Biochemical Pharmacology, Vol. 35, No. 21, pp. 3733–3738, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00
Pergamon Journals Ltd.

RAT LUNG AND LIVER MICROSOMAL CYTOCHROME P-450 ISOZYMES INVOLVED IN THE HYDROXYLATION OF *n*-HEXANE

RUNE TOFTGÅRD,*† TAPIO HAAPARANTA,* LARS ENG* and JAMES HALPERT‡

*Department of Medical Nutrition, Karolinska Institute, Huddinge University Hospital F69, S-141 86 Huddinge, Sweden; and ‡Department of Pharmacology and Toxicology, College of Pharmacy, University of Arizona, Tucson, AZ 85721, U.S.A.

(*Received* 16 *December* 1985; *accepted* 7 *March* 1986)

Abstract—The primary metabolism of *n*-hexane in rat lung and liver microsomes was investigated. In liver microsomes from untreated animals the formation of each of the metabolites, 1-, 2- and 3-hexanol, was best described kinetically by a two-enzyme system, whereas for lung microsomes a one-enzyme system was indicated for each metabolite. Cytochrome P-450-PB-B, the major cytochrome P-450 isozyme induced in rat liver by phenobarbital, appeared to be responsible for the formation of 2- and 3-hexanol in lung microsomes from untreated rats as judged by antibody inhibition studies. The presence of this isozyme was confirmed by immunoblotting. In contrast, formation of 1-hexanol in rat lung was catalyzed by a cytochrome P-450 isozyme different from the major isozymes induced by either phenobarbital or β-naphthoflavone. Similarly, formation of 2,5-hexanediol from 2-hexanol was catalyzed by a P-450 isozyme different from cytochrome P-450-PB-B and present in liver but not in lung microsomes. Furthermore, alcohol dehydrogenase activity with hexanols or hexanediol as the substrate was found exclusively in liver cytosol. These results suggest that inhaled *n*-hexane must be transported to the liver either intact or in the form of 2-hexanol before the neurotoxic metabolite 2,5-hexanedione can be formed.

Chronic exposure of humans and experimental animals to the organic solvent *n*-hexane is associated with peripheral neuropathy. A metabolite, 2,5-hexanedione, has been identified as the probable neurotoxic agent [1, 2]. Formation of 2,5-hexanedione involves an initial hydroxylation in the 2-position, in a reaction catalyzed by cytochromes P-450 present in both rat liver and lung microsomes [1–4]. Cytochromes P-450 also hydroxylate *n*-hexane in the 1- and 3-positions, leading to the detoxification of the compound. Previous studies from this laboratory have shown that pretreatment of rats with either phenobarbital or xylene induces the metabolism of *n*-hexane to 2,5-hexanedione *in vivo* [5] as well as the conversion of *n*-hexane to 2-hexanol by rat liver microsomes *in vitro* [4, 6]. However, detailed information on the role of specific enzymes in the conversion of *n*-hexane to 2,5-hexanedione and on the nature of the rate-limiting step is lacking. In the present study we have investigated the importance of certain cytochrome P-450 isozymes in the pulmonary and hepatic *in vitro* metabolism of *n*-hexane in the rat, and we present evidence for an obligatory role of hepatic enzymes in the formation of 2,5-hexanedione.

† To whom all reprint requests should be addressed.

§ Abbreviations: PB, phenobarbital; BNF, β-naphthoflavone; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; and SDS, sodium dodecyl sulfate.

MATERIALS AND METHODS

Materials. The following chemicals were purchased: *n*-hexane p.a. and sodium deoxycholate, Merck, Darmstadt, West Germany; *m*-xylene, 1-hexanol, 2-hexanol, 3-hexanol, 4-heptanol, 2,5-hexanediol, and 1,6-hexanediol, Fluka AG Buchs, Switzerland; dilauryl L-3 phosphatidylcholine, NADPH, NAD, and NADP, Sigma Chemical Co., St. Louis, MO, U.S.A.; protein A-Sepharose, Pharmacia Fine Chemicals, Uppsala, Sweden; nitrocellulose filters (GSWP 304 FO, 0.22 μm), Millipore, Bedford, MA; and ^{125}I-labeled protein A (89 μCi/μg), New England Nuclear, Dreieich, West Germany.

Animal treatments and preparation of microsomes. Male Sprague–Dawley rats (200–300 g) were obtained from Anticimex (Sweden) and were kept in cages for 5 days prior to treatment. The animals had free access to food and water and were kept in a room with controlled temperature and light (12 hr light/12 hr dark). Groups of five rats were administered phenobarbital (PB§) (80 mg/kg in 0.5 ml saline) or β-naphthoflavone (BNF) (80 mg/kg in 0.5 ml corn oil) intraperitoneally each day for 3 days. No food was allowed during the 24 hr preceding sacrifice. Liver and lung microsomes were prepared as described previously [4] and pooled within each group. The concentration of microsomal protein was determined by the method of Lowry *et al.* [7] using bovine serum albumin as the standard. The concentration of cytochrome P-450 was determined according to Omura and Sato [8] and Matsubara *et*

al. [9] for liver and lung microsomes respectively. When necessary, the microsomes were frozen as a suspension under a nitrogen atmosphere at −70°.

Enzyme assays. *n*-Hexane hydroxylation was assayed as described earlier [4]. Hydroxylation of 2-hexanol was assayed in 1-ml incubations containing 50 mM HEPES buffer, pH 7.5, 15 mM $MgCl_2$, 0.1 mM EDTA, 2 mg microsomal protein, 2 mM substrate and 1 mM NADPH. The reaction was allowed to proceed for 10 min and was terminated by the addition of 0.1 ml of saturated copper sulfate solution. Internal standard, 1,6-hexanediol (30 nmoles), was added, and the samples were centrifuged. The formation of 2,5-hexanediol was determined by gas–liquid chromatographic analysis of the supernatant fraction, on a column packed with 10% Carbowax 20 M on 100/120 Supelcoport. The oven temperature was 180° and the nitrogen flow 38 ml/min. The retention time for 2,5-hexanediol was 2.3 min.

The assay of hexanols as substrates for cytosolic alcohol dehydrogenase was performed according to Patel *et al.* [10] in incubations containing 3 mg cytosolic protein, 10 mM substrate, and 0.4 mM NAD or NADP in a final volume of 3 ml. The reduction of NAD or NADP was followed spectrophotometrically at 340 nm. The measurements were performed at 23°.

Purification of enzymes and preparation of antibodies. The major phenobarbital-inducible isozymes of rat liver cytochrome P-450 were isolated as described previously [5, 11] by modifications of the method of Guengerich and Martin [12]. As discussed previously [11], the preparations used correspond to isozymes PB-B and PB-D of Guengerich *et al.* [13], and are referred to as such. The major β-naphthoflavone-inducible isozyme of rat liver cytochrome P-450 was also purified as described previously [14] and is referred to as BNF-B according to the nomenclature of Guengerich *et al.* [13]. Antibodies to cytochromes PB-B and BNF-B were raised in New England rabbits, and immunoglobulin G fractions were isolated using a protein-A Sepharose CL-4B column as described previously [14]. As discussed by us and others, the anti-PB-B also recognized isozyme PB-D [11, 13, 14].

For inhibition studies, the desired amount of IgG was added to the incubation mixtures, which were incubated for 20 min at room temperature before the reactions were started as described in the previous section. A constant IgG concentration in each experimental series was achieved by addition of preimmune IgG when necessary. Data are expressed as a percentage of the metabolite formation in similar incubations containing preimmune IgG only.

Assay of n-hexane metabolism with purified enzymes. Incubations with purified enzyme fractions contained 0.05 nmoles P-450, 0.3 units NADPH-cytochrome P-450 reductase, 30 μg dilaurylphosphatidylcholine, 100 μg sodium deoxycholate, and 0.2 mM NADPH and were allowed to proceed for 3–9 min. In all other respects the assay was performed as for the microsomal assay.

Immunoquantitation of cytochrome P-450 isozymes. Microsomes and known amounts of purified cytochrome P-450 isozymes were electrophoresed on 8% SDS-polyacrylamide gels, transferred to nitrocellulose sheets, and incubated with the appropriate antibody [15]. Quantitation of specific P-450 isozymes was performