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Diethyl Ether as a Substrate for Acetone/Ethanol-Inducible Cytochrome P-450 and as an Inducer for Cytochrome(s) P-450

JOHN F. BRADY, M. J. LEE, MARY LI, HIROYUKI ISHIZAKI and CHUNG S. YANG

Department of Biochemistry, University of Medicine and Dentistry of New Jersey-New Jersey Medical School, Newark, New Jersey 07103

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SUMMARY

The ability of diethyl ether to serve as a substrate for microsomal and purified cytochrome P-450 (P-450) and as an inducer for rat hepatic microsomal monooxygenase activities was examined. Microsomal oxidation of ether to acetaldehyde, as monitored by high pressure liquid chromatography, was elevated 3- to 5-fold by treatment of rats with acetone or ethanol, 1.5- to 2-fold by treatment with ether, and only slightly by phenobarbital treatment. Ether also induced *N*-nitrosodimethylamine demethylase by up to 2-fold and 7-pentoxyresorufin dealkylation by up to 10-fold. These trends agreed with immunoblot experiments in which ether was a weak inducer of the P-450 isozyme IIE1 (encoded by the rat gene *P450IIE1*), but a stronger inducer of IIB1. A monoclonal antibody against IIE1 inhibited the deethylation by 78% in microsomes from acetone-treated rats and by 45% in controls. *N*-Nitrosodimethylamine, as well as common inhibitors of IIE1 such as hexane, benzene, pyrazole, and phenylethylamine, strongly inhibited ether deethylation. Using microsomes from acetone-induced rats, the apparent K_m for deethylation was 13.4 ± 2.4 μM and the V_{max} was 8.2 ± 0.2 (nmol of acetaldehyde/min/nmol of P-450). The K_m for the controls was 71.3 ± 9.5 μM. The rates of deethylation at 1 mM ether by purified, reconstituted IIE1 and IIB1 were 4.2 and 0.42 (nmol of acetaldehyde/min/nmol of P-450), respectively. Cytochrome b_5 stimulated the rate due to IIE1 apparently by a decrease in the K_m. These findings, along with previous work showing marked inhibition by ether of IIE1-dependent reactions, strongly support a major role for this isozyme in ether metabolism.

The metabolic fate of diethyl ether has received considerable attention since it is a widely used anesthetic agent and is an important industrial solvent. Although the P-450[1]-dependent oxidation of the compound to acetaldehyde has been demonstrated in microsomes (3), more recent studies have implicated specific isozymes. Ether anesthesia inhibits the metabolism of hexobarbital (4), aminopyrine (5), pentobarbital (6), and diphenylhydantoin (7). Ether has also been shown to inhibit the metabolism of *p*-nitroanisole and antipyrine in rat hepatocytes and liver microsomes (8, 9). Keefer *et al.* (10) demonstrated that short-term exposure of rats to this anesthetic leads to inhibition of NDMA metabolism. Recent work by Tan *et al.* (11) indicated that ether is a competitive inhibitor of the low K_m (K_mI) form of the microsomal NDMA demethylase and suggested that the inhibited clearance of NDMA in ether-anesthetized rats is due to the inhibitory action of ether present in the animals. The hepatic postmitochondrial fraction from rats given a similar treatment exhibited lower NDMA demethylase activity than that from untreated rats, possibly due to the presence of ether in the liver homogenate (11). Because NDMA is a high affinity substrate for IIE1, these results suggest that ether may also have a high affinity for this isozyme. Form IIE1 is inducible by acetone, ethanol, fasting, diabetes, and numerous other factors and is active in the metabolism of a variety of small organic compounds, including ethanol (12), acetone (13), and *n*-pentane (14), which may be considered structurally similar to ether. These findings suggest the possible role of this isozyme in catalyzing the oxidation of diethyl ether.

Long-term ether treatment of rats leads to induction of hepatic microsomal enzymes and to proliferation of smooth endoplasmic reticulum (15). It has been suggested that these effects are qualitatively similar to those produced by phenobarbital treatment (15, 16). Since it is often observed that a substrate for a particular form of P-450 is also an inducing agent of the isozyme, the possibility that the level of IIE1 is elevated by prolonged ether treatment is worth investigating. In the present work, we investigated the ability of ether to serve as a substrate for microsomal oxidation as well as its ability to induce P-450. The results indicated that this compound is efficiently oxidized by microsomal and purified IIE1 and is an inducer of this isozyme and of other isozymes of P-450.

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[1] The nomenclature of the P-450 isozymes follows the convention in Ref. 1. Isozyme IIE1, encoded by the rat gene *P450IIE1*, has also been referred to as P-450$_w$ by our laboratory and as P-450$_j$ by Ryan *et al.* (2).

ABBREVIATIONS: P-450, cytochrome P-450; NDMA, *N*-nitrosodimethylamine; BHT, butylated hydroxytoluene; MAb, monoclonal antibody.

Experimental Procedures

Materials. Anhydrous diethyl ether (analytical reagent grade) and acetaldehyde were obtained from J. T. Baker Chemical Co. (Phillipsburg, NJ). Isoflurane (1-chloro-2,2,2-trifluoroethyl difluoromethyl ether) was purchased from Ohio Medical Products (Madison, WI). BHT was a product of Nutritional Biochemical Corp. (Cleveland, OH). Ethylmorphine hydrochloride was purchased from Mallinckrodt Chemical Works (St. Louis, MO). Resorufin and 7-pentoxyresorufin were from Pierce Chemical Co. (Rockford, IL). Benzphetamine hydrochloride was a gift from The Upjohn Co. (Kalamazoo, MI). Aminopyrine was a gift from the Sterling-Winthrop Research Institute (Rensselaer, NY). Antibodies against IIB1 and MAbs against IIE1 were kindly provided by Drs. F. J. Gonzalez, S. S. Park, and H. V. Gelboin (Laboratory of Molecular Carcinogenesis, National Cancer Institute, Bethesda, MD). All other chemicals were obtained from sources indicated previously (17).

Animals and microsomes. Male Sprague-Dawley rats (90–100 g initial body weight) were maintained as described (17). Phenobarbital was administered by intraperitoneal injection at 75 mg/kg in saline, daily for 3 days. BHT was given intragastrically at a daily dose of 400 mg/kg in corn oil for 3 days. Acetone was administered intragastrically as a 25% aqueous solution in a single dose of 2.5 ml/kg, 18 hr before sacrifice. The ethanol treatment consisted of replacing the water with 15% (v/v) ethanol for 3 days. For treatment with ether, the animal was placed in a closed jar (15 cm × 15 cm diameter) containing paper towels which had been wetted with 10 ml of diethyl ether. The rat was removed after loss of its righting reflex. The treatment was repeated either three (3×) or five (5×) times daily between 9:00 am and 6:00 pm for 3 days and the animals were sacrificed at 9:00 am on the fourth day. Isoflurane was administered similarly three times daily for 3 days. Liver microsomes were prepared as described (18) and stored at −80° until use. Isozyme IIE1, NADPH/P-450 reductase, and cytochrome b_5 were purified and reconstituted as described (19). Isozyme IIB1 was purified by the method of West *et al.* (20). Polyclonal antibodies against IIE1 (anti-IIE1 IgG) were prepared as described previously (21).

Demethylase assays. The microsomal demethylase activities with NDMA, benzphetamine, ethylmorphine, and *p*-nitroanisole as substrates were determined using a colorimetric assay as described previously (17).

Ether deethylase assay. The incubations were modified when ether was the substrate due to the volatility of ether and acetaldehyde. After initiation of the incubation by the addition of ether, the tubes were capped with rubber septa to minimize evaporation. (The amount of evaporation of ether was estimated as described below.) The 5-min incubations were terminated by injecting 0.1 ml of a 1:1 mixture of 25% zinc sulfate and 0.8 M semicarbazide which served to quench the reaction and to trap the acetaldehyde as its semicarbazone derivative. Saturated barium hydroxide solution (0.05 ml) was added with mixing, and the tubes were centrifuged. In the following steps, the 2,4-dinitrophenylhydrazone derivative of acetaldehyde was formed and assayed by a modification of the method of Farrelly *et al.* (22). In brief, 0.35 ml of the supernatant was added to 1 ml of water, 0.1 ml of dinitrophenylhydrazine solution (0.25% in 6 N HCl) plus 1.5 ml of hexane, and mixed in capped test tubes for 1 hr. One ml of the hexane layer was mixed with 0.35 ml of acetonitrile. A portion of the acetonitrile layer was analyzed using a Waters high pressure liquid chromatography system (Waters Associates, Milford, MA). The column (5 mm × 10 cm) was Radial-Pak C_{18} on 10-μm silica. The mobile phase was 55% acetonitrile at a flow rate of 1 ml/min. The absorbance at 340 nm was monitored using a model 440 detector. The peak due to the 2,4-dinitrophenylhydrazone derivative of acetaldehyde gave a retention time of 5.5 min and its area was compared to the standard. For the standard, a known quantity of acetaldehyde was added to an incubation lacking ether and terminated after 2.5 min. In the kinetic studies, at the lowest substrate concentration, less than 20% of the substrate was utilized. Plots of P-450 content versus rate were linear. The evaporative loss of ether was estimated by gas chromatography using a Hewlett-Packard 5710A apparatus. At 37°, 75–85% of the initial concentration was retained in 20 min incubations in the absence of the NADPH-generating system.

The apparent kinetic parameters were obtained by curve-fitting to a simple Michaelis-Menten kinetic model using the PENNZYME nonlinear regression program (23).

Other assays and analyses. The *O*-dealkylation of 7-pentoxyresorufin was assayed as described by Lubet *et al.* (24) using a Perkin-Elmer model 512 double beam fluorescence spectrophotometer, with the excitation wavelength set at 522 nm, the emission wavelength set at 586 nm, and the entrance and exit slits set at 10 mm.

The gel electrophoresis and immunoblot analyses were performed using previously described methods (21, 25). Densitometry was conducted using a Shimadzu CS-930 TLC Scanner (Shimadzu Corp. Kyoto, Japan).

Results

Effect of inducing agents on microsomal ether metabolism. It has been demonstrated that diethyl ether inhibits IIE1-dependent demethylation of NDMA (11). In the present work, the possibility that ether is also a substrate for this isozyme was examined by determining the deethylase activity of hepatic microsomes from rats treated with inducers for P-450. The effect of treatments with acetone, ethanol, phenobarbital, BHT, and ether on microsomal deethylase activity is shown in Table 1. Treatment of rats with acetone and ethanol generated microsomes with the highest deethylase activities, showing 3- to 4-fold induction when expressed per mg of protein or per nmol of P-450. BHT, which is not known to induce IIE1, or phenobarbital treatment did not increase the activity when expressed on a per mg basis. Phenobarbital treatment slightly elevated the activity per nmol of P-450. An enhancement of 1.5- to 2-fold was also noted when ether itself was used as an inducing agent. Other changes produced by the ether treatment will be discussed in a later section.

The formation of acetaldehyde as a product of ether deethylation was also observed by a colorimetric method using 3-methyl-2-benzothiazolone hydrazone (26) (results not shown). The results observed using this method were similar to those obtained using high pressure liquid chromatography but were less reproducible.

Inhibition of ether deethylase activity by MAbs against IIE1 and by other compounds. An MAb against IIE1 has recently been characterized. This MAb preparation was shown to inhibit the NDMA demethylase activity of mi-

TABLE 1

Effect of inducing agents on the ether deethylase activity of rat liver microsomes

Microsomes (0.2 nmol of P-450) from control or treated rats were incubated for 5 min at a final volume of 0.5 ml with ether at an initial concentration of 0.8 mM. Each value represents the average from two separate incubations.

Microsomes	Ether deethylase activity	
	Per mg of protein	Per nmol of P-450
	nmol of acetaldehyde/min	
Experiment I		
Control	1.9	2.0
Acetone-induced	6.1	7.0
Ethanol-induced	7.2	7.9
Phenobarbital-induced	1.3	3.0
BHT-induced	1.8	1.6
Experiment II		
Control	2.1	1.9
Ether-induced (5×)	4.2	4.0

crosomes from acetone-treated rats and of the reconstituted NDMA demethylase with purified IIE1, by 77% and 92%, respectively (27). In the present study, addition of 0.40 mg of the ascites fluid containing MAb 1-91-3 (see Ref. 27) inhibited the microsomal deethylase activity by 45% in control and by 78% in acetone-treated animals (Fig. 1). This suggested that a portion of the deethylase activity in control microsomes was catalyzed by isozymes other than IIE1. Addition of control ascites fluid (HyHel-9) gave no detectable inhibition.

The deethylation reaction was also inhibited by previously described inhibitors of NDMA deethylase activity (Table 2). Hexane and benzene, each at 1%, essentially completely inhibited the ether deethylase activity in agreement with their effects on NDMA demethylase (17). Pyrazole and phenylethylamine, each at 1 mM, inhibited deethylation by greater than 80%, in agreement with their reported effects on NDMA demethylase (28). NDMA itself was a potent inhibitor, giving about 50% inhibition at 4 mM.

Apparent kinetic parameters for diethyl ether oxidation. Since the preceding data suggested that diethyl ether was primarily metabolized by IIE1, the apparent K_m and V_{max} values for acetaldehyde formation were determined. Using livers from acetone-treated rats, the observed kinetic parameters varied slightly depending upon the microsomal preparation. The mean (±SD) V_{max} value was 8.2 ± 0.2 (nmol of acetaldehyde/min/nmol of P-450) and the K_m value was 13.4 ± 2.4 μM. A representative plot is shown in Fig. 2. The activity was highest at approximately 0.4–0.8 mM and was 25% less at 10 mM, possibly resulting from substrate inhibition (data not shown). The V_{max} for the microsomal deethylase activity from acetone-treated rats was 3- to 5-fold higher than that for the control microsomes for which an apparent K_m value of 71.3 ± 9.5 μM was observed. This K_m value was higher than that for the microsomes from acetone-treated rats, possibly reflecting the presence of a higher proportion of isozymes with lower affinity for ether in the control microsomes. Correlation coefficients from Hanes-Woolf plots (not shown) of the data in Fig. 2 were 0.995 for acetone-induced and 0.935 for control microsomes. The results indicated the involvement of a single isozyme in the acetone-induced microsomes. A slight curvature in the plot for the control microsomes suggested the possible participation of more than one isozyme.

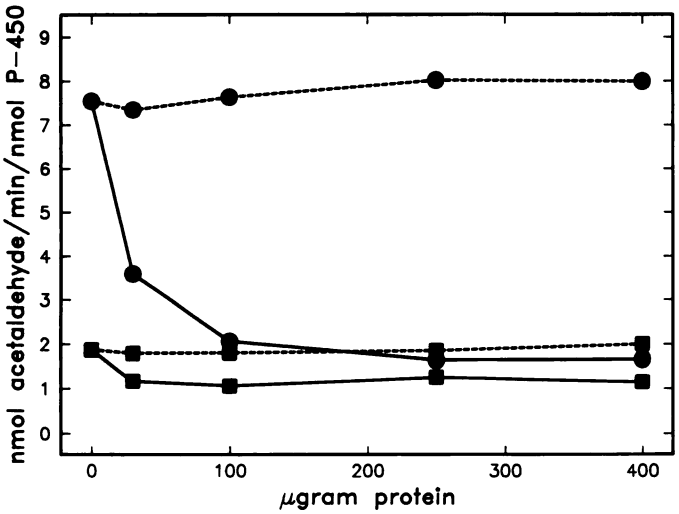

Fig. 1. Effect of monoclonal antibodies on ether deethylation by microsomes. Microsomes [0.15 nmol of P-450 from either control (■) or acetone-treated (●) rats] were preincubated with MAb 1-91-3 (——) or control ascites fluid (HyHel-9, – – –), added as μl portions from the stock ascites fluids, for 15 min at room temperature. The tubes were then transferred to a 37° bath for 1 min and the reaction (lasting 5 min) was initiated by addition of the NADPH-generating system and the substrate (0.8 mM final ether concentration). Each point represents the average from two separate incubations.

TABLE 2

Effect of inhibitors on microsomal ether metabolism

The 0.5-ml incubations, lasting 5 min, contained microsomes from acetone-treated rats (0.15 nmol of P-450) and 0.8 mM ether. The activity in the absence of inhibitors was 7.9 nmol of acetaldehyde/min/nmol of P-450. Each value represents the average from two separate incubations.

Inhibitor	Percentage of control activity
Control	100.0
NDMA (1 mM)	60.0
NDMA (4 mM)	53.7
Hexane 0.2% (15 mM)	20.2
Hexane 1.0% (77 mM)	<1.0
Benzene 0.2% (22 mM)	13.6
Benzene 1.0% (112 mM)	<1.0
Pyrazole (1 mM)	17.8
Phenylethylamine (1 mM)	<1.0

Studies with purified IIE1 and IIB1. The rate of deethylation by purified IIE1 and IIB1, and the effect of cytochrome b_5, are shown in Table 3. In the absence of cytochrome b_5, the rates due to IIE1 were 82-, 10-, and 3.7-fold higher at 0.1, 1, and 10 mM compared to the respective rates catalyzed by IIB1. This suggests that IIE1 has a lower K_m than IIB1 in catalyzing the oxidation of ether. Cytochrome b_5 had a pronounced stimulatory effect on the IIE1 deethylase activity at the lower ether concentrations, giving 4- and 2.4-fold stimulation at 0.1 and 1.0 mM ether, respectively, but no stimulation at 10 mM ether. These results are consistent with a cytochrome b_5-dependent decrease in the K_m for the deethylation reaction and are in

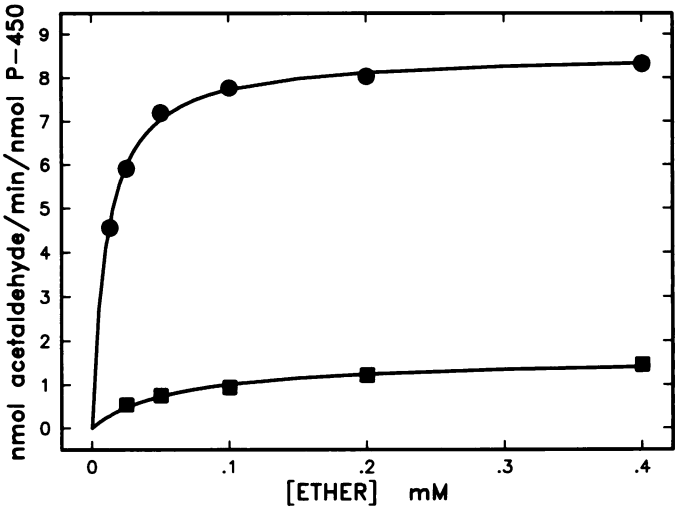

Fig. 2. Rate of acetaldehyde formation by microsomes from control and acetone-induced rat liver versus ether concentration. The incubations contained microsomes from either control (0.2 nmol of P-450, ■) or acetone-induced (0.1 nmol of P-450, ●) rats. The curves were generated using the following constants derived from nonlinear regression analysis: control, K_m = 58.2 μM, V_{max} = 1.60 (nmol of acetaldehyde/min/nmol of P-450); acetone-induced, K_m = 10.8 μM, V_{max} = 8.56 (nmol of acetaldehyde/min/nmol of P-450). Each point represents the average from two separate incubations. The concentrations of ether have not been corrected for the slight evaporation mentioned under Experimental Procedures.

TABLE 3

The effect of cytochrome b_5 on the metabolism of ether by reconstituted cytochrome P-450 isozymes

The activity is expressed as nmol of acetaldehyde/min/nmol of P-450. The incubations (0.25 ml total volume) contained 36.2 nmol of dilauroylphosphatidylcholine and either 0.2 nmol of IIB1 or 0.1 nmol of IIE1. The concentrations of the buffer and NADPH-generating system components were the same as for the microsomal incubations as described under Experimental Procedures. The molar ratio of reductase to P-450 was 4.2:1. The molar ratio of cytochrome b_5 (when present) to P-450 was 1:1. Each value represents the average from two separate incubations.

Ether	Activity			
	IIB1		IIE1	
	$-b_5$	$+b_5$	$-b_5$	$+b_5$
mM				
0.1	0.01	0.03	0.82	3.29
1.0	0.42	0.55	4.17	10.15
10.0	4.96	7.65	18.14	18.00

TABLE 4

Effect of diethyl ether and isoflurane treatments on microsomal P-450, reductase, and NDMA demethylase (NDMAd)

Rats were treated with ether three (3×) or five (5×) times daily for 3 days before sacrifice. The values are the mean ± standard deviation of four microsomal preparations in each group.

Treatment	P-450	Reductase	NDMAd
	nmol/mg	*nmol/min/mg*	
Experiment I			
1. Control	1.26 ± 0.18	205 ± 53	2.12 ± 0.13
2. Ether (3×)	1.67 ± 0.13[a]	227 ± 64	3.04 ± 0.28[a]
3. Ether (5×)	1.63 ± 0.24[b]	309 ± 35[b]	4.32 ± 0.71[a]
Experiment II			
4. Control	0.93 ± 0.04	219 ± 1	1.90 ± 0.08
5. Isoflurane	1.00 ± 0.07	238 ± 11[b]	2.78 ± 0.30[a]

[a] Values are significantly different from those of the corresponding control, $p < 0.01$.

[b] Values are significantly different from those of the corresponding control, $p < 0.05$.

agreement with trends noted for the demethylation of NDMA by IIE1 (19, 29). Because of the presence of glycerol, an inhibitor of IIE1, in the reconstituted system, the K_m values in this system were not determined. The substrate concentration-dependent elevation of the deethylation rate by cytochrome b_5 was not as pronounced with IIB1, giving 3-, 1.3-, and 1.5-fold stimulation at 0.1, 1.0, and 10 mM ether, respectively.

Induction of microsomal enzyme activities by diethyl ether and isoflurane. Since the preceding data (Table 1) had indicated an enhancement of microsomal deethylase by ether treatment, the ability of this anesthetic to induce other enzyme activities was considered. The specific content of P-450 (in nmol of P-450/mg of protein) and cytochrome *c* reductase activity, as well as the rate of NDMA demethylation, are shown in Table 4. Ether treatment, three times a day (3×) or five times a day (5×) for 3 days, significantly increased the specific content of P-450 relative to control microsomes. The NDMA demethylase activity was enhanced 43 and 104% when expressed on a per mg basis with 3× and 5× treatments, respectively. It was also noted that microsomal NADPH/cytochrome *c* reductase activity, which reflects the quantity of NADPH/P-450 reductase, was also increased by 50% due to the 5× ether treatment.

The effect of another ether anesthetic, isoflurane, as an inducer was also studied. The NDMA demethylase activity was increased by 1.5-fold after isoflurane treatment (three times daily for 3 days). This induction was accompanied by a slight increase in reductase activity (Table 4).

The effect of diethyl ether treatment on the induction of various oxygenase activities, which reflect the presence of different forms of microsomal P-450, was determined. The rates of metabolism of NDMA, benzphetamine, ethylmorphine, and *p*-nitroanisole are listed in Table 5. As was the case in Table 4, NDMA demethylase activity was increased by ether treatment. The enhancement was 1.6-fold by the 3× ether treatment and was 2-fold by the 5× treatment. The enhancement of benzphetamine and ethylmorphine demethylation was negligible after the 3× and 5× treatments. The *p*-nitroanisole demethylation was elevated slightly after the 5× treatment.

A more specific substrate for the phenobarbital-inducible forms of P-450 is 7-pentoxyresorufin (24). The rates of *O*-dealkylation by microsomes derived from ether- and phenobarbital-treated rats are also shown in Table 5. The dealkylation rate was enhanced by 2.6-fold after the 3× ether treatment and by 9.8-fold after the 5× treatment. The rate was induced to a much greater extent by phenobarbital treatment, showing a 110-fold enhancement, consistent with the report by Lubet *et al.* (24). The results suggest that one or more of the phenobarbital-inducible forms of P-450 were induced by ether.

Immunoblot analysis. Since the foregoing experiments demonstrated an increase in microsomal enzyme activities associated with both IIE1 and the phenobarbital-inducible forms, the presence of these isozymes was analyzed with anti-IIE1 IgG and anti-IIB1 IgG using an immunoblot technique (Fig. 3). Enhancement of the intensity of the IIE1 band after ether treatment was slight, but was consistently observed upon repetition of the immunoblot analysis. In contrast, a much greater increase in the intensity of the IIB1 band was observed [compare in Fig. 3 *lane O* (control) and *lane P* (5× ether treatment), for example]. The enhancement in the IIB1 intensity appeared greater at higher doses of ether [compare in Fig. 3 *lanes O* (control) and *P* (5× treatment) to *lanes S* (control) and *T* (3× treatment)] in agreement with the trend noted above for 7-pentoxyresorufin metabolism.

Discussion

Hepatic IIE1 catalyzes the oxidation of a variety of low molecular weight compounds including NDMA, butanol, ethanol, aniline, isopropanol, acetone, carbon tetrachloride, and *n*-pentane (13, 14, 19, 30, 31). An analogous isozyme is present in hamsters (21), rabbits (12), and humans (32). In the rat, IIE1 was predicted to have a high affinity for diethyl ether since this compound strongly inhibits the metabolism of NDMA *in vivo* (10) and *in vitro* (11). The present findings provide further evidence for the role of IIE1 in the oxidation of ether. Treatment by either acetone or ethanol, both known to induce microsomal NDMA demethylase (33), also significantly enhanced ether deethylase activity. An MAb toward IIE1 that inhibits microsomal NDMA demethylase by 77% (27) inhibited deethylase activity by 78% in the present study.

The apparent kinetic constants reported here indicated a high efficiency for the deethylation reaction catalyzed by acetone-induced microsomes. The K_m value of 13.4 ± 2.4 μM for diethyl ether is lower than the K_mI for NDMA (40–50 μM) (17) and is comparable to the K_m for *n*-pentane (9 μM) (14). Using a purified, reconstituted enzyme system, IIE1 catalyzed the deethylation reaction more efficiently than did IIB1 (Table 3).

