MOLECULAR PHARMACOLOGY, 23:265–271

Xylene Induces a Cytochrome P-450 Isozyme in Rat Liver Similar to the Major Isozyme Induced by Phenobarbital

RUNE TOFTGÅRD,[1,2] JAMES HALPERT,[1] AND JAN-ÅKE GUSTAFSSON[1]

Department of Medical Nutrition, Karolinska Institute, Huddinge University Hospital F69, S-141 86 Huddinge, Sweden, and Department of Pharmacology, Karolinska Institute, S-104 05 Stockholm, Sweden

Received June 24, 1982; Accepted September 14, 1982

SUMMARY

Rats pretreated with xylene or phenobarbital, and then exposed to *n*-hexane, exhibited a markedly increased peak serum concentration of the neurotoxic metabolite 2,5-hexanedione. In order to elucidate the mechanism underlying this synergistic effect, the major liver microsomal cytochrome P-450 isozymes induced by xylene and phenobarbital, respectively, were purified. In a reconstituted system both isozymes showed a high enzymatic activity with *n*-hexane as the substrate. Turnover numbers for the formation of 2-hexanol were 24 and 27 for the xylene- and phenobarbital-induced isozyme, respectively. The turnover numbers for 7-ethoxycoumarin, benzo[*a*]pyrene, and 1,1,2,2-tetrachloroethane were also in the same range for the two cytochrome P-450 preparations. The isozyme induced by xylene had an amino acid composition very similar to that of the phenobarbital-induced isozyme, and the purified proteins had identical electrophoretic mobilities on polyacrylamide gels in the presence of sodium dodecyl sulfate. Furthermore, similar peptide maps were obtained following digestion with α-chymotrypsin and papain, and each isozyme yielded a single immunoprecipitin band upon reaction with the immunoglobulin G fraction from rabbits immunized with the phenobarbital-induced enzyme. We conclude that xylene induces a rat liver microsomal cytochrome P-450 isozyme very similar to the major isozyme induced by phenobarbital and that this induction is the probable explanation for the enhanced formation of 2,5-hexanedione from *n*-hexane *in vivo*.

INTRODUCTION

Many toxic compounds, including several chemical carcinogens, require metabolic activation in order to exert an adverse effect (1). Of major importance with regard to this activation process is the cytochrome P-450-dependent enzyme system, which is composed of a family of isozymes with different but often overlapping substrate specificities (2, 3). The level of individual isozymes can be modified by exogenous chemicals which possess inductive or repressive properties (2, 3). Such a modification of the isozyme pattern may have a profound influence on the preferred metabolic pathways of potentially toxic compounds (4, 5).

Recently, we and others have shown that the widely used industrial organic solvent xylene can induce the cytochrome P-450 enzyme system in rat liver after inhalation exposure (6–8).

The present study was prompted by our observation that pretreatment with xylene gives rise to a markedly increased *in vivo* serum concentration of the neurotoxic metabolite 2,5-hexanedione when rats are subsequently exposed to *n*-hexane. In an effort to investigate the biochemical basis for this effect and to evaluate which cytochrome P-450 isozyme(s) is induced, we now report the purification and characterization of the major cytochrome P-450 isozyme in rat liver induced by xylene.

METHODS

Materials

[1,2-^{14}C]1,1,2,2-tetrachloroethane (9.4 mCi/mmole > 99% pure) was purchased from New England Nuclear Corporation (Boston, Mass.) and [G-^{3}H]benzo[*a*]pyrene (26 Ci/mmole) from the Radiochemical Centre (Amersham, United Kingdom). Dilauryl L-3-phosphatidylcholine, Lubrol PX, NADPH, and papain were purchased from the Sigma Chemical Company (St. Louis, Mo.). DEAE-Sephacel and Sepharose 4B were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden) and 2′,5′-ADP agarose and 2′-AMP from P-L Biochemicals (Milwaukee, Wisc.). Sodium deoxycholate, sodium cholate, diethyl ether p.a., and *n*-hexane p.a. were purchased from Merck (Darmstadt, West Germany). The sodium cholate

This study was supported by a grant from the Swedish Work Environment Health Fund.

[1] Department of Medical Nutrition.

[2] Department of Pharmacology.

0026-895X/83/010265-07$02.00/0

was used without recrystallization. 1,8-Diaminoctane, cyanogen bromide, 1-hexanol, 2-hexanol, 3-hexanol, 4-heptanol, and 2,5-hexanedione were obtained from Fluka AG (Buchs, Switzerland), and 2,4-hexanedione from Pfaltz & Bauer Inc. (Stamford, Conn.). Bio-Beads SM-2 were purchased from Bio-Rad Laboratories (Richmond, Calif.), and 7-ethoxyresorufin and resorufin from Pierce Eurochemie BV (Rotterdam, Holland). 7-Ethoxycoumarin and 7-hydroxycoumarin were obtained from Aldrich Chemical Company (Milwaukee, Wisc.), and reference benzo[*a*]pyrene metabolites were provided by the National Cancer Institute Chemical Repository under a contract to ITT Research Institute (Chicago, Ill.). α-Chymotrypsin was purchased from Worthington Biochemical Corporation (Freehold, N.J.), and *Staphylococcus aureus* V_8 protease from Miles Laboratories Inc. (Elkhart, Ind.) Xylene p.a. was obtained from BDH Chemicals Ltd. (Poole, United Kingdom). Upon gas-liquid chromatographic analysis the xylene was shown to contain 2% *o*-xylene, 64.5% *m*-xylene, 10% *p*-xylene, 23% ethyl benzene, 0.5% toluene, and 4 ppm of benzene.

