[CANCER RESEARCH 88, 1499–1504, March 15, 1988]

Metabolism of *N*-Nitrosodialkylamines by Human Liver Microsomes[1]

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ABSTRACT

The metabolism of *N*-nitrosodimethylamine (NDMA), *N*-nitrosodiethylamine, *N*-nitrosobenzylmethylamine, and *N*-nitrosobutylmethylamine was investigated in incubations with human liver microsomes. All of the 16 microsomal samples studied were able to oxidize NDMA to both formaldehyde and nitrite at NDMA concentrations as low as 0.2 mM; the rates of product formation of the samples ranged from 0.18 to 2.99 nmol formaldehyde/min/mg microsomal protein (median, 0.53 nmol). At a concentration of 0.2 mM NDMA, the rates of denitrosation (nitrite formation) were 5 to 10% (median, 6.3%) those of demethylation (formaldehyde formation); the ratio of denitrosation to demethylation increased with increases in NDMA concentration, in a similar manner to rat liver microsomes. Immunoblot analysis with antibodies prepared against rat P-450$_{ac}$ (an acetone-inducible form of cytochrome P-450) indicated that the P-450$_{ac}$ [P-450$_j$ (isoniazid-inducible form)] orthologue in human liver microsomes had a slightly higher molecular weight than rat P-450$_{ac}$ and the amounts of P-450$_{ac}$ orthologue in human liver microsomes were highly correlated with NDMA demethylase activities (r = 0.971; $P < 0.001$). Analysis of four selected microsomal samples showed that human liver microsomes exhibited at least three apparent K_m and corresponding V_{max} values for NDMA demethylase. This result, suggesting the metabolism of NDMA by different P-450 enzymes, is similar to that obtained with rat liver microsomes, even though most of the human samples had lower activities than did the rat liver microsomes. The high affinity K_m values of the four human samples ranged from 27 to 48 μM (median, 35 μM), which were similar to or slightly lower than those observed in rat liver microsomes, indicating that human liver microsomes are as efficient as rat liver microsomes in the metabolism of NDMA. The human liver microsomes also catalyzed the dealkylation and denitrosation of other nitrosamines examined. The rates of product formation and the ratios of denitrosation to dealkylation varied with the structures and concentrations of the substrates as well as with the microsomal samples tested. The results indicate that human liver microsomes are capable of metabolizing *N*-nitrosodialkylamines via the pathways that have been established with rat liver microsomes.

INTRODUCTION

Nitrosamines, carcinogenic compounds occurring widely in the environment, require metabolic activation for their cytotoxic and carcinogenic actions. The major activation step for *N*-nitrosodialkylamines is believed to be the oxygenation of the α-carbon catalyzed by a P-450[3]-dependent enzyme system. In previous work, we have demonstrated that rat liver microsomes metabolize NDMA and exhibit at least three different K_m values for NDMAd (1, 2). The high affinity K_m (K_mI) form has been shown to be inducible by various factors such as fasting, diabetes, consumption of ethanol, and pretreatment with acetone or isopropanol (1, 3–5). The P-450 enzyme corresponding to the K_mI form of NDMAd has been purified and characterized (6). This form, referred to as P-450$_{ac}$ (6) or P-450$_{et}$ (7) in our laboratory, is probably identical to P-450$_j$ (8). P-450$_{ac}$ has been shown to be responsible mainly for the activation of NDMA to a mutagen for mammalian cells (9).

Because of the potent carcinogenicity of nitrosamines and their frequent exposure to humans as well as the species variability in nitrosamine tumorigenicity, knowledge of nitrosamine metabolism in human liver is of considerable importance. Information concerning the P-450$_{ac}$ orthologue in human liver microsomes is of particular interest because this form is likely to be the enzyme responsible for the metabolism of the low level of NDMA present in the human body due to environmental exposure and endogenous synthesis. Recently, the cDNA for a human P-450$_{ac}$ orthologue (referred to as human P-450$_{ac}$) was isolated and sequenced (10). The human P-450$_{ac}$ cDNA shared 75% nucleotide and 78% predicted amino acid similarities to rat P-450$_{ac}$ cDNA; the calculated molecular mass of the predicted human P-450$_{ac}$ was slightly greater (56,916 daltons) than that of rat P-450$_{ac}$ (56,634 daltons). It was not known whether this human enzyme would display the same catalytic activities as the rat enzyme. Although the metabolism of nitrosamines in human tissues and microsomes has been studied previously (11–14), the enzymology has not been thoroughly investigated. Also of great interest is the possible existence in human tissues of the metabolic pathway which leads to the denitrosation of nitrosamines. This metabolic pathway, which presumably leads to the inactivation of *N*-nitrosodialkylamines, has been previously established in rats (15–17) but has not been documented for humans. The present study was thus undertaken to investigate the enzymology of NDMA metabolism as well as the metabolism of other nitrosamines such as NDEA, NBzMA, and NBuMA in human liver microsomes. We report herein that the K_mI value of human liver microsomes is similar to or slightly lower than that observed in rat liver microsomes, suggesting that human liver microsomes are as efficient as rat liver microsomes in the metabolism of NDMA. The metabolism of NDEA, NBzMA, and NBuMA varied with the structures and concentrations of the substrates as well as with the microsomal samples tested.

MATERIALS AND METHODS

Chemicals. NDMA, sulfanilamide, and *N*-(1-naphthyl)ethylenediamine dihydrochloride were purchased from Aldrich Chemical Co. (Milwaukee, WI). NDEA, NBuMA, cytochrome *c*, NADP, NADPH, glucose-6-phosphate (disodium salt), glucose-6-phosphate dehydrogenase (Type XV), and semicarbazide-HCl were obtained from Sigma Chemical Co. (St. Louis, MO). NBzMA was from Ash Stevens, Inc. (Detroit, MI). Freund's complete and incomplete adjuvants were purchased from Difco Laboratories (Detroit, MI). Nitrocellulose membranes were obtained from Bio-Rad Laboratories (Richmond, CA). Phosphatase-labeled goat anti-rabbit IgG, 5-bromo-4-chloro-3-indolylphosphate, and nitroblue tetrazolium were purchased from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, MD). Reagents for electrophoresis were obtained from sources described previously (18), and all other chemicals were reagent grade from commercial sources.

Human Liver Microsomes. Human liver samples were obtained, through the Nashville Regional Organ Procurement Agency, from

Received 8/20/87; revised 12/8/87; accepted 12/15/87.

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[1] Supported by Grants CA-37037, ES-03938, GM-38336, and CA-30907 from NIH.

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[3] The abbreviations used are: P-450, cytochrome P-450; NDMA, *N*-nitrosodimethylamine; NDMAd, *N*-nitrosodimethylamine demethylase; P-450$_{ac}$, an acetone-inducible form of P-450 that may be identical to P-450$_j$ (isoniazid-inducible form); NDEA, *N*-nitrosodiethylamine; NBzMA, *N*-nitrosobenzylmethylamine; NBuMA, *N*-nitrosobutylmethylamine; cDNA, complementary DNA.

organ donors who met accidental deaths and donated other tissues for transplant. Livers were removed, perfused, and chilled on ice within 15–30 min of death, and small portions (1–6 cm^3) were frozen in liquid nitrogen and stored at −70°C (19). Individual microsomal fractions were prepared from these liver samples and stored at −70°C as described (20). Glycerol in the microsomal preparations was removed by dialysis against 0.25 M sucrose before use. Subject code numbers refer to individual livers for which donor age, sex, and cause of death have been given elsewhere in many cases (20–23).

Enzyme Assays. Protein and P-450 contents were determined as described previously (24). NADPH-P-450 reductase was assayed at room temperature using cytochrome *c* as an artificial electron acceptor (25). One unit of reductase activity corresponds to the NADPH-dependent reduction of 1 nmol cytochrome *c*/min/mg protein. The metabolism of NDMA was measured as formation of formaldehyde and nitrite for demethylation and denitrosation, respectively (2, 4, 26). In brief, the assay mixture contained (in a total volume of 1.0 ml) 50 mM Tris-HCl (pH 7.0 at 37°C), 10 mM $MgCl_2$, 150 mM KCl, an NADPH-generating system (0.4 mM $NADP^+$, 10 mM glucose-6-phosphate, and 0.4 units glucose-6-phosphate dehydrogenase), human liver microsomes (0.7–0.8 mg protein), and NDMA as indicated. Blanks were prepared in the absence of either NDMA or the NADPH-generating system. For standards, HCHO and $NaNO_2$ (66 and 10 nmol/ml incubation, respectively) were added, at around the midpoint of the incubation, to the assay mixture in the absence of either NDMA or the NADPH-generating system. At the termination of the 20-min reaction, the assay mixture was centrifuged, and 0.35 ml of supernatant was used for the determination of formaldehyde for NDMAd activity (4). Another 0.35 ml of the supernatant was used for the determination of nitrite (2) as modified from the method of Appel and Graf (27). The 0.35-ml aliquot was mixed with 0.075 ml of 100 mM sulfanilamide in 3 N HCl. After 5 min, 0.075 ml of 1 mM *N*-(1-naphthyl)ethylenediamine dihydrochloride in 3 N HCl was added, followed by immediate mixing with a vortex. Absorbance at 546 nm was measured after the mixture was kept at room temperature for 10 min.

The dealkylations of NDEA, NBzMA, and NBuMA were measured as formation of acetaldehyde, benzaldehyde and formaldehyde, and butyraldehyde and formaldehyde, respectively. The denitrosation of these nitrosamines was assayed as nitrite formation. The formation of aldehydes was measured by a modification of the method of Farrelly (28). Incubation conditions were identical to that used for the NDMA metabolism except that after initiation of the incubation, test tubes were capped with rubber septa (Fisher Scientific, Springfield, NJ) to minimize evaporation of the metabolites (aldehydes). The incubation was terminated by injecting 0.1 ml of a 1:1 mixture of 50% $ZnSO_4$ and 0.1 M semicarbazide into the tubes, followed by mixing with a vortex; this procedure served to quench the reaction as well as to trap the aldehydes as their semicarbazone derivatives. To the tubes 0.1 ml saturated $Ba(OH)_2$ was then added and mixed, preceding centrifugation. For aldehyde determination, a 0.35-ml aliquot of the resulting supernatant was added to a 16- × 125-mm tube containing 1 ml H_2O, 0.1 ml of 0.25% 2,4-dinitrophenylhydrazine reagent in 6 N HCl, and 1.5 ml hexane, and the tube was capped and shaken for 1 h. One ml of the hexane layer was then added to 0.35 ml acetonitrile followed by mixing with a vortex for 20–30 sec. Finally, 0.125 ml of the acetonitrile layer was transferred to sample vials which were subsequently loaded onto a WISP autoinjector (Waters Associates, Milford, MA) and 50 μl of sample were injected onto a high performance liquid chromatography column. The column (5 × 100 mm) was Rad-Pak C_{18} on 10-μm silica fitted in an RCM-100 Module (Waters). The mobile phase was 65% acetonitrile (in H_2O) at a flow rate of 1.1 ml/min. The peaks due to the dinitrophenylhydrazone derivatives of the aldehydes had retention times of 3.5, 4.5, 8.2, and 9.5 min for formaldehyde, acetaldehyde, butyraldehyde, and benzaldehyde, respectively. They were monitored by a model 440 UV detector (Waters) at a wavelength of 340 nm and quantified by their peak areas which were determined with an integrator. For the standards, known quantities of aldehydes were added to incubation mixtures that were run in parallel to the assays. A time-course study showed that under these assay conditions, rates of aldehyde production were linear for at least 20 min. Plots of protein content *versus* rate were linear. No appreciable amounts of the aldehydes were lost during the incubation.

Immunoblot Analysis. Purified P-450$_{ac}$ and polyclonal antibodies against P-450$_{ac}$ were prepared as described previously (2, 6). Immunoblot analysis of human liver microsomal proteins using anti-P-450$_{ac}$ IgG was performed by a modification of the method of Guengerich *et al.* (29) as described previously (2). For the quantitation of P-450$_{ac}$, microsomal samples and known amounts of purified rat P-450$_{ac}$ (which were predetermined to be in linear ranges) were applied to the same gel and immunoblotted, and resulting immunostained bands of the samples were quantified as compared with those of the standard. Intensities of the immunostained bands were measured using a Shimadzu Dual-wavelength Thin-layer Chromato Scanner (Model CS-930; Shimadzu Corporation, Kyoto, Japan).

RESULTS

NDMA Metabolism and P-450$_{ac}$ Orthologue in Human Liver Microsomes. It was recently observed that glycerol was a competitive inhibitor of the K_mI form of rat liver microsomal NDMAd (26). Sixteen human liver microsomal samples originally stored in 20% glycerol (20) were, therefore, dialyzed against 0.25 M sucrose shortly before the initiation of the present study and the dialyzed samples were used throughout the study. The history of some of the 16 subjects has been reported elsewhere (20–23). Based on our experience obtained from the study of NDMA metabolism in rat liver microsomes, which exhibit multiple K_m values for NDMAd, three substrate concentrations (0.2, 4, and 100 mM NDMA) were selected to determine the profiles of NDMA metabolism, and the results are presented in Table 1. All 16 microsomal samples were able to metabolize NDMA to produce both formaldehyde and nitrite at NDMA concentrations as low as 0.2 mM; the oxidation rates of the 16 samples ranged from 0.18 to 2.99 nmol HCHO/min/mg protein (median, 0.53 nmol) and from 0.01 to 0.25 nmol NO_2^-/min/mg protein (median, 0.03 nmol). At 0.2 mM NDMA, the rates of denitrosation (NO_2^- formation) were 5 to 10% (median, 6.3%) those of demethylation (HCHO formation). The ratio of denitrosation to demethylation increased with increases in NDMA concentration; *e.g.*, the median values of the ratios were 8 and 24% at 4 and 100 mM NDMA, respectively. These properties of NDMA metabolism in human liver microsomes are in general similar to those in rat liver microsomes (2), although the rates of metabolism for both demethylation and denitrosation in most of the former were about half those in the latter.

The result that human microsomes metabolized NDMA effectively at a low concentration (0.2 mM NDMA) strongly suggested the possible existence of a P-450$_{ac}$ orthologue in human microsomes. We, therefore, subjected the microsomal samples to sodium dodecyl sulfate-polyacrylamide slab-gel electrophoresis and then transferred the proteins electrophoretically to nitrocellulose paper. When the resolved proteins were treated with anti-P-450$_{ac}$ IgG in an immunoblot analysis, only one major band (human P-450$_{ac}$) at an electrophoretic mobility of 54,400 daltons, which is slightly higher than rat P-450$_{ac}$ (52,000 daltons) on the same immunoblot, was detected (Fig. 1). The intensity of the human P-450$_{ac}$ bands was measured using a densitometer. The peak area of sample number 15 was arbitrarily set at 100 units and relative values of the other samples were obtained. The human P-450$_{ac}$ content was highly correlated with the NDMAd activity at 0.2 mM NDMA (r = 0.971; $P < 0.001$), whereas the NDMAd activity was rather weakly correlated with the total P-450 content (r = 0.769; $P <$ 0.01) or the reductase activity (r = 0.539; $P < 0.05$) (Table 1).

Table 1 *NDMA metabolism in human liver microsomes*

The assay mixture contained (in a total volume of 1.0 ml) buffer, an NADPH-generating system, human liver microsomes (0.7–0.8 mg protein), and NDMA as indicated. After a 20-min reaction at 37°C, HCHO and NO_2^- formed were measured and expressed as nmol/min/mg protein.

Sample no.[a]	Microsomal P-450 (nmol/mg)	Reductase activity (units)	P-450$_{ac}$ (units)	Rates of product formation at substrate (NDMA) concentrations					
				0.2 mM		4 mM		100 mM	
				HCHO	NO_2^-	HCHO	NO_2^-	HCHO	NO_2^-
1	0.33	197	2.1	0.41	0.03	0.55	0.05	1.20	0.30
2	0.33	136	9.1	0.55	0.03	0.72	0.06	1.27	0.31
3	0.33	268	13.9	0.63	0.05	0.86	0.08	1.53	0.34
4	0.15	181	5.8	0.37	0.02	0.50	0.05	0.91	0.25
5	0.29	181	16.0	0.51	0.04	0.72	0.07	1.44	0.35
6	0.24	169	8.3	0.18	0.01	0.28	0.02	0.98	0.26
7	0.26	127	5.2	0.61	0.03	0.83	0.07	1.73	0.43
8	0.41	137	22.2	1.00	0.05	1.32	0.11	2.48	0.53
9	0.32	219	15.2	0.90	0.06	1.07	0.08	1.59	0.32
10	0.16	160	3.0	0.32	0.02	0.45	0.03	1.02	0.27
11	0.57	260	10.8	0.65	0.04	0.96	0.10	2.95	0.65
12	0.10	196	4.2	0.38	0.02	0.55	0.04	1.09	0.25
13	0.14	196	3.6	0.34	0.02	0.45	0.03	1.10	0.28
14	0.44	222	12.6	0.40	0.01	0.60	0.04	1.60	0.32
15	0.74	307	100.0	2.99	0.25	3.63	0.33	4.32	0.72
16	0.32	129	5.4	0.66	0.03	0.94	0.08	2.22	0.54

[a] Each sample number has its own subject code and the information for some of the subjects has been reported (20–23): sample numbers 1 through 16 correspond to subject codes 29, 33, 34, 35, 39, 93, 94, 95, 97, 98, 99, 100, 103, 104, 105, and 106, respectively.

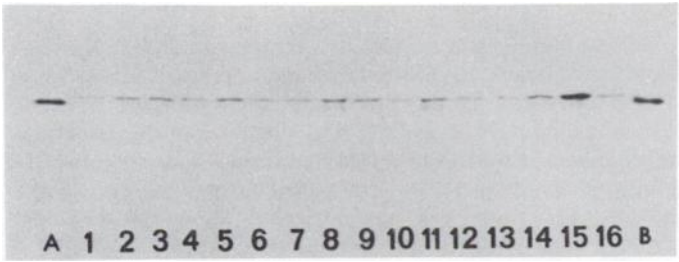

Fig. 1. Immunoblots of human liver microsomal proteins. *Lanes A* and *B*, purified rat P-450$_{ac}$ (0.25 μg/lane); *lanes* 1 to 16, human liver microsomal sample numbers 1 to 16 (9.3 μg protein/lane) corresponding to Table 1.

Microsomal sample number 15 had 0.20 nmol P-450$_{ac}$/mg microsomal protein, which is equivalent to 27% of total P-450 content (0.74 nmol/mg protein). It can be thus estimated that 1 nmol of human P-450$_{ac}$ can oxidize NDMA (when present at 0.2 mM) to produce 15 nmol HCHO/min.

Kinetic Parameters of NDMA Metabolism in Human Liver Microsomes. The results obtained in the preceding section led us to examine in detail the enzymology of NDMA metabolism in four selected microsomal samples. Fig. 2 shows NDMA metabolism in one microsomal sample (sample no. 15): *a*, demethylation and *b*, denitrosation. The date are expressed in Lineweaver-Burke double-reciprocal plots. The kinetic constants were obtained by linear regression analyses of the double-reciprocal plots and were very similar to those obtained from Eadie-Hofstee plots. Because the K_m values of NDMA metabolism are known to be affected by a host of experimental conditions, the presently observed parameters can only be considered apparent K_m and V_{max} values. The high affinity K_mI values of demethylation and denitrosation were 30 and 24 μM, respectively. Judging from the experimental variation of the denitrosation assay, these values may be considered to be similar rather than different. In previous studies with rat and hamster microsomes, we have demonstrated that the high affinity K_m (K_mI) values for demethylation and denitrosation are the same (2, 30). At higher NDMA concentrations the rate of demethylation was quite different from that of denitrosation, resulting in different low affinity K_mII values between the two reactions; this property is similar to that observed in rat liver microsomes (2).

Table 2 summarizes the kinetic parameters of NDMA de-

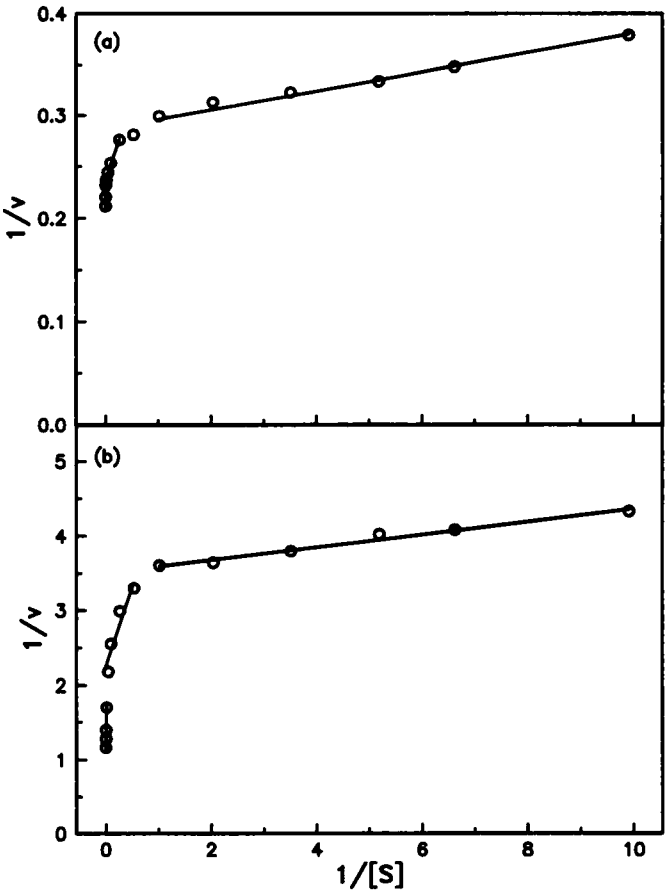

Fig. 2. NDMA metabolism in human liver microsomes. The demethylation (HCHO formation) and denitrosation (NO_2^- formation) were assayed in the presence of microsomes (0.75 mg protein/ml) of sample number 15 as described in Table 1. *Points*, average of a duplicated assay at the substrate concentration indicated; the difference between the duplicates was usually < 5%. Kinetic constants were obtained by linear regression analysis. *a*, Double-reciprocal plot of NDMA demethylation: K_mI = 0.030 mM (V_{max} = 3.43 nmol/min/mg protein), K_mII = 0.63 mM (V_{max} = 4.24 nmol/min/mg protein), and K_mIII = 20.5 mM (V_{max} = 5.23 nmol/min/mg protein). *v*, nmol HCHO/min/mg protein and *S*, mM NDMA. *b*, Double-reciprocal plot of NDMA denitrosation: K_mI = 0.024 mM (V_{max} = 0.29 nmol/min/mg protein), K_mII = 0.95 mM (V_{max} = 0.45 nmol/min/mg protein), and K_mIII = 33.2 mM (V_{max} = 0.98 nmol/min/mg protein). *v*, nmol NO_2^-/min/mg protein and *S*, mM NDMA.

Table 2 *Apparent kinetic constants of NDMA demethylation in human liver microsomes*

The apparent K_m (designated as I, II, and III) and corresponding "corrected" V_{max} values were calculated by the linear regression analysis of the double-reciprocal plots of the NDMA demethylation data from sample numbers 8, 14, 15, and 16. Each K_mI value was calculated from 5 to 6 data points (r = 0.97). Each value for K_mII and K_mIII was calculated from 3 or 4 data points (r = 0.93–0.99). The corrected V_{max} values were obtained by subtracting the contribution from the higher affinity K_m forms as described previously (2). Similar results were also obtained from Eadie-Hofstee plots.

Sample no.	K_m (mM)	V_{max} (nmol/min/mg)
8	K_mI 0.040	1.20
14	0.027	0.45
15	0.030	3.43
16	0.048	0.82
8	K_mII 0.60	0.41
14	0.59	0.29
15	0.64	0.81
16	0.99	0.43
8	K_mIII 32.4	1.80
14	47.1	1.72
15	20.5	0.99
16	49.9	2.21

methylation in four selected microsomal samples. Each of the four samples exhibited at least three different K_m values. The K_mI values of the four human samples ranged from 27 to 48 μM (median, 35 μM), which are similar to or slightly lower than the K_mI value (40–50 μM) observed in rat liver microsomes (26). The results suggest the existence of a P-450$_{ac}$ orthologue in human microsomes. It is not known, however, why the K_mI values vary among different microsomal samples. Although there were experimental errors in such determinations, the variations among different experiments with the same sample were usually less than the range (27–48 μM) observed. The variation, therefore, may reflect some unknown inherited differences among different microsomes. The K_mII form, ranging from 0.59 to 0.99 mM, displayed 24–64% of the activity of the K_mI form as judged by corresponding V_{max} values. The K_mIII form ranging from 20 to 50 mM displayed V_{max} values from 0.99 to 2.21 nmol/min/mg and these values were independent of the V_{max} values for the K_mI form. The K_mII and K_mIII values reflect a composite of different P-450 enzymes existing in the microsomes, and can only be considered operational terms rather than real kinetic constants. The presently observed K_mII values were slightly higher than the value (0.22–0.36 mM) observed in rat liver microsomes, but the K_mIII values for human and rat microsomes were in the same range.

Metabolism of Other Nitrosamines by Human Liver Microsomes. Previous studies of nitrosamine metabolism in rat liver microsomes have demonstrated that acetone- or ethanol-induced microsomes have a high activity for demethylation and deethylation and that phenobarbital-induced microsomes have a high activity for debenzylation and debutylation (31), suggesting that P-450$_{ac}$ and P-450$_b$ (a major phenobarbital-inducible form of P-450) have different affinities for different alkyl groups of nitrosamines. In the present study, the metabolism of NDEA, NBzMA, and NBuMA in four selected human liver microsomes was examined using three substrate concentrations (0.2, 2, and 20 mM). The results for NDEA are presented in Table 3. When assayed at 0.2 mM NDEA the rates of deethylation with different microsomes were parallel with, and were about 60% of, the rates of demethylation obtained with 0.2 mM NDMA. The rates of deethylation with sample numbers 8, 15, and 16 increased as NDEA concentration increased to 2 mM, and then leveled off or slightly decreased at 20 mM NDEA, probably due to the saturation of substrate binding to P-450$_{ac}$

Table 3 *Metabolism of NDEA by human liver microsomes*

The assay mixture contained human liver microsomes (0.7–0.8 mg protein) and NDEA as indicated in a total volume of 1.0 ml. After a 20-min incubation at 37°C, acetaldehyde and nitrite formed were measured and expressed as nmol/min/mg protein, as described in "Materials and Methods."

Sample no.	Rates of product formation at substrate (NDEA) concentrations					
	0.2 mM		2 mM		20 mM	
	CH_3CHO	NO_2^-	CH_3CHO	NO_2^-	CH_3CHO	NO_2^-
8	0.52	0.09	0.68	0.14	0.60	0.20
14	0.24	0.05	0.38	0.09	0.65	0.23
15	1.69	0.30	2.42	0.42	1.96	0.44
16	0.44	0.08	0.59	0.11	0.61	0.17

and substrate inhibition. In comparison, the activity of sample number 14 was lower at 0.2 mM NDEA, but the activity increased by 1.6- and 2.7-fold when assayed at 2 and 20 mM, respectively. The results suggest that, in addition to P-450$_{ac}$, NDEA is also metabolized by other P-450 forms which display higher K_m values. The denitrosation of NDEA followed a similar pattern to that of the deethylation. The ratios of denitrosation to deethylation at 0.2 and 2 mM NDEA were about 0.2, approximately 3-fold higher than the denitrosation to demethylation ratio in NDMA metabolism.

For the metabolism of NBzMA, sample number 15 was still most active among the four microsomal samples studied (Table 4), but other features of the metabolism were quite different from those of NDMA and MDEA. The rates of the demethylation increased by 1.6- to 2-fold upon increasing the substrate concentration from 0.2 to 2 mM, but further increase in the demethylation rate was not observed upon elevating the NBzMA concentration to 20 mM. In contrast, such a saturation behavior was not seen with the debenzylation reaction. Upon increasing NBzMA concentration from 0.2 to 2 mM, the debenzylation rates were enhanced 3- to 4-fold; and at 20 mM NBzMA, the rates were increased about 10-fold for sample numbers 8, 15, and 16 and 20-fold for sample number 14. As a consequence, the ratio of the rates of debenzylation to demethylation was 2.7 at 0.2 mM NBzMA and increased about 7-fold upon increasing NBzMA concentration to 20 mM. The substrate saturation behavior of the denitrosation reaction fell in between the patterns of the demethylation and debenzylation reactions. The ratios for the denitrosation rates of 0.2, 2, and 20 mM NBzMA were 1, 1.6–1.9, and 3.7–6.6, respectively. The ratio of denitrosation to dealkylation was 0.24 at 0.2 mM NBzMA and decreased gradually with increases in NBzMA concentration due to rather marked elevation of debenzylation rate.

The metabolism of NBuMA in human liver microsomes was somewhat similar to that of NBzMA but with substantial differences (Table 5). The demethylation rate was several-fold higher than the debutylation rate at 0.2 and 2 mM NBuMA. However, the former reaction appeared easily saturable but the latter was not. Upon increasing the substrate concentration to 20 mM, the demethylation rate changed only slightly whereas the debutylation rate increased markedly and surpassed the demethylation rate. The substrate saturation pattern of the denitrosation, again, fell in between the demethylation and debutylation reactions.

DISCUSSION

During the past decades the properties of hepatic NDMAd have been extensively studied. It has been demonstrated that at least two NDMAd enzyme systems, one with a K_m value of 0.2

Table 4 *Metabolism of NBzMA by human liver microsomes*

The assay conditions were the same as those described in Table 3 except for the substrate. The metabolites formed were expressed as nmol/min/mg protein.

Sample no.	Rates of product formation at substrate (NBzMA) concentrations								
	0.2 mM			2 mM			20 mM		
	C_6H_5CHO	HCHO	NO_2^-	C_6H_5CHO	HCHO	NO_2^-	C_6H_5CHO	HCHO	NO_2^-
8	0.30	0.10	0.11	0.90	0.16	0.18	3.01	0.17	0.41
14	0.26	0.12	0.10	1.07	0.23	0.19	5.53	0.23	0.66
15	1.02	0.59	0.28	3.59	0.92	0.53	11.13	0.92	1.43
16	0.27	0.07	0.09	0.99	0.14	0.16	3.04	0.14	0.37

Table 5 *Metabolism of NBuMA by human liver microsomes*

The assay conditions were the same as those described in Table 3 except for the substrate. The metabolites formed were expressed as nmol/min/mg protein.

Sample no.	Rates of product formation at substrate (NBuMA) concentrations								
	0.2 mM			2 mM			20 mM		
	C_3H_7CHO	HCHO	NO_2^-	C_3H_7CHO	HCHO	NO_2^-	C_3H_7CHO	HCHO	NO_2^-
8	0.06	0.50	0.05	0.13	0.54	0.06	0.48	0.49	0.13
14	0.07	0.33	0.04	0.18	0.51	0.08	1.02	0.51	0.23
15	0.59	1.92	0.15	0.77	2.49	0.23	2.49	2.19	0.47
16	0.03	0.30	0.04	0.03	0.26	0.04	0.42	0.30	0.12

to 0.3 mM and the other with a K_m value of 44 to 51 mM, are responsible for the demethylation of NDMA (32). Recently, one of our laboratories has demonstrated the presence of an even lower K_m (higher affinity) form of NDMAd (K_m = 40 to 50 μM) in rat liver microsomes and this form has been shown to be important in the bioactivation of NDMA (1, 2, 9, 26, 33). The P-450 species responsible for this high affinity (K_mI) form, P-450$_{ac}$, has been purified from rat liver microsomes and characterized (6). The cDNA for both the rat and human P-450$_{ac}$ genes have been sequenced (10) the human P-450$_{ac}$ cDNA shares 75% nucleotide and 78% predicted amino acid sequence identities to rat P-450$_{ac}$ cDNA. Wrighton *et al.* (34) reported that a human P-450$_j$ orthologue resembled P-450$_j$ and P-450$_{LM3a}$ (an ethanol-inducible form of rabbit P-450) in its NH_2-terminal amino acid sequence. From this information, it might be expected that the enzymology of NDMA metabolism in human liver microsomes is similar to that in rat liver microsomes. The present study showed that human liver microsomal samples catalyzed NDMA metabolism efficiently at low NDMA concentrations and that the activity correlates well (r = 0.971) with the quantity of P-450$_{ac}$ as determined by immunoblot analysis using anti-P-450$_{ac}$ IgG. This result is similar to the recent report of Wrighton *et al.* (34) showing that the NDMAd activity (measured at 1 mM NDMA) was well correlated (r = 0.87) with the level of a P-450$_j$ orthologue in human liver microsomes and that this enzyme activity was inhibited (70–80%) by anti-P-450$_j$ IgG.

In the present work we further characterized the kinetic parameters and demonstrated the existence of the K_mI form of NDMAd in human liver microsomes. The observed value of K_mI is similar to or slightly lower than the K_mI value of rat liver microsomal NDMAd. This indicates that human liver microsomes contain an enzyme similar to that in rat liver microsomes for the metabolism of NDMA. The human microsomal NDMAd activity, however, was not inhibited by a monoclonal antibody preparation (designated as 1-91-3 in Ref. 35) which has been shown previously (35) and in the present work to inhibit more than 90% of the NDMAd activity in rat liver microsomes (data not shown). This result suggests that the rat P-450$_{ac}$ possesses an antigenic site which is absent or inaccessible in human microsomal P-450$_{ac}$. Judging from the possible low cellular level of NDMA that humans are most likely to be exposed to under normal conditions, the high affinity K_m form of NDMAd is probably the most important enzyme to consider in the metabolism of this carcinogen. By assaying the activity with a substrate concentration of 0.2 mM, we believe that we have accurately assayed the activity of this enzyme with little interference from the activity of the low affinity K_mII form.

Using rat liver microsomes, we have previously demonstrated the specificity of P-450 enzymes in the metabolism of different alkyl groups of nitrosamines (31). The present study demonstrated that human liver microsomes have similar properties. The rates of demethylation and deethylation of NDMA, NDEA, NBzMA, and NBuMA were rather high at low substrate concentrations and easily saturable; whereas the rates of the debenzylation and debutylation of NBzMA and NBuMA increased with increasing substrate concentrations and were not saturated at 20 mM. In both human and rat liver microsomes, the rate of NDEA deethylation was about half that of NDMA demethylation and, in NBzMA metabolism, the debenzylation rate was several-fold higher than the demethylation rate. On the other hand, the metabolism of NBuMA in human liver microsomes appeared to be quite different from that observed in rat liver microsomes; in the former the rate of demethylation was several-fold greater than that of debutylation at substrate concentrations < 2 mM whereas the latter showed only slightly higher rates of demethylation than debutylation.

We have previously studied the mechanisms of denitrosation of low concentrations of NDMA by rat liver microsomes and postulated that it may share the initial α-oxygenation step with the dealkylation pathway (2, 7, 16, 17, 30). It is reasonable, therefore, to demonstrate that such a pathway exists in human tissues for the metabolism of *N*-nitrosodialkylamines and that the relationship between this pathway and the dealkylation pathway is similar to that observed in rat liver microsomes. However, the ratio of the rates of denitrosation to dealkylation increases upon increasing the substrate concentrations. It appears that other mechanisms may also be involved in the denitrosation of high concentrations of NDMA. Because denitrosation may be a detoxificating metabolic pathway for *N*-nitrosodialkylamines, it is important to consider the substrate concentration (or dosage) factor when we assess the relative importance of these pathways. The same considerations should also be applied when we weigh the relative importance of one dealkylation reaction *versus* another (*e.g.*, demethylation *versus*

debenzylation) in the metabolism of an asymmetrical *N*-nitrosodialkylamine.

These results are consistent with the postulate that human P-450$_{ac}$ plays an important role in catalyzing the demethylation and denitrosation of NDMA as well as other *N*-nitrosodialkylamines especially when they are present in low concentrations. The regulation of this P-450 enzyme in humans is relatively unknown. It may be speculated that human P-450$_{ac}$ could be induced by alcohol consumption, fasting, diabetes, and other factors that are known to induce this enzyme in rats (36). This postulate, however, remains to be substantiated. It was noted in this work that sample number 15 had the highest content of P-450$_{ac}$ and metabolic activities. The case history of this donor did not provide sufficient information for interpreting the high activity. The reason for the individual variabilities in the metabolism of nitrosamines is an important issue and remains to be systematically investigated.

ACKNOWLEDGMENTS

The authors thank S. M. Ning for the immunoblot analysis and M. J. Lee for the high performance liquid chromatography analysis.

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