TABLE 5

Effect of diethyl ether and phenobarbital (PB) treatments on microsomal monooxygenase activities

Treatment	Demethylase activity[a]				7-Pentoxyresorufin O-dealkylation[b]
	NDMA	Benzphetamine	Ethylmorphine	p-Nitroanisole	
	nmol formaldehyde/min/nmol P-450				pmol resorufin/min/mg
Experiment I					
Control	2.40	6.78	12.72	4.79	6.2 ± 1.8[c]
Ether (3×)	3.80	6.65	13.10	5.45	16.1 ± 6.6[c]
Ether (5×)	4.89	7.40	13.48	5.78	61.4 ± 14.1[c]
Experiment II					
Control		7.55[d]			8.3[d]
PB		12.26[d]			922.1[d]

[a] The reaction mixture contained microsomes (0.5 mg of protein) from control or ether-induced rats in a final volume of 0.5 ml. The three microsomal samples were selected from groups 1–3 shown in Table 4. The substrate concentrations were 4 mM for NDMA and 1 mM for the others.

[b] The incubations contained microsomes in a total volume of 2 ml. The substrate concentration was 10 μM. The protein content was 0.14 mg for the microsomes from the phenobarbital-treated rats and 1–1.5 mg for the others.

[c] The values are the mean ± standard deviation of three microsomal preparations. The microsomal preparations were distinct from those used in the demethylase assays.

[d] Each value represents the average from two separate incubations; the differences between the duplicates were usually < 5%.

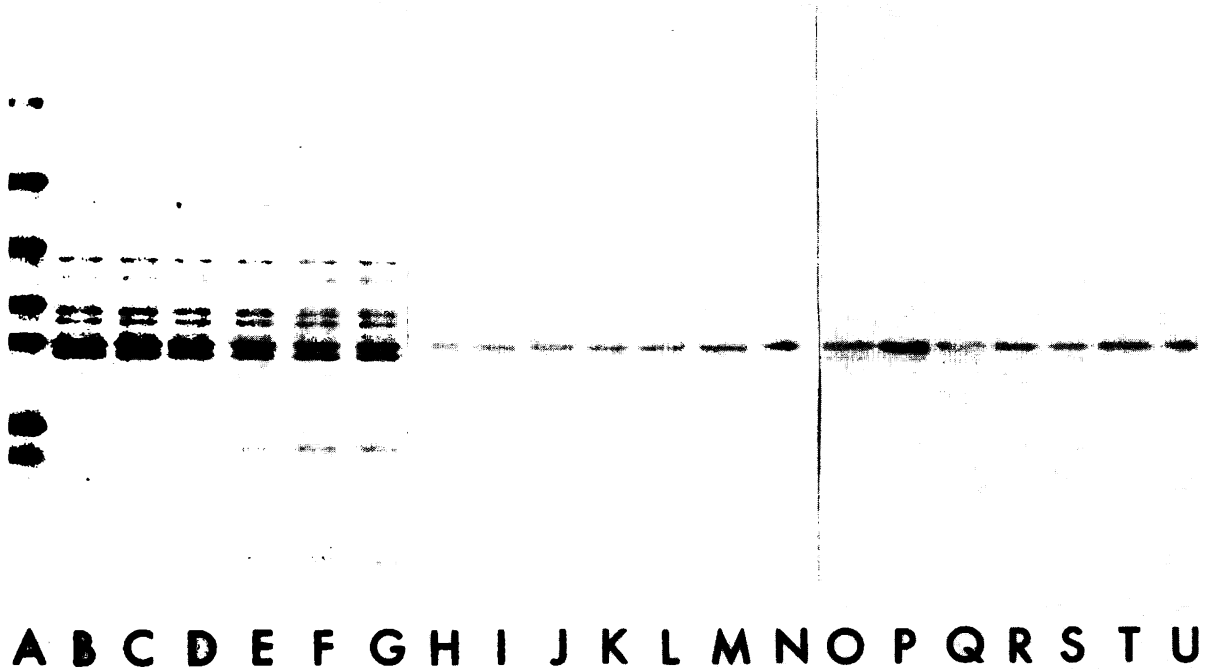

Fig. 3. Sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis (SDS-PAGE) and immunoblot analysis of hepatic microsomes from control and ether-induced rats. *Lanes A–G*, SDS-PAGE; *lanes H–N*, immunoblots using anti-IIE1 IgG; *lanes O–U*, immunoblots using anti-IIB1 IgG. *Lane A* (18.8 μg total protein applied) contained seven protein standards (of the indicated subunit molecular weight): from *top* to *bottom*, α_2-macroglobulin (180,000), β-galactosidase (116,000), fructose-6-phosphate kinase (84,000), pyruvate kinase (58,000) fumarase (48,500), lactic dehydrogenase (36,500), and triose phosphate isomerase (26,600). *Lanes B* and *C* contained microsomal proteins from a control and a 5× ether-treated rat, respectively. *Lanes D* and *E* represent a repeat of *lanes B* and *C*, respectively, using a separate set of rats. *Lanes F* and *G* represent a repeat of *lanes B* and *C*, respectively, except that the treated rat was subjected to 3× ether treatment. *Lanes H–M* and *lanes O–T* both correspond to *lanes B–G*, respectively. Purified IIE1 (0.13 μg) was applied to *lane N*. *Lane U* contained 0.4 μg of purified IIB1. *Lanes B–G* contained 5.3 μg of microsomal protein each. Densitometry indicated that the 3× and 5× ether treatments led to an average of a 7 and a 22% increase in IIE1, respectively, and a 40 and 104% increase in IIB1, respectively.

This agrees with the study of microsomal induction (Table 1) and provides further evidence for the role of IIE1. Cytochrome b_5 has been reported to lower the K_m by 10-fold and to elevate the V_{max} by 1.3-fold for the demethylation of NDMA by purified IIE1 (19). An effect of cytochrome b_5 consistent with an enhancement of substrate affinity was also observed in the present study since the stimulation of IIE1 due to cytochrome b_5 was more pronounced at the lower substrate concentrations.

Several of the substrates for IIE1 are also inducers of this enzyme. A moderate induction of NDMA demethylase activity by diethyl ether as well as increased P-450 content and reductase activities were observed in the present work. Previous investigators have reported ether-dependent increases in P-450 content, reductase, and cytochrome b_5 levels, as well as proliferation of smooth endoplasmic reticulum (15). An approximately 56% increase in ethylmorphine *N*-demethylase activity, expressed per mg of protein, has also been described (16), which agrees with the present results. However, differences in the treatment protocol make this comparison difficult, since the rats were exposed to ether 7 hr/day for 10 days in the previous

study (16). A lack of specificity toward the commonly used substrates also leads to ambiguity in the assignment of a particular isozyme. However, a recently characterized substrate, 7-pentoxyresorufin, is a rather specific substrate for phenobarbital-inducible isozymes (24). The approximately 10-fold elevation in the rate of the *O*-dealkylation observed after the 5× ether treatment (Table 5) indicated induction of the phenobarbital-inducible forms. This observation is consistent with the results of the immunoblot analysis showing a marked increase in IIB1 in the 5×-treated group. It should be noted that the polyclonal antibody against IIB1 used in the present work probably cross-reacts with the closely related IIB2 (24, 34); therefore, it was not possible to distinguish between these two forms. The immunoblot analysis also demonstrated a slight increase in intensity of the IIE1 band in agreement with the observed induction of NDMA demethylase activity.

Isoflurane, an anesthetic structurally related to ether, has been noted to inhibit NDMA demethylation *in vitro* (11) and was also found to moderately induce this activity (Table 4). An interaction between isoflurane and IIE1 is further indicated by the demonstration that metabolism of this anesthetic is elevated during streptozotocin-induced diabetes (35), and also by ethanol (36) and isoniazid (37) treatments.

An alternative explanation for the induction of NDMA demethylase by ether is possible anorexia induced by this treatment, producing a fasting effect. However, the rats in the present study seemed to recover from the anesthesia within 5 min. The weights of the treated animals increased in parallel with those of the controls during the treatment period, and all of the rats appeared normal at the time of sacrifice. An increase in the concentration of ketone bodies in blood after ether anesthesia, as reported by Woodside and Heimberg (38), may have contributed to the observed induction since other treatments that generate ketosis elevate hepatic activities associated with IIE1 (33).

The mechanism of induction of IIE1 is unknown but is apparently complex. Recent studies have demonstrated increased levels of rat hepatic IIE1-specific mRNA after fasting, but no change in this mRNA after acetone administration, concomitant with an elevation of microsomal NDMA demethylase activity by both treatments (39). The induction of IIE1 (40), as well as of phenobarbital-inducible forms (41), appears to be regulated, at least in part, by hormonal factors.

In conclusion, hepatic IIE1 efficiently catalyzed the oxidation of diethyl ether and was slightly induced by ether treatment. Such a treatment also induced IIB1. These points should be particularly noted in the design of experimental protocols utilizing compounds that are substrates for these cytochromes. Since ether is commonly used in industrial and therapeutic settings, its potential for altering the pharmacokinetics of other xenobiotics should also be emphasized.

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Send reprint requests to: Dr. Chung S. Yang, Department of Biochemistry, University of Medicine and Dentistry of New Jersey-New Jersey Medical School, 185 South Orange Avenue, Newark, NJ 07103-2757.