Evaluation of Octamethylcyclotetrasiloxane (D₄) as an Inducer of Rat Hepatic Microsomal Cytochrome P450, UDP-Glucuronosyltransferase, and Epoxide Hydrolase: A 28-Day Inhalation Study

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Repeated inhalation exposure to octamethylcyclotetrasiloxane (D₄) produces a reversible and dose-related hepatoxic reduction and proliferation of hepatic endoplasmic reticulum in rats. However, the effects of D₄ on the expression of cytochrome P450 enzymes have not been evaluated. In the present study, the time course for changes in hepatic microsomal cytochrome P450 enzyme expression following repeated inhalation exposure to D₄ vapors was determined in male and female Fischer 344 rats. Animals were exposed to D₄ vapor at concentrations of 70 and 700 ppm, via whole body inhalation for 6 h/day, 5 days/week for 4 weeks. Specified animals were euthanized on exposure days 3, 7, 14, 21, and 28. Microsomal fractions were prepared from fresh liver by differential centrifugation. Enzyme activity as well as immunoreactive protein levels of several cytochrome P450 enzymes (CYP), epoxide hydrolase, and UDP-glucuronosyltransferase (UDPGT) were evaluated. The time course for enzyme induction was monitored by measuring 7-ethoxyresorufin O-deethylase (EROD) and 7-pentoxyresorufin O-depentylase (PROD) activities on days 3, 7, 14, 21, and 28. CYP1A1/2 activity, as determined by EROD activity, was increased approximately 2- to 3-fold over the exposure period. Only slight changes in the expression and activity of UDPGT were detected, and these did not appear to be dose related. Thus, repeated inhalation exposure to D₄ induces CYP enzymes and epoxide hydrolase in a manner similar to that observed for phenobarbital (PB). Therefore, D₄ can be described as a "PB-like" inducer of hepatic microsomal enzymes in the Fischer 344 rat.

Oxamethylcyclotetrasiloxane (D₄) is a clear, odorless, synthetically derived silicone fluid with a molecular weight of 296. Chemically, D₄ consists of alternating silicon-oxygen bonds connected in a ring (cyclic) arrangement with two methyl groups covalently bonded to each silicon atom (-(CH₃)₂SiO—). The structure of D₄ is shown in Fig. 1. D₄ is used principally as an intermediate in the industrial manufacture of polydimethylsiloxane, a silicone polymer that is used widely in industrial and consumer applications (Stark et al., 1982). Secondarily, D₄ is used as an ingredient in selected personal care products such as antiperspirants and shampoos. Because of these widespread uses and potential human exposure, studies have been initiated to examine the biological fate and effects of this material.

The environmental fate of D₄, including its toxicity in aquatic organisms, biocaccumulation in fish, and fate in the atmosphere and soil, has been well characterized (Annelin and Frye, 1989; Annelin 1990, 1992, 1993; Lehmann et al., 1994; Sousa et al., 1995; Fackler et al., 1995; Mueller et al., 1995; Hobson and Silberhorn 1995). In comparison, less
Figure 1. Molecular structure of octamethylcyclotetrasiloxane (D₄).

information exists on the fate and effect of D₄ in higher vertebrates; however, some studies have been reported. The genotoxicity of unmodified D₄ was evaluated in a battery of in vitro assays with and without the addition of liver S9 fractions as a metabolic activation system. D₄ was negative in all mutagenicity test systems, but was slightly positive at the highest dose in the sister chromatid exchange assay in the presence of the S9 liver fraction (Isquith et al., 1988). In a subsequent study, D₄ was negative in the SCE assay. In addition, recent studies have demonstrated that repeated inhalation exposure of rats to D₄ results in transient liver enlargement and proliferation of hepatic endoplasmic reticulum (Meeks et al., 1995).

High-molecular-weight silicone polymers such as Antifoam-A have been shown to interact with P450 producing a type-I binding spectrum (Franklin, 1972). When administered to rats, phenyltrimethylsilane and phenyltrimethylsilolane undergo biotransformation to hydroxylated and glucuronidated products that are excreted in urine (Fessenden and Hartman, 1970). Oral administration of phenyltrimethylsilane to rats resulted in the formation of a hydroxymethyl derivative and silanol (Fessenden and Hartman, 1970). When rats, dogs, and humans were dosed orally with 4-[(3-methoxyphenyl)methyl]-2,2,6,6-tetramethyl-1-oxa-4-aza-2,6-disilacyclohexane (MPSC) hydrochloride, the major route of elimination was renal and was preceded by substantial metabolism of parent material (Dain and Nicoletti, 1995). These studies provide examples of silicone agents that interact with cytochrome P450 enzymes and suggest that other silicon-containing compounds may be subject to oxidative metabolism via the cytochrome P450 system.

The effects of D₄ on the expression of specific members of the cytochrome P450 family have not been evaluated. Chemicals, such as phenobarbital (PB) and 3-methylcholanthrene (3MC), produce a pleiotropic response in hepatic gene expression that has been well defined in rats and mice (Parkinson, 1996; Lubet et al., 1990, 1992). Identification of specific hepatic enzymes induced and the magnitude of induction relative to controls allows new agents to be classified into chemical groups that produce similar changes. This information can then be used for determining and evaluating interspecies differences between rodents and humans. Moreover, when these data are combined with rodent and human pharmacokinetic data, dose-response information, and human exposure assessment data, meaningful safety assessment can be performed. Thus, the primary objective of the present study was to identify the effects of D₄ on the expression of hepatic microsomal cytochrome P450 (CYP) enzymes, epoxide hydrolase and UDP-glucuronosyltransferase, in Fischer 344 rats following repeated whole-body inhalation exposure.

In the present study, changes in the relative abundance and activity of CYP enzymes from four subfamilies that are highly inducible and primarily responsible for the metabolism of xenobiotics in animals were examined. In addition to the CYP enzymes, the activities of epoxide hydrolase and UDP-glucuronosyltransferase are also affected by exposure to various agents. Thus, an evaluation of these important enzymes provides a profile of changes that may then be compared to profiles obtained following exposure to other agents.

MATERIALS AND METHODS

Chemicals. 7-Ethoxyresorufin, 7-pentoxyresorufin, resorufin, cytochrome c (horse heart), phenylmethylsulfonyl fluoride (PMSF), bicinchoninic acid, sodium cholate, Triton N101, and PB were purchased from Sigma Chemical Co. (St. Louis, MO). Trizma base, EDTA, phosphate salts, glycine, and KCN were from Fisher Scientific (Pittsburgh, PA). β-NADPH was from Boehringer Mannheim (Indianapolis, IN). D₄ was obtained from Dow Corning and was >99% pure.

Animals. Male (175–200 g) and female (130–145 g) Fischer 344 rats were purchased from Charles River. Upon receipt all animals were placed in quarantine for at least 7 days. The animals were randomized into the three treatment groups (0, 70, and 700 ppm D₄) based on weight, ear-tagged, and housed individually in wire mesh stainless steel cages. The animal rooms were maintained on a 12-h light/dark cycle at a temperature of 18–26°C and a humidity of 40–70%. The air exchange rate in the room was 10–15 changes per hour. Animals were allowed free access to food (Purina certified rodent diet) and reverse-osmosis-treated water (except during the 6-h exposure periods) for the duration of the study. Body weights and clinical observations were taken at least weekly as an indication of general health.

Inhalation exposure conditions. Before each exposure, animals were transferred to wire mesh cages designed to be placed inside the exposure chambers. The test and negative control animals were exposed 6 h a day (after a T₀ (Silver, 1946) of approximately 25 min) 5 days a week, for 4 weeks. After each exposure, the animals were returned to their nonexposure housing. The exposure chambers used were Rochester style stainless steel and glass, whole-body chambers (2 m³) operated under dynamic conditions and slight negative (~0.6 inches of H₂O) pressure (Leach and Spiegel, 1958). Room air was treated prior to entering the chamber by a HEPA filter and activated carbon. Chamber temperature and relative humidity values were determined every 5 min with daily mean values of 24 ± 0.7°C and
The procedure is briefly described below:

**Preparation of microsomes.** Animals were euthanized by decapitation and exsanguinated. Hepatic microsomes were prepared essentially as described by Lu and Levin (1972). The procedure is briefly described below:

**Inhalation Study of Octamethylcyclotetrasiloxane**

**TABLE 1**

Summary of Enzyme Activities on Day 28 in Liver Microsomes from Male and Female Fischer 344 Rats Exposed to 0, 70, and 700 ppm Octamethylcyclotetrasiloxane (D4) for 4 Weeks

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Assay/substrate</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>70</td>
</tr>
<tr>
<td>Total P450</td>
<td>Difference spectra</td>
<td>1.0 (0.09)</td>
<td>1.3* (0.07)</td>
</tr>
<tr>
<td>NADPH-cytochrome P450 reductase</td>
<td>Cytochrome c reduction</td>
<td>292 (29)</td>
<td>365* (33)</td>
</tr>
<tr>
<td>CYP1A</td>
<td>EROD</td>
<td>0.8 (0.05)</td>
<td>1.7 (0.17)</td>
</tr>
<tr>
<td>CYP2B</td>
<td>PROD</td>
<td>0.06 (0.01)</td>
<td>0.9* (0.15)</td>
</tr>
<tr>
<td>CYP3A</td>
<td>Testosterone 6β-OH</td>
<td>2.0 (0.65)</td>
<td>3.0* (0.91)</td>
</tr>
<tr>
<td>CYP4A</td>
<td>Larnc acid 12-β-OH</td>
<td>1.4 (0.09)</td>
<td>1.4 (0.13)</td>
</tr>
<tr>
<td>Epoxide hydrolase</td>
<td>cis-Stilbene oxide</td>
<td>8.1 (1.6)</td>
<td>11* (0.20)</td>
</tr>
<tr>
<td>UDPGT</td>
<td>Chloramphenicol</td>
<td>2.6 (0.30)</td>
<td>3.0* (0.18)</td>
</tr>
<tr>
<td>UDPGT</td>
<td>para-Nitrophenol</td>
<td>30 (2.5)</td>
<td>33 (0.8)</td>
</tr>
</tbody>
</table>

**Note.** Values represent the mean (±SD) of 3–6 animals. Values for enzyme assays are expressed as nmol/min/mg protein; values for total P450 are expressed as nmol/mg protein. EROD, ethoxyresorufin O-deethylation; PROD, pentoxyresorufin O-depentylase. UDPGT, UDP-glucuronosyltransferase.

*Statistically different from controls p < 0.05

43 ± 2.5%, respectively. Airflow through the chambers was maintained at 360 ± 12 liters/min. The test article was introduced into the chambers through specially designed heated glass J-tubes (Miller et al., 1980). To facilitate more efficient vaporization of test article, the J-tubes were wrapped with heat tape. D4 was metered into the J-tubes at 30 liters/min. The airflow mixture was then passed into the inlet port at the top of the chambers where it was mixed with chamber supply air to achieve the target concentration (70 or 700 ppm). Actual chamber concentrations of D4 were determined by functional assays, using substrates considered relatively specific for various CYP subfamilies, and immunochemical techniques. In addition to CYP enzymes, microsomal epoxide hydrolase and UDP-glucuronosyltransferase were also evaluated. In order to determine the time course for enzyme induction during exposure to D4, 7-ethoxyresorufin O-deethylase (EROD) and 7-pentoxyresorufin O-depentylase (PROD) assays were performed on microsomal samples from each time point. All other enzyme assays were performed on day 28. It should be noted that microsomes from PB-treated rats were always included as intra assay positive controls. In addition, positive control microsomes were used as appropriate in Western blot analyses (see figure legends).
FIG. 2. Changes in hepatic microsomal 7-ethoxyresorufin O-deethylase (EROD) activity with time in male and female Fischer 344 rats. The upper panels represent EROD specific activity and the lower panels represent fold induction relative to controls. Values are means ± SEM for N = 6–9 animals. Asterisks indicate values that are statistically different from controls at p < 0.05.

addition of NADPH and the change in absorbance at 550 nm was recorded for at least 3 min at 30°C. The reduction of cytochrome c was analyzed with a Beckman DU640i single-beam spectrophotometer. Enzyme activity expressed as nmol cytochrome c reduced/min/mg protein was determined by dividing the ΔAbs550 by 0.0295, the extinction coefficient for cytochrome c derived from horse heart.

Dealkylation of 7-ethoxy- and 7-pentoxyresorufin. The assays were performed using the principles established by Prough et al. (1978), Burke et al. (1985), Lubet et al. (1985), Dutton and Parkinson (1989), and Rutten et al. (1992) with minor modifications. Reaction mixtures were prepared in 3-ml cuvettes and contained the following: 50 mM potassium phosphate buffer, pH 7.7, 5 mM MgCl₂, 10–250 μg/mg microsomal protein, either 7-ethoxyresorufin or 7-pentoxyresorufin (10 μM), and 100 μM NADPH, in a final volume of 2.0 ml. The amount of phosphate buffer added depended on the amount of microsomal protein added. Reactions were started with the addition of NADPH. All reactions were performed at 37°C. Direct measurement of the production of resorufin was monitored with a Perkin-Elmer Model LS50B fluorescence spectrophotometer at excitation and emission wave lengths of 530 and 585 nm, respectively.

Testosterone 6β-hydroxylase activity. The products of testosterone hydroxylation catalyzed by liver microsomes were determined by HPLC, as described by Pearce et al. (1996). Liver microsomes were incubated 8 to 10 min at 37°C in 1-ml incubation mixtures containing potassium phosphate buffer (50 mM, pH 7.4), MgCl₂ (3 mM), EDTA (1 mM), NADP (1 mM), glucose-6-phosphate (5 mM), glucose-6-phosphate dehydrogenase (1 U/ml), the steroid 5α-reductase inhibitor 17β-N,N-diethylnitroso-4-methyl-4-aza-5α-androstan-3-one (1 μM), and testosterone (250 μM), at the final concentrations indicated.

Hydroxylation of lauric acid. Hydroxylation of lauric acid at positions 11 and 12 were determined by combining the methods of radiometric partitioning (Giera and Van Lier, 1991) and radiometric HPLC analysis (Romano et al. 1988). This assay measures the rate of conversion of [¹⁴C]lauric acid to the water soluble hydroxylated metabolites and has recently been reported by Pearce et al. (1996). Briefly, the reaction mixture contained the following in a final volume of 1 ml: 80 μl of microsomal protein (0.2 mg), 80 μl of the NADPH generating system (1 mM, NADP, 5 mM glucose-6-phosphate, 1 U/ml glucose-6-phosphate dehydrogenase), and 840 μl of substrate solution (50 mM potassium phosphate, pH 7.4, 3 mM MgCl₂, 1 mM EDTA, 4 mM [¹⁴C]lauric acid (20 μCi/ml) dissolved in 5 mM sodium carbonate). An aliquot representing 12.5% (105 nCi) of the substrate solution was placed into scintillation fluid and the total radioactivity measured. The reaction was started with the addition of the NADPH generating system and incubations were carried out at 37°C for 20 min. All units represent final values in the 1-ml reaction mixture. Reactions were stopped with the addition of 1% sulfuric acid (400 μl). Microsomes from clofibric-acid-treated rats were included as intraassay positive controls. The completed reactions were vortexed three times for 1-min and centrifuged at 2000g for 10 min. The upper organic layer, containing unreacted substrate, was aspirated and discarded...
Portions of the lower aqueous phase, containing the 11- and 12-hydroxy metabolites, were analyzed by HPLC. An aliquot representing 12.5% of the lower aqueous phase (200 μl) was analyzed for total radioactivity.

**Epoxide hydrolase activity.** Epoxide hydrolase activity was determined using cis-stilbene oxide as substrate according to the principles described by Hammock et al. (1985) and Gill et al. (1983). Reaction mixtures contained the following: 100 μl of 100 mM Tris–HCl, pH 9.0, at ambient temperature containing 10 μg of liver microsomal protein; the reaction was started with the addition of 1 μl of cis-[3H]stilbene oxide (50 μM, 24,000 cpm) dissolved in acetone. The reaction mixture was incubated at 37°C for 5 min. The reaction was stopped by the addition of 200 μl dodecane.

**UDP-glucuronosyltransferase activity.** Glucuronidation of chloramphenicol was determined essentially as described by Young and Lietman with modifications reported by Arloto et al. (1986) and Dutton et al. (1981). The reaction mixtures were prepared as follows: Microsomal protein (5 mg/ml) was solubilized for 15 min with an equal volume of 10 mM Chaps (3-(3-cholamidopropyl)-dimethyl-ammonio)-1-propane) in 0.25 M sucrose and 400 mM Tris–HCl (pH 8.0 at ambient temperature). Following solubilization 50-μl aliquots were added to the incubation mixtures. The final incubation volume was 500 μl and contained 200 mM Tris–HCl buffer, pH 8.0, 10 mM MgCl₂, 1 mM EDTA, 100 μM p-nitrophenol, 0.8 mg/ml microsomal protein, 4 mM UDP-glucuronic acid, 0.5 mM Chaps, 2 mM 1[14]C]chloramphenicol (0.4 μCi/ml), and solubilized microsomal protein (0.25 mg/ml) at the final concentrations indicated. Reactions were started with addition of UDP-glucuronic acid and stopped after a 20-min incubation at 37°C with 6 ml of isooamyl acetate, followed by 1.1 ml of water. The reaction mixture was vortexed for 1-min two times and the aqueous and organic phases separated by centrifugation at 2000g for 10 min.

Glucuronidation of para-nitrophenol was determined essentially as described by Guengerich (1994). Microsomal protein was diluted to a final concentration of 4 mg/ml with 100 mM Tris–HCl buffer (pH 7.4, at 37°C). The following were placed into a glass tube. 0.05% (v/v) Triton X-100, 5 mM MgCl₂, 100 mM Tris–HCl buffer, 0.5 mM p-nitrophenol, 0.8 mg/ml protein. The mixture was allowed to incubate for 3 min at 37°C. Reactions were started with the addition of UDP-glucuronic acid (3 mM). The final reaction volume was 0.5 ml and incubations were carried out for 10 min at 37°C. Aliquots (100 μl) of the incubation mixture were removed and placed into microcentrifuge tubes containing 1 ml TCA (5%) to stop the reaction. The reaction mixture was centrifuged at −1500g (3000 rpm) for about 4 min to pellet precipitated protein. In order to determine the amount of glucuronidation that had occurred, a 1-ml aliquot was removed and mixed with 250 μl of 2 N NaOH. Samples were quantified by reading their absorbance at 405 nm in a Beckman DU640i spectrophotometer. All concentrations shown are final in the 0.5-ml reaction volume.

**Detection of immunoreactive CYP enzymes by Western blot analysis.** Microsomal proteins were separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) essentially as described by Laemmli (1970), with minor modifications as described by Arloto et al. (1989). The separating gel was 0.75 mm × 12.5 cm and composed of 7.5% acrylamide. Following SDS–PAGE, the proteins were electrophoretically transferred to an Immobilon membrane for immunochemical detection of target proteins. The immuno blotting procedure was done according to the principles described by Towbin et al. (1979). Nonspecific binding sites were blocked by incubating the membrane and transferred proteins in a solution containing 5% nonfat dried milk in 10 mM Tris–HCl, pH 7.4, 50 mM NaCl, 1 mM EDTA, and 30 mM sodium azide. Detection of immunoreactive proteins was accomplished with polyclonal antibodies raised against rat liver microsomal CYP1A1/2, CYP2B1/2, CYP3A1/2, and epoxide hydrolase as described by Parkinson and Gemzik (1991). Analysis of CYP4A immunoreactive proteins was accomplished with an ELISA kit purchased from Amersham Inc. (Arlington Heights, IL). Changes in the relative abundance of proteins were determined by laser scanning densitometry. Arbitrary densitometric units were used to determine changes in treated groups relative to controls.

**Statistical analyses.** Some data were evaluated using the general linear models method (Proc GLM, SAS V. 6.11). When significant treatment effects were indicated, p < 0.05, subsequent comparisons of means were assessed using Tukey's method for all possible pairwise comparisons with a family-wise error rate of 5%. In addition, some data were tested using ANOVA (Plot It V3.1, Scientific Programming Enterprises) followed by Duncan's New Multiple Range test for significance at p < 0.05.

**RESULTS**

In order to assess the quality of the microsomal fraction obtained and to evaluate overall changes in total cytochrome P450 enzymes, selected microsomal preparations were analyzed for total P450 and cytochrome-resistant NADPH-cytochrome c reductase activity. On day 28, total P450 in microsomes isolated from control (0 ppm) and D₄-treated (70 and 700 ppm) groups was determined (Table 1). The 0 ppm group had values of approximately 1.0 nmol/mg protein. Exposure to D₄ resulted in a slight increase in total P450, but this change did not appear dose-related. Contamination with ferrous hemoglobin and conversion of cytochrome P450 to P420 was minimal. On day 28, NADPH–cytochrome c reductase activity increased slightly (Table 1) in both D₄ treatment groups.

**7-Ethoxyresorufin O-deethylase activity is considered relatively specific for CYP1A1/2 enzymes.** Changes in EROD activity over the course of the study are presented in Fig. 2. On day 3, a modest but statistically significant increase in EROD activity was detectable in both male (1.7- and 2.5-fold) and female (1.5- and 2.8-fold) 70 ppm and 700 ppm
FIG. 4. Changes in 7-pentoxyresorufin O-depentylase (PROD) activity with time in male and female Fischer 344 rats following repeated inhalation exposure to \( \text{D}_4 \). The upper panels represent PROD specific activity and the lower panels represent fold-induction relative to controls. Phenobarbital (PB) intraassay positive control animals received 80 mg/kg PB for 4 days prior to being euthanized. Male PB PROD activity was 5388 ± 453 pmol/min/mg protein and female PB PROD activity was 3493 ± 391 pmol/min/mg protein. Values represent the mean ± SEM for \( N = 6-9 \) animals. Asterisks indicate values that are statistically different from corresponding controls at \( p < 0.05 \).

\( \text{D}_4 \) groups, respectively. By day 14, EROD induction in both male and female 70 and 700 ppm \( \text{D}_4 \) groups was near maximum and remained relatively constant to day 28. However, immunochemical analysis (Fig. 3) showed no increase in the amount of CYP1A1 immunoreactive protein, and an apparent suppression in CYP1A2 immunoreactive protein in microsomes from \( \text{D}_4 \)-treated animals.

7-Pentoxyresorufin O-depentylase activity is relatively specific for CYP2B1/2 enzymes. Changes in PROD activity over the course of the study are presented in Fig. 4. Exposure to \( \text{D}_4 \) resulted in a pronounced dose-related increase in PROD (Fig. 4). On day 3, PROD in the male 70 and 700 ppm \( \text{D}_4 \) groups was induced 6- and 23-fold, respectively. Male PROD activity and induction remained relatively constant throughout the study, reaching a maximum of 27-fold on day 28 in the 700 ppm \( \text{D}_4 \) group. For female 70 and 700 ppm \( \text{D}_4 \)-treated groups the induction in PROD was 11- and 35-fold, respectively, on day 3. Maximum PROD induction (46-fold) in females occurred on day 7 (1000 pmol/min/mg protein). Between days 7 and 14, PROD activity declined to about 30-fold over controls (665 pmol/min/mg) and then remained relatively constant for the remainder of the exposure period. The increase in female PROD activity correlated with an increase in CYP2B1/2 immunoreactive protein (Fig. 5). Due to an insufficient amount of sample, male Western blot analyses were not performed in this study. However, subsequent studies in our group have confirmed that male CYP2B1/2 immunoreactive protein also increases following inhalation exposure to 700 ppm \( \text{D}_4 \) (data not shown). During the 14-day postexposure period, PROD activity recovered significantly in both male and female 70 and 700 ppm \( \text{D}_4 \) treatment groups, but remained slightly elevated in the 700 ppm \( \text{D}_4 \) group even after 14 days of recovery.

The effects of \( \text{D}_4 \) exposure on the expression of CYP3A
enzymes following exposure to D₄ was investigated by measuring testosterone 6β-hydroxylase activity and CYP3A1/2 immunoreactive protein levels in day 28 microsomal fractions. The data in Fig. 6 (top) show that 6β-hydroxylase activity was modestly induced (two- to fourfold) in a dose-related manner in males and females, with an increase in CYP3A1/2 immunoreactive protein (Fig. 6, bottom).

Changes in CYP4A enzymes can be used as an indication that an agent is acting as a peroxisome proliferator. In the present study, CYP4A1/2 was evaluated by measuring lauric acid 12-hydroxylation and by changes in immunoreactive protein levels as determined by ELISA. No change in CYP4A activity or protein levels were detected in the D₄ treatment groups (Table 1).

FIG. 5. (Top) Effects of D₄ on hepatic microsomal CYP2B1/2 immunoreactive protein levels in female Fischer 344 rats on day 28. The values are expressed as a fold induction relative to controls and represent the mean ± SEM of N = 3 animals. Asterisks indicate that the value is statistically different from corresponding controls at p < 0.05. (Bottom) Comparison of CYP2B1/2 immunostaining of D₄-treated microsomes (4 μg) and PB-treated microsomes (2, 1, and 0.5 μg). The intensity of the D₄ 700 ppm 4-μg scan is similar to the intensity of the PB (1.0 μg) scan. Standard errors and statistical significance have been omitted for clarity, but can be seen in the upper panel.

FIG. 6. Effects of D₄ on hepatic microsomal CYP3A1/2 activity and immunoreactive protein levels on day 28. Microsomes from male and female Fischer 344 rats were analyzed for 6β-hydroxylation of testosterone (top) which is considered relatively specific for CYP3A1/2 activity. Changes in the abundance of CYP3A1/2 immunoreactive protein (bottom) were assessed by Western blot analysis. The stained blots were scanned with a laser densitometer. CYP3A1/2 associated activity values represent the mean ± SEM of N = 6–9 animals. CYP3A1/2 immunoreactive protein values are expressed as the mean ± SEM of fold induction relative to controls for N = 6–9 animals. Asterisks indicate values that are statistically different from corresponding controls at p < 0.05.

Microsomal epoxide hydrolase (EH) activity and immunoreactive protein were increased in a dose-related manner on day 28 as determined by metabolism of cis-stilbene oxide, and by changes in the abundance of immunoreactive EH protein. The inductive effect of D₄ treatment on EH was similar in both males and females with a maximum induction of approximately 1.5-fold in the 70 ppm D₄ groups and 2-fold in the 700 ppm D₄ groups relative to controls (Fig. 7).

UDP-glucuronosyltransferase activity was measured using chloramphenicol and para-nitrophenol (Table 1) as substrates. There was a small but statistically significant increase in UDPGT (chloramphenicol) at the 70 ppm exposure concentration, but not at the 700 ppm level. There was no detectable change in the activity of UDPGT when para-nitrophenol was used as substrate (Table 1).
FIG. 7. Effects of D₄ on hepatic microsomal epoxide hydrolase activity (top) and immunoreactive protein levels (bottom) on day 28 in male and female Fischer 344 rats. Values represent the mean ± SEM of N = 3–6 animals. Asterisks indicate values that are statistically different from corresponding controls at p < 0.05.

Representative Western blots are shown in Fig. 8. Figure 9 compares the enzyme induction profile obtained following treatment with PB to that obtained following exposure to D₄. Enzyme activity is expressed relative to controls (fold induction) for male rats exposed to either PB (positive interassay control), or D₄. The profile of enzyme induction is nearly identical for both compounds. A similar pattern was observed for females.

DISCUSSION

A considerable amount of information exists concerning the chemical and physical properties of cyclic siloxanes; however, little is known about the fate and effect of these compounds in biological systems. Recent studies have shown that repeated inhalation exposure to D₄ produces a dose-related increase in liver size and proliferation of smooth endoplasmic reticulum (Meeks et al., 1995). Although many chemicals produce hepatomegaly, changes in the expression of liver enzymes vary between different classes of organic agents. Identification of the cytochrome P450 induction profile following treatment with D₄ can provide important information on how it interacts with cell macromolecules, place D₄ into a group of well-characterized organic compounds that produce similar effects, identify mechanisms underlying gross observations, uncover interspecies differences in biochemical events, and provide necessary components for research-based risk characterization.

EROD and PROD activities measured in control microsomes (Figs. 2 and 4) were 2 to 3 times higher than those reported in some earlier reports (Prough et al., 1978; Dutton and Parkinson, 1989; Linder and Prough, 1993), while the magnitude of induction, relative to controls, was comparable in the PB and 3-methylcholanthrene (3MC) groups. The higher activity values obtained can be attributed to the assay chosen (direct fluorometric measurement versus extraction) and optimization of several assay parameters. Several investigators conduct the reaction at pHs between 7.4 and 7.8 (Rutten et al., 1992). Highest rates were obtained at a pH of 7.7 in the current study. Typically, a potassium phosphate or Tris buffer is used at a concentration of 100 mM (Rutten et al., 1992); however, it has been shown that the ionic strength of the reaction buffer can inversely affect CYP enzyme activity (Gemzik et al., 1990). A concentration of 50 mM potassium phosphate produced optimum results in this study. Another important influence on the rate of EROD and PROD reactions is substrate concentration. Concentrations of 7-ethoxy- and 7-pentoxyresorufin ranging from 1.5 to 10 μM have been reported (Rutten et al., 1992). A substrate concentration of 10 μM nearly doubled the activity seen at 1.5 μM (data not shown) in the present study. Finally, reaction temperature can also affect reaction rates. For the assays performed in this study, all buffers were warmed to 37°C prior to use and the fluorescent spectrophotometer was equipped with a temperature-controlled cuvette holder. Thus, the reaction temperature was maintained at 37°C throughout the incubation. The conditions described in this study are similar to those reported by Burke et al. (1985) and Lubet et al. (1985) and the EROD and PROD activities obtained and reported here are in close agreement with these studies.

Induction of PROD activity in the Fischer 344 rat reached near maximum levels after 3 days of D₄ exposure in both males and females (Fig. 4). Activity remained relatively constant from the time of maximal effect to the last day of exposure. This plateau effect suggests saturation of the system and or establishment of steady state conditions. In females, there was an apparent decrease in PROD activity on day 14 which was not observed in males (Fig. 4). During the postexposure recovery period PROD activity rapidly returned to near control values. Recovery was complete in the 70 ppm group, while in the 700 ppm groups PROD remained
FIG. 8. Representative Western blot analyses of CYP1A1/2, CYP2B1/2, CYP3A1/2, and epoxide hydrolase. For CYP1A1/2, the amount of microsomal protein loaded was 20 μg for the 0, 70, and 700 ppm D4-treated groups and 2 μg for the 3-MC positive control group. For CYP2B1/2, 4 μg of microsomal protein from the 0, 70, and 700 ppm D4 groups was loaded. For CYP3A1/2, 8 μg of microsomal protein from the 0, 70, and 700 ppm D4 groups was loaded for males and 24 μg from each group for females. The positive control, pregnenolone-16α-carbonitrile (PCN), was loaded at 2 μg. For epoxide hydrolase 8 μg was loaded for both male and female 0, 70, and 700 ppm D4 and PB-treated microsomes.

slightly elevated relative to controls on day 14 of the postexposure period.

Although the present study was not designed to address gender differences at a mechanistic level, some statements can be made. In general, there were only minor gender-related differences in enzyme activities. This is to be expected since the most significant gender differences are seen in constitutively expressed enzymes such as the CYP2C subfamily. One exception, however, was observed in the testosterone 6β-hydroxylation assay used for assessing CYP3A1/2 activity (Fig. 6). These data indicate a much higher activity in male versus female rats. In adult rats the male has much higher levels of CYP3A2 compared to CYP3A1 (Parkinson, 1996). Moreover it is unlikely that CYP3A1 and CYP3A2 possess identical catalytic rates of testosterone metabolism. Therefore, one explanation for the gender differences shown in Fig. 6 is that male rats possess more CYP3A2 than CYP3A1 and that CYP3A2 has a higher rate of testosterone metabolism.

Inhalation exposure to D4 increased hepatic EROD activity (an indicator of CYP1A1/2 activity) two- to fourfold. Subsequent analysis by immunoblotting techniques showed that the inducible enzyme CYP1A1 was not affected and that the relative abundance of the constitutively expressed CYP1A2 was decreased. These observations have been reported in animals exposed to PB as well as other classical inducing agents (Thomas et al., 1983; Yeowell et al., 1985). The small increase in EROD activity observed following treatment with PB-like agents has been attributed to a low rate of metabolism by CYP2B1 and CYP2C6 (Burke et al.,...
In addition, repeated exposure to D₄ did not increase CYP4A enzymes, suggesting that D₄ is not a peroxisome proliferating agent.

In untreated rat liver CYP2B1 is present at or below detectable levels, while CYP2B2 is constitutively expressed, but at very low levels (Parkinson, 1996). The primary CYP enzymes induced by PB and PB-like agents are CYP2B1/2 in rats and mice. Inhalation exposure to 70 and 700 ppm D₄ vapors resulted in a pronounced (>10- and >20-fold, respectively) dose-related increase in hepatic CYP2B activity and immunoreactive protein in the Fischer 344 rat.

In terms of CYP2B1/2 specific activity, as determined by PROD assays, male rats exposed to D₄ had 1.5- to 2-fold higher activity than females. However, when changes in PROD were expressed as fold increase relative to controls, female rats showed the greatest response. This difference in total response is most likely due to the fact that control male rat microsomes possess PROD activity 3-fold greater than female controls. Thus, although activity in males is slightly greater, the overall response is less when normalized to controls. This trend has also been reported in Fischer 344 rats treated with phenobarbital (Nims et al., 1993).

D₄ is not unique in its PB-like activity in rat liver. Examples of other agents that produce these effects include oxazepam, loratadine, and doxylamine succinate. All of these agents have been shown to induce CYP2B1/2 and are considered PB-like inducers based on changes in hepatic cytochrome P450 enzyme levels (Langer et al., 1991; Griffin et al., 1996; Parkinson et al., 1992; Bookstaff et al., 1995; Skare et al., 1995).

When possible, it is helpful to make comparisons between inducers based on equimolar doses. Previous studies have shown that rats exposed to 700 ppm D₄ vapors for 6 h/day received a systemic dose of approximately 100–125 mg/kg/day or 338–422 μmol/kg/day based on 5% absorption of the total D₄ exposure over a 6-h period (Plotzke et al., 1995, 1996). In comparison, 80 mg/kg/day of sodium PB administered by ip injection (a dose known to produce maximal induction of CYP2B1/2) was equivalent to 315 μmol/kg/day. Thus, on a molar basis the doses received were of similar magnitude. PROD activity in microsomes isolated from female rats exposed to 700 ppm D₄ by whole-body inhalation for 3 consecutive days was approximately one-third to one-fourth the PROD activity measured in microsomes from animals treated with PB by ip injection over the same time period (Table 2). This difference in induction was also observed in the Western blot data for D₄ on day 28 (Fig. 5, bottom). Therefore, under the conditions described in the present study and assuming similar absorption and distribution kinetics, PB was approximately three to four times more effective at inducing CYP2B1/2 than D₄.

As with any chemical that induces CYP enzymes, it is important to know whether the enzymes induced are also involved with the metabolism (auto-inducers) of the chemical. Exposure to D₄ vapors via whole-body inhalation produces hepatic biochemical changes that are similar to those observed following treatment with PB. Interestingly, PB is not metabolized by CYP2B1/2 enzymes (Whysner et al., 1996). In comparison, when Fischer 344 rats were preinduced with PB prior to administering radiolabeled D₄, there was an increase in the rate of urinary elimination and metabolism of D₄. In contrast, D₄ elimination from 3-methylcholanthrene-induced animals was not different from that of controls (Salyers et al., 1996). These data suggest that the enzymes induced by PB and by D₄ are also capable of metabolizing D₄.

It is important to be able to predict whether or not the biochemical events observed in animal models would also

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**TABLE 2**

<table>
<thead>
<tr>
<th>Gender</th>
<th>PROD activity, day 3 (D₄, 700 ppm)</th>
<th>PROD activity, day 3 (PB)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>1614 [304]</td>
<td>5388 [453]</td>
</tr>
<tr>
<td>Female</td>
<td>968 [210]</td>
<td>3493 [391]</td>
</tr>
</tbody>
</table>

*Note.* Values represent the mean and standard error of N = 6 to 7 animals and are expressed as pmol resorufin produced/min/mg protein. Values in brackets are ± standard error. PB, Na-phenobarbital administered ip for 3 consecutive days at 80 mg/kg/day or 315 μmol/kg/day; D₄, octamethylcycloptotetrasiloxane (700 ppm) for 6 h is equivalent to approximately 110 mg/kg/day or 372 μmol/kg/day in males and 122 mg/kg/day or 412 μmol/kg/day in females. For changes in the relative abundance of CYP2B1/2 protein see Fig. 5.
occurs in humans. To this end, some studies have evaluated the effects of PB treatment on human liver size and enzyme induction. There was an increase in the clearance of antipyrine, but no change in liver weight in healthy humans exposed to PB (3 mg/day) for 3 weeks (Roberts et al., 1976). In vitro studies employing human and rat hepatocytes to examine the effects of PB on enzyme induction demonstrated that the concentration of PB required to reach an ED50 was higher in human cells than in rat cells. These findings suggest that enzyme induction in humans may be more resistant to PB than rodents and that different mechanisms as well as different CYP enzymes may be involved in the response (Donato et al., 1990; Kocarek et al., 1990).

In general, compounds that cause significant PB-like effects also have a high probability of producing thyroid and liver tumors in rodent 2-year bioassays. Although chronic exposure to PB produces hepatic tumors in several strains of mice (Thorpe and Walker, 1973; Williams et al., 1980) there is significant interspecies sensitivity to this effect. Hepatocellular adenomas may develop in some strains of rats after chronic exposure to PB; as with the mouse, this response can vary depending on strain and age. For example, when Fischer 344 rats were exposed to PB in their diet during chronic studies no increase in the development of hepatic neoplasms was observed, although nodular foci of hyperplasia were present in some animals (Butler, 1978; Saito et al., 1990). In another study, chronic exposure to PB failed to produce any type of tumor in hamsters (Diwan et al., 1986). It is clear from the previous examples that rodents show considerable interspecies and interstrain differences in their susceptibility to PB-induced hepatic tumor formation. The question still remains as to whether PB or PB-like compounds would increase the incidence of tumors in humans. In extensive human epidemiological studies, patients using PB for the treatment of epileptic seizures were monitored over several years, and no increase in tumor incidence related to PB treatment was observed (Clemmesen and Jensen, 1978; Shirts et al., 1986; Olsen et al., 1989, 1990, 1993; Whysner et al., 1996). Thus, based on biochemical differences between rodents and humans as well as epidemiological evidence, PB and PB-like agents have not been classified as human carcinogens (Whysner et al., 1996).

In conclusion, the results of the present study provide compelling evidence that the cyclic siloxane D₄ is a PB-like inducer of hepatic microsomal enzymes in the Fischer 344 rat. This conclusion is based on the enzyme induction profile produced during exposure to D₄ compared to that obtained following treatment with PB (Fig. 9). In the rat, it is well known that PB treatment produces a pleiotropic response typified by a large increase in the expression of hepatic CYP2B1/2 (Nims et al., 1993; Burke et al., 1994; Whysner et al., 1996), and a modest (two- to threefold) induction of CYP3A1/2 (Sonderfan et al., 1987; Lubet et al., 1992), epoxide hydrolase (Lubet et al., 1992), UDP-glucuronosyl-transferase (Bock et al., 1983), and glutathione S-transferase (Whysner et al., 1996). In the present study, and with the exception of GST which was not measured, repeated inhalation exposure of rats to D₄ resulted in a cytochrome P450 enzyme induction profile nearly identical to that described for PB.

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