Treatment of Animals and Preparation of Microsomes

Adult male Sprague-Dawley rats (200 g) were treated with phenobarbital or xylene. Phenobarbital treatment consisted of the addition of 0.1% (w/v) sodium phenobarbital to the drinking water for 5 days or of single daily i.p. injections of 80 mg/kg for 3 days. Treatment with xylene vapor (2000 ppm, 6 hr daily for 3 days) was performed as described by Toftgård *et al.* (7). Microsomes were prepared essentially as described by van der Hoeven and Coon (9).

For the determination of serum concentrations of 2,5-hexanedione, animals were exposed to 2000 ppm of *n*-hexane for 1 hr in the morning the day after the last xylene or phenobarbital treatment. At different time points after *n*-hexane exposure the animals were killed by decapitation and serum was collected.

Purification of Enzymes

Epoxide hydrolase, NADPH-cytochrome P-450 reductase, and the major form of cytochrome P-450 were purified from liver microsomes of phenobarbital- or xylene-treated rats according to the method of Guengerich and Martin (10), with the following modifications. After sample application the octylamino Sepharose column was washed with buffer containing 0.5% rather than 0.42% sodium cholate. The cytochrome P-450 was then eluted with buffer containing 0.08% rather than 0.06% Lubrol PX and 0.40% rather than 0.33% sodium cholate. The reductase was eluted with buffer containing 0.40% rather than 0.35% cholate and 0.20% rather than 0.15% sodium deoxycholate. The cytochrome P-450 fraction from octylamino Sepharose was chromatographed on a column (2 × 90 cm) of DEAE-Sephacel.

A 1.5-liter linear gradient of 25 mM–100 mM NaCl in buffer was used to eluate the various P-450 forms. The altered conditions were necessary to separate the major P-450 fraction (B_2) from a later fraction of slightly higher molecular weight (B_3) found in the microsomes from both the phenobarbital- and xylene-treated rats. The B_3 isozyme from the phenobarbital-treated rats cross-reacts with antibodies to the B_2 isozyme but has a much lower turnover number toward a number of different substrates.[3] In these respects the B_2 and B_3 fractions from Sprague-Dawley rats appear similar to isozymes *b* and *e* isolated by Ryan *et al.* (11) from Long-Evans rats.

The cytochrome P-450 B_2 fractions, the epoxide hydrolase Fraction II, and the reductase used in this study were ≥95% pure as judged by sodium dodecyl sulfate/polyacrylamide gel electrophoresis according to the method of Laemmli (12). The specific content of the P-450 preparations was 15 nmoles/mg of protein based on the protein concentration determined by the method of Lowry *et al.* (13), using bovine serum albumin as the standard. The specific activity of the reductase was 44 μmoles of cytochrome *c* reduced/min/mg of protein as assayed in 300 mM potassium phosphate buffer (pH 7.7) at 25°. The specific activity of the epoxide hydrolase used was 215 nmoles of styrene glycol per minute per milligram (14).

Assays of Metabolism

General. All assays were performed in 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (pH 7.5) containing 15 mM $MgCl_2$, 0.1 mM EDTA, dilauryl phosphatidylcholine (30 μg/ml), and sodium deoxycholate (100 μg/ml). Blanks contained all components except NADPH. Samples were preincubated for 3 min at 37° prior to the addition of NADPH to start the reaction. Individual assays were carried out as follows.

7-Ethoxycoumarin. The *O*-deethylation of 7-ethoxycoumarin was assayed by the method of Greenlee and Poland (15). Incubations were carried out for 5 min at 37° using 0.05 nmole of cytochrome P-450, 0.3 units of reductase, 300 μM substrate, and 0.2 mM NADPH in a final volume of 1 ml.

1,1,2,2-Tetrachloroethane. The conversion of [^{14}C]1,-1,2,2-tetrachloroethane to soluble metabolites was assayed as described by Halpert (16). Incubations were carried out for 2 min at 37° using 0.5 nmole of cytochrome P-450, 1.7 units of reductase, 100 μM substrate, and 0.2 mM NADPH in a final volume of 0.5 ml.

7-Ethoxyresorufin. The *O*-deethylation of 7-ethoxyresorufin was assayed as described earlier (17). Incubations were carried out directly in the fluorometric cuvette at 37° using 0.1 nmole of cytochrome P-450, 0.5 units of reductase, 4 μM substrate, and 0.2 mM NADPH in a final volume of 2.5 ml.

n-Hexane. Assays of *n*-hexane hydroxylation were performed as previously described (17). Incubations were carried out for 5, 7.5, and 10 min at 37° using 0.05 nmole of cytochrome P-450, 0.25 units of reductase, 10.2 mM substrate, and 0.2 mM NADPH in a final volume of 1 ml.

Benzo[a]pyrene. The hydroxylation of [^{3}H]benzo[*a*]pyrene was assayed by the method of Holder *et al.* (18). In the high-pressure liquid chromatographic separation of metabolites, phenol Fraction I and Fraction II eluted with the same retention times as 3-hydroxy- and 9-hydroxybenzo[*a*]pyrene, respectively. Incubations were carried out for 5, 7.5, 10, and 12.5 min at 37° using 0.1

[3] J. Halpert, unpublished observations.

nmole of cytochrome P-450, 1 unit of reductase, 0.1 mM substrate (2×10^6 dpm) and 0.2 mM NADPH in a final volume of 1 ml. Incubations were carried out both in the presence and absence of 50 μg of epoxide hydrolase.

Peptide mapping. Limited proteolysis of cytochromes P-450 in sodium dodecyl sulfate and analysis by gel electrophoresis were performed according to the method of Cleveland *et al.* (19). The purified cytochrome P-450 was concentrated to 0.5 mg/ml by precipitation with trichloroacetic acid. This procedure was shown in a separate experiment not to influence the peptide maps. Cytochrome P-450 (9 μg) was incubated with 2, 2, and 0.2 μg of α-chymotrypsin, *Staphylococcus aureus* V_8 protease, and papain, respectively, in a total volume of 55 μl. The peptides were separated on 12.5% sodium dodecyl sulfate/polyacrylamide gels according to the method of Laemmli (12).

Amino Acid Analysis

Samples were dialyzed for 24 hr against three 1-liter volumes of distilled water, and triplicate aliquots containing 50–100 μg of protein each were removed and lyophilized. Hydrolysis was performed in sealed, evacuated tubes for 24 or 72 hr at 110° with 6 N HCl containing phenol (10 mg/ml). The hydrolysates were rotary-evaporated to dryness and analyzed with a Durrum D-500 analyzer. Threonine and serine were calculated by linear extrapolation to zero-time of the values from the 24- and 72-hr hydrolyses. Valine and isoleucine were determined from the 72-hr hydrolysis only. Cysteine was determined as cysteic acid after performic acid oxidation and 24-hr hydrolysis. The number of residues was calculated on the basis of a molecular weight of 53,000 for the protein moiety excluding tryptophan, which was not determined.

Other Assay Methods

Antibodies to the cytochrome P-450 B_2 fraction from the phenobarbital-treated rats were raised in New Zealand White rabbits. Immunoglobulin G fractions were prepared from the antisera by chromatography on Protein A Sepharose (20). The immunoglobulin G fractions were used for Ouchterlony double-diffusion analysis, which was performed as described by Guengerich *et al.* (21).

Serum concentrations of 2,5-hexanedione were determined by gas-liquid chromatography after extraction with ethyl acetate. Prior to extraction, 2,4-hexanedione was added as an internal standard. Separation was performed on a column packed with 10% Carbowax 20M on 100/120 Supelcoport. The retention time was 4.5 min and the detection limit was 0.1 μg/ml.

RESULTS

Influence of Xylene and Phenobarbital Pretreatment on Serum Concentrations of 2,5-Hexanedione

When rats pretreated with xylene or phenobarbital were exposed to *n*-hexane and the serum concentration of 2,5-hexanedione determined, it was apparent that much higher peak concentrations were found after both pretreatments as compared with untreated rats (Fig. 1). The highest concentration was observed after phenobarbital pretreatment, which is consistent with the fact that

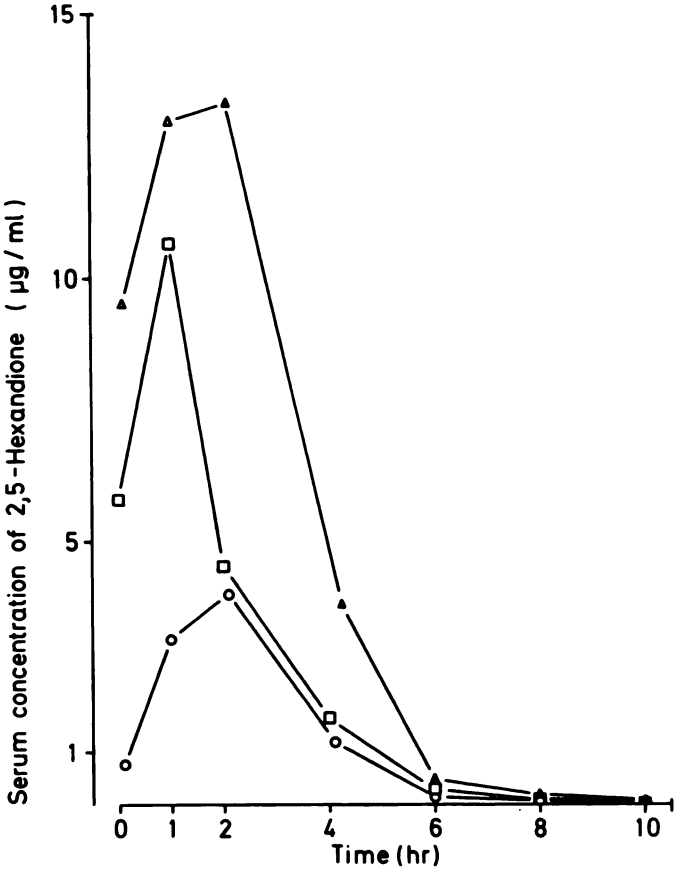

FIG. 1. *Influence of xylene and phenobarbital treatment on serum concentrations of 2,5-hexanedione*

Rats were pretreated by exposure to xylene, 2000 ppm 6 hr daily for 3 days, or by i.p. injections of phenobarbital, 80 mg/kg daily for 3 days. In the morning of the day after the last pretreatment the animals were exposed to 2000 ppm of *n*-hexane for 1 hr. At the indicated time points after cessation of the *n*-hexane exposure the rats were decapitated, and serum was collected and analyzed for 2,5-hexanedione as described under Methods. Each value represents the mean from three rats. △, Phenobarbital-treated rats; □, xylene-treated rats; ○, untreated rats.

phenobarbital is a more effective inducer of hepatic cytochrome P-450 than xylene. The maximal serum concentration was reached 1–2 hr after cessation of the *n*-hexane exposure. The half-life for the rapid elimination of 2,5-hexanedione from serum was approximately 1 hr for both pretreated and untreated rats.

Purification and Chacterization of Liver Microsomal Cytochrome P-450

Exposure of rats to xylene according to the schedule used in this study (2000 ppm, 6 hr daily for 3 days) causes an increase in the total liver microsomal content of cytochrome P-450 from 0.56 to 1.03 nmoles/mg of microsomal protein as compared with 1.7 nmole/mg of microsomal protein after phenobarbital treatment.

The elution profiles obtained upon DEAE-Sephacel chromatography of the octylamino Sepharose fractions derived from microsomes from phenobarbital- or xylene-treated rats were very similar. In both cases the B_2 fractions were quantitatively the most prominent, and these fractions were then compared by several different biochemical methods.

Amino acid composition. As seen in Table 1, the amino acid compositions of the cytochrome P-450 B_2 fractions from the xylene- and phenobarbital-treated rats were strikingly similar. The only noteworthy incongruity is the slightly higher number of cysteine residues in the preparation from the xylene-treated rats. This could easily reflect a slight degree of contamination by a cysteine-rich protein. A difference index of 1.67 for the two proteins indicates that they could be the same enzyme (22).

Peptide maps. The purified cytochrome P-450s have identical mobilities during sodium dodecyl sulfate/polyacrylamide gel electrophoresis (Fig. 2) and an estimated subunit molecular weight of 54,000. As shown in Fig. 2 the peptide maps after digestion with α-chymotrypsin and papain seem identical, and both cytochrome P-450 preparations are quite resistant to digestion by *S. aureus* V_8 protease.

Reactivity with antibodies. As seen in Fig. 3, the cytochrome P-450 B_2 fractions from the phenobarbital- and xylene-treated rats each yielded a single immunoprecipitin band upon reaction with the immunoglobulin G fraction from rabbits immunized with the phenobarbital-induced enzyme, whereas the corresponding P-450 fraction from rats treated with β-naphthoflavone gave no band. Furthermore, the immunoprecipitin bands produced by the B_2 fractions from the phenobarbital- and xylene-treated rats formed a line of identity; the absence of spurs at the intersection of the lines indicates that these two cytochrome P-450 enzymes have identical antigenic sites.

Substrate specificity. Both cytochrome P-450 fractions had high and similar turnover numbers using 7-ethoxycoumarin and *n*-hexane as substrates, as can be seen in Table 2. With *n*-hexane primarily 2-hexanol was formed. The turnover numbers for 1,1,2,2-tetrachloroethane and benzo[*a*]pyrene were lower but also in the same range for the two P-450 fractions. Generally the cytochrome P-450 B_2 fraction from xylene-treated rats showed the same substrate specificity as the corresponding fraction from phenobarbital-treated rats, but the specific activities were approximately 20% lower. The one apparent exception from this was the *O*-deethylation of 7-ethoxyresorufin, for which the P-450 fraction from xylene-treated rats was clearly more active.

When incubations with benzo[*a*]pyrene were carried out in the absence of epoxide hydrolase, primarily the 4,5-epoxide and phenols were formed. In the presence of epoxide hydrolase the 4,5-epoxide was converted to the corresponding dihydrodiol, and small amounts of the 7,8-dihydrodiol were also detected.

TABLE 1

Amino acid compositions of cytochrome P-450 B_2

The amino acid analyses were performed as described under Methods

Amino acid	Residues/molecule	
	Phenobarbital	Xylene
Aspartic acid	37	37
Threonine	24	25
Serine	33	33
Glutamic acid	48	47
Proline	27	28
Glycine	34	32
Alanine	24	24
Cysteine	6	8
Valine	24	24
Methionine	11	12
Isoleucine	26	26
Leucine	58	56
Tyrosine	12	14
Phenylalanine	36	34
Histidine	15	15
Lysine	24	25
Arginine	30	29
Total	469	469

DISCUSSION

n-Hexane is a known neurotoxic agent, and the metabolite 2,5-hexanedione has been identified as the probable mediator of this effect (23). This metabolite is also capable of causing atrophy of testicular germinal epithelium (24) and is the main metabolite found in the urine of workers exposed to *n*-hexane (25). Our results show that the peak serum concentration of 2,5-hexanedione is markedly increased after pretreatment of rats with either xylene or phenobarbital and that the formation of 2-hexanol, a metabolic precursor to the 2,5-hexanedione, is effectively catalyzed by the major cytochrome P-450 isozyme induced by either agent. Since phenobarbital does not induce alcohol dehydrogenase (26), it is suggested that the induction of this isozyme is of major importance with regard to the increased formation of 2,5-hexanedione *in vivo*. A second possibility, although less likely, is that xylene and phenobarbital induces (an)other enzyme(s) involved in the conversion of 2-hexanol to 2,5-hexanedione and that this enzyme is the one which is rate-limiting.

The present results strongly indicate that xylene induces a cytochrome P-450 isozyme very similar to, if not identical with, the major isozyme induced by phenobarbital. This conclusion is supported by the results of amino acid analysis, peptide mapping, reactivity with antibodies, and substrate specificity studies. The only difference worthy of notice between the two cytochrome P-450 fractions is that the 7-ethoxyresorufin *O*-deethylase activity is higher for the xylene-induced cytochrome P-450 fraction. The reason for this discrepancy is not known, but two possibilities are the existence of slight structural differences between the two proteins or a minor contamination of the xylene-induced P-450 fraction with another isozyme. It should be noted that the catalytic turnover for this substrate is quite low, especially as compared with what has been reported for partially purified cytochrome P-450 fractions from β-naphthoflavone-treated rats (27).

It is interesting to note that the cytochrome P-450 enzymes induced by both phenobarbital and xylene have considerable activity toward benzo[*a*]pyrene, which is primarily metabolized to phenols and the 4,5-epoxide. The latter is effectively hydrolyzed to the 4,5-dihydrodiol by epoxide hydrolase. The turnover number for formation of benzo[*a*]pyrene metabolites obtained with the present assay method are significantly higher than those

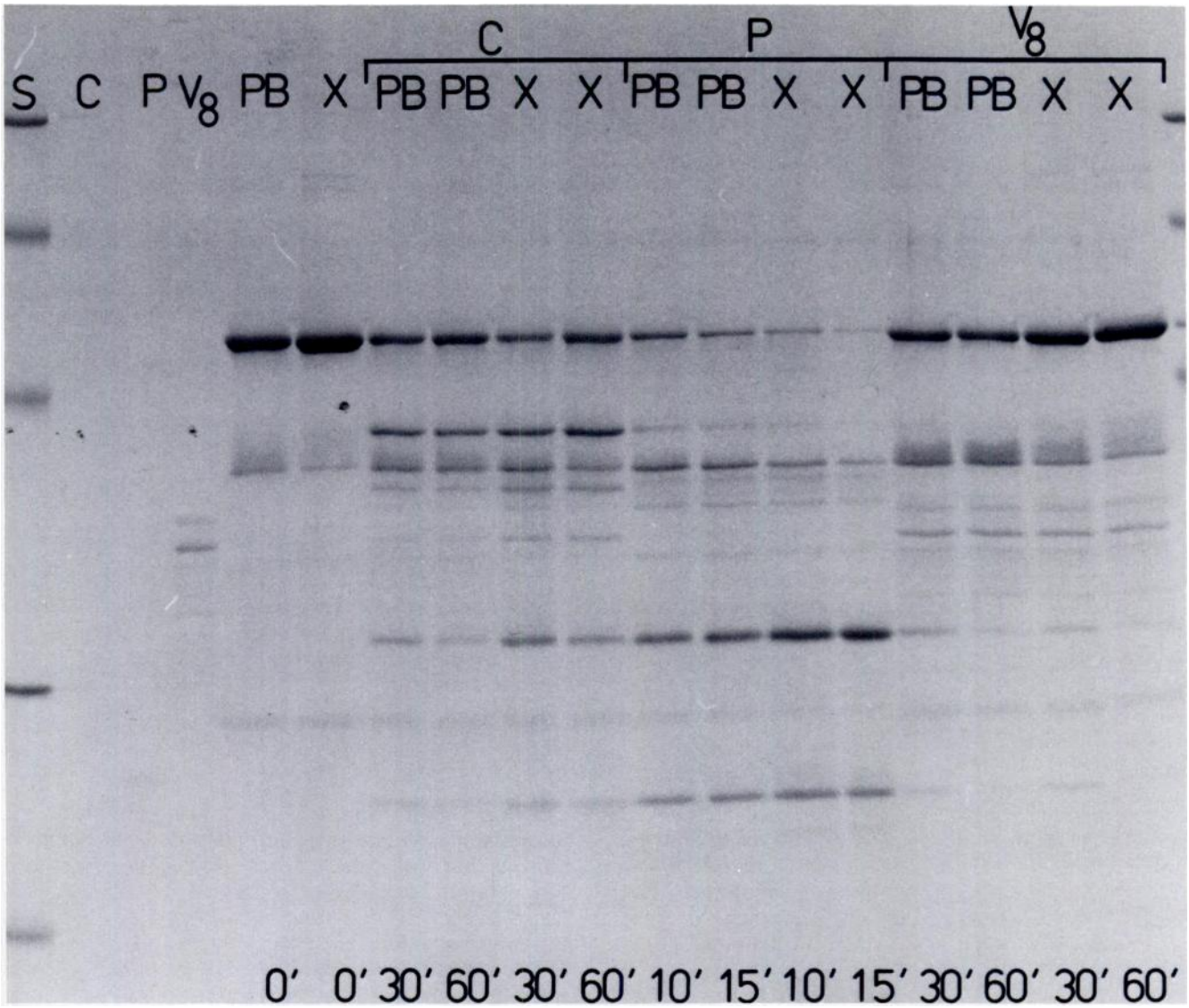

FIG. 2. *Sodium dodecyl sulfate/polyacrylamide gel (12.5%) electrophoresis of peptides generated by digestion of cytochrome P-450 B_2 fractions with α-chymotrypsin, papain, and Staphylococcus aureus V_8 protease*

Digestion and electrophoresis were performed as described under Methods. Molecular weight standards included the following: phorphorylase B, 94,000; bovine serum albumin, 68,000; ovalbumin, 45,000; carbonic anhydrase, 30,000; and soybean trypsin inhibitor, 21,000. Samples were digested for the times indicated. *S*, molecular weight standards; *C*, α-chymotrypsin; *P*, papain; *V_8*, *S. aureus* V_8 protease; *PB*, cytochrome P-450 B_2 fraction from phenobarbital-pretreated rats; *X*, cytochrome P-450 B_2 fraction from xylene-treated rats. Three micrograms of the cytochrome were applied to each well.

reported for the phenobarbital-induced isozyme utilizing the fluorimetric assay (28). As compared with the phenobarbital-inducible cytochrome P-450 isozymes in rabbit liver, the rat isozyme is approximately 10 times more active (17)—although in both cases the dominating dihydrodiol formed upon incubation of benzo[*a*]pyrene in the presence of epoxide hydrolase is the 4,5-isomer.

Using immunological techniques it has been shown that in rat liver the phenobarbital-inducible isozyme represents between 30% and 60% of the total microsomal cytochrome P-450 in induced animals, whereas it is quite low in untreated animals (29, 30). Based on the rate of formation of 2- and 3-hexanol from *n*-hexane and of benzo[*a*]pyrene-4,5-dihydrodiol from benzo[*a*]pyrene relative to the total cytochrome P-450 content in liver microsomes from rats treated with phenobarbital or xylene (7), it appears that after xylene exposure this isozyme represents a similar percentage of the total cytochrome P-450. Phenobarbital is a more effective inducer than xylene, which, under the conditions used in this study, caused a 2-fold increase in the cytochrome P-450 content. However, an inducing effect on the liver microsomal cytochrome P-450 enzyme system has been reported even after exposure to concentrations of xylene as low as 75 ppm for 3 days (31) or to 300 ppm 5 days per week for 18 weeks (6).

The observed induction of a specific cytochrome P-450 isozyme by xylene forms a molecular basis for possible synergistic effects with other organic solvents to which a person might be exposed concomitantly. Examples are

TABLE 2

Metabolic activity of the cytochrome P-450 B_2 fractions from phenobarbital- and xylene-treated rats

Assays were performed as described under Methods. The data represent the means of two or three separate experiments. The activity of the cytochrome P-450 B_2 fraction from xylene-treated rats as a percentage of the corresponding value from phenobarbital-treated rats is given in parentheses. Incubations with benzo[*a*]pyrene were performed with and without added epoxide hydrolase (EPH) as indicated.

Substrate	Turnover number	
	Phenobarbital	Xylene
	nmoles metabolite/nmole P-450/min	
7-Ethoxycoumarin	28	24.5 (88)
7-Ethoxyresorufin	0.17	0.45 (265)
1,1,2,2-Tetrachloroethane	1.9	1.6 (84)
n-Hexane		
1-Hexanol	1.2	1.3 (108)
2-Hexanol	27	24 (89)
3-Hexanol	6	5 (83)
4,5-Dihydrodiol	ND[a]	ND
4,5-Dihydrodiol, + EPH	0.9	0.7 (78)
7,8-Dihydrodiol	ND	ND
7,8-Dihydrodiol, + EPH	0.2	0.1 (50)
Benzo[*a*]pyrene[b]		
4,5-epoxide	1.2	1.0 (83)
4,5-epoxide, + EPH	ND	ND
quinones	0.2	0.2 (100)
quinones, + EPH	0.2	0.1 (50)
phenol fraction I	0.8	0.7 (88)
phenol fraction I, + EPH	0.6	0.5 (83)
phenol fraction II	4.9	3.7 (76)
phenol fraction II, + EPH	3.9	3.2 (82)
Total metabolites	7.1	5.6 (79)
Total metabolites, + EPH	5.8	4.6 (79)

[a] ND, Not detectable.

[b] Formation of pre-9,10-dihydrodiol metabolites was less than 0.01 in all instances. Small amounts of benzo[*a*]pyrene-9,10-dihydrodiol were detected at one time point in the presence of EPH only, for both cytochrome P-450 fractions.

the described increase in the formation of 2,5-hexanedione *in vivo* and the enhancement of the *in vitro* metabolic activation of 1,1,2,2-tetrachlorethane by pretreatment of rats with xylene (16). As this isozyme seems to be very similar to the major isozyme induced by phenobarbital, the vast literature on the enhancement or suppression of toxic effects by pretreatment with phenobarbital may be relevant also for xylene. In this context, a good knowledge of the dose-response relationship for the induction of cytochrome P-450 by xylene is of course essential, and such studies are in progress.

In conclusion, this study has shown that xylene induces a rat liver microsomal cytochrome P-450 isozyme very similar to the major isozyme induced by phenobarbital, and that this induction is the probable explanation on the molecular level for the enhanced formation of the neurotoxic metabolite, 2,5-hexanedione from *n*-hexane *in vivo* and for the enhanced *in vitro* metabolic activation of 1,1,2,2-tetrachloroethane.

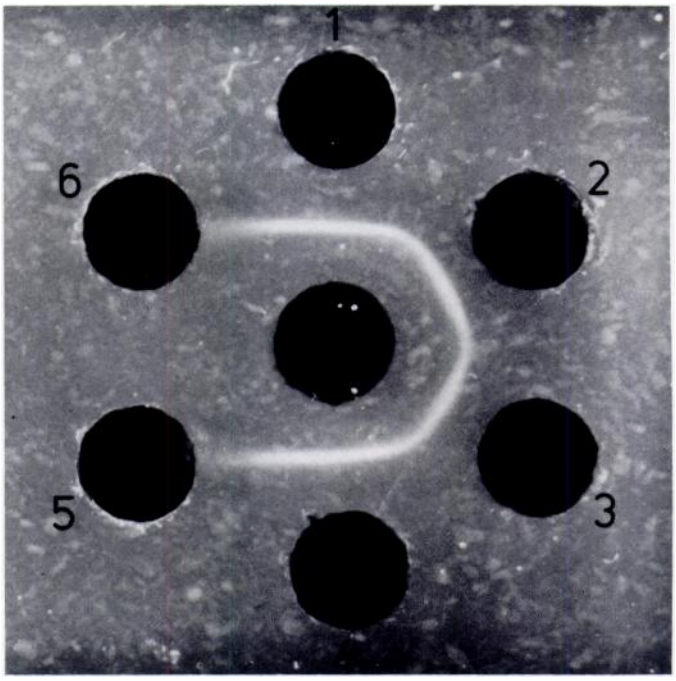

FIG. 3. *Ouchterlony double-diffusion analysis of cytochrome P-450 B_2 fractions purified from liver microsomes of rats treated with phenobarbital, xylene, or β-naphthoflavone*

The preparation of the cytochrome P-450 B_2 fraction from β-naphthoflavone-treated rats is described in ref. 10. The *center well* contained 0.21 mg of immunoglobulin G from rabbits immunized with the phenobarbital-induced enzyme. The *outer wells* contained the following: *Wells 1* and *2*, 0.03 nmole of phenobarbital-induced enzyme; *Wells 3* and *4*, 0.03 nmol of xylene-induced enzyme; *Wells 5* and *6*, 0.03 nmol of β-naphthoflavone-induced enzyme.

ACKNOWLEDGMENTS

We are grateful to Dr. Tapio Haaparanta for performing the antibody-reactivity studies, and to Mrs. Kerstin Svensson, Ms. Elisabeth Sjöholm, and Mr. Ingvar Betnér for their expert assistance.

REFERENCES

1. Miller, E.C., and J.A. Miller. Biochemical mechanisms of chemical carcinogenesis, in *The Molecular Biology of Cancer* (H. Busch, ed.). Academic Press, New York, 377–402 (1974).
2. Haugen, D. A., and M. J. Coon. Properties of electrophoretically homogeneous phenobarbital-inducible and β-naphthoflavone-inducible forms of liver microsomal cytochrome P-450. *J. Biol. Chem.* **251**:7929–7939 (1976).
3. Guengerich, F. P. Isolation and purification of cytochrome P-450, and the existence of multiple forms. *Pharmacol. Ther.* **6**:99–121 (1979).
4. Holder, G., H. Yagi, P. Dansette, D. M. Jerina, W. Levin, A. Y. H. Lu, and A. H. Conney. Effects of inducers and epoxide hydrase on the metabolism of benzo(*a*)pyrene by liver microsomes and a reconstituted system: analysis by high pressure liquid chromatography. *Proc. Natl. Acad. Sci. U. S. A.* **71**:4356–4360 (1974).
5. Gillette, J. R. Effects of induction of cytochrome P-450 enzymes on the concentration of foreign compounds and their metabolites and on the toxicological effects of these compounds. *Drug Metab. Rev.* **10**:59–87 (1979).
6. Elovaara, E., Y. Collan, P. Pfäffli, and H. Vainio. The combined toxicity of technical grade xylene and ethanol in the rat. *Xenobiotica* **10**:435–445 (1980).
7. Toftgård, R., O. G. Nilsen, and J.-Å. Gustafsson. Changes in rat liver microsomal cytochrome P-450 and enzymatic activities after the inhalation of *n*-hexane, xylene, methyl ethyl ketone and methylchloroform for four weeks. *Scand. J. Work. Environ. Health* **7**:31–37 (1981).
8. Toftgård, R., and O. G. Nilsen. Effects of xylene and xylene isomers on cytochrome P-450 and in vitro enzymatic activities in rat liver kidney and lung. *Toxicology* **23**:197–212 (1982).
9. van der Hoeven, T. A., and M. J. Coon. Preparation and properties of partially purified cytochrome P-450 and reduced nicotinamide adenine dinucleotide phosphate cytochrome P-450 reductase from rabbit liver microsomes. *J. Biol. Chem.* **249**:6302–6310 (1974).

10. Guengerich, F. P., and M. V. Martin. Purification of cytochrome P-450, NADPH-cytochrome P-450 reductase, and epoxide hydratase from a single preparation of rat liver microsomes. *Arch. Biochem. Biophys.* **205**:365–379 (1980).
11. Ryan, D. E., P. E. Thomas, and W. Levin. Purification and characterization of a minor form of hepatic microsomal cytochrome p-450 from rats treated with polychlorinated biphenyls. *Arch. Biochem. Biophys.* **216**:272–288 (1982).
12. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* **227**:680–685 (1970).
13. Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
14. Jerina, D. M., P. M. Dansette, A. Y. H. Lu, and W. Levin. Hepatic microsomal epoxide hydrase: a sensitive radiometric assay for hydration of arene oxides of carcinogenic aromatic hydrocarbons. *Mol. Pharmacol.* **13**:341–351 (1977).
15. Greenlee, W. F., and A. Poland. An improved assay of 7-ethoxycoumarin *O*-deethylase activity: induction of hepatic enzyme activity in C57BL/6J and DBA/2J mice by phenobarbital, 3-methylcholanthrene and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *J. Pharmacol. Exp. Ther.* **205**:596–605 (1978).
16. Halpert, J. Cytochrome P-450-dependent covalent binding of 1,1,2,2-tetrachloroethane *in vitro*. *Drug. Metab. Dispos.* **10**:465–468 (1982).
17. Nilsen, O. G., R. Toftgård, and J.-Å. Gustafsson. Regio-selectivity of purified forms of rabbit liver microsomal cytochrome P-450 in the metabolism of benzo(*a*)pyrene, *n*-hexane and 7-ethoxyresorufin. *Acta Pharmacol. Toxicol.* **48**:369–376 (1981).
18. Holder, G. M., H. Yagi, D. M. Jerina, W. Levin, A. Y. H. Lu, and A. H. Conney. Effect of substrate concentration and 3-methylcholanthrene pretreatment on hepatic metabolism by microsomes from rats and mice. *Arch. Biochem. Biophys.* **170**:557–566 (1975).
19. Cleveland, D. W., S. G. Fischer, M. W. Kirschner, and U. K. Laemmli. Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. *J. Biol. Chem.* **252**:1102–1106 (1977).
20. Goding, J. W. Conjugation of antibodies with fluorochromes: modifications of the standard methods. *J. Immunol. Methods* **13**:215–225 (1976).
21. Guengerich, F. P., P. Wang, P. S. Mason, and M. B. Mitchell. Immunological comparison of rat, rabbit and human microsomal cytochromes P-450. *Biochemistry* **20**:2370–2376 (1981).
22. Metzger, H., M. B. Shapiro, J. E. Mosimann, and J. E. Vinton. Assessment of compositional relatedness between proteins. *Nature (Lond.)* **219**:1166–1168 (1968).
23. Schaumburg, H. H., and P. S. Spencer. Environmental hydrocarbons produce degeneration in cat hypothalamus and optic tract. *Science (Wash. D. C.)* **199**:199–200 (1978).
24. Krasavage, W. J., J. L. O'Donoghue, G. D. DiVicenzo, and C. J. Terhaar. The relative neurotoxicity of methyl-*n*-butyl ketone, *n*-hexane and their metabolites. *Toxicol. Appl. Pharmacol.* **52**:433–441 (1980).
25. Perbellini, L., F. Brugnone, and I. Pavan. Identification of the metabolites of *n*-hexane, cyclohexane and their isomers in men's urine. *Toxicol. Appl. Pharmacol.* **53**:220–229 (1980).
26. Patel, J. M., C. Harper, and R. T. Drew. The biotransformation of *p*-xylene to a toxic aldehyde. *Drug. Metab. Dispos.* **6**:368–374 (1978).
27. Burke, M.D., and R. T. Mayer. Inherent specificities of purified cytochrome P-450 and P-448 toward biphenyl hydroxylation and ethoxyresorufin deethylation. *Drug. Metab. Dispos.* **3**:245–253 (1975).
28. Ryan, D. E., P. E. Thomas, and W. Levin. Hepatic microsomal cytochrome P-450 from rats treated with isosafrole. *J. Biol. Chem.* **255**:7941–7955 (1980).
29. Thomas, P. E., L. M. Reik, D. E. Ryan, and W. Levin. Regulation of three forms of cytochrome P-450 and epoxide hydrolase in rat liver microsomes. *J. Biol. Chem.* **256**:1044–1052 (1981).
30. Pickett, C. B., R. J. Jeter, J. Morin, and A. Y. H. Lu. Electroimmunochemical quantitation of cytochrome P-450, cytochrome P-448, and epoxide hydrolase in rat liver microsomes. *J. Biol. Chem.* **256**:8815–8820 (1981).
31. Toftgård, R., O. G. Nilsen, H. Glaumann, and J.-Å. Gustafsson. Induction of hepatic cytochrome P-450 in the rat after xylene exposure: dose-response relationship and dependence on endocrine factors. *Toxicology*, in press (1982).

Send reprint requests to: Dr. Rune Toftgård, Department of Medical Nutrition, Karolinska Institutet, Huddinge Hospital F69, S-141 86 Huddinge, Sweden.