrate for PB-induced P450 isoenzymes than NDMA. It was namely shown that P450 2E1 (cytochrome $P450_j$) is the major isoenzyme responsible for NDMA oxidation *in vivo* as well as *in vitro* (Yang et al. 1985, 1990). However, the K_m value obtained for P450 2B1 (which is more effective with respect to NDMA oxidation than P450 2B2) was very close to that obtained for P450 2E1 *in vitro* (around 3 mmol/l, see Yang et al. 1985, 1990; Patten et al. 1986). But the value of V_{max} for P450 2B1 was three times smaller than that for P450 2E1 (Yang et al. 1985). Maximal velocities obtained with P450 2B1 and P450 2B2 are comparable with the maximal rate obtained with the PB-induced isoenzyme of rabbit livers, namely, P450 LM_2 (Yang et al. 1985).

The alfa-hydroxylation reaction is supposed to be an important activation pathway for many nitrosamines (Dipple et al. 1987). It leads to 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 microsomal metabolism of nitrosamines (Lorr et al. 1982; Yang et al. 1990; Scheper et al. 1991). It may represent a detoxication pathway for nitrosamines.

NDMA and NMA are readily demethylated by isolated P450 isoenzymes (P450 2B1 and P450 2B2) to produce formaldehyde. As second products, methyldiazonium and benzenediazonium ions have to be formed, as it was determined previ-

ously in the system with crude microsomal enzymes (Kroeger-Koepke et al. 1981; Koepke et al. 1990; Yang et al. 1990). Thus the results of the present study suggest that there are isoenzymes P450 2B1 and P450 2B2 which may play an important role in the metabolism of NDMA and NMA, leading to their activation to ultimate carcinogens.

The relationship between demethylation and denitrosation, and the mechanism of the two reactions in reconstituted P450 2B1 and P450 2B2 systems, remain to be elucidated.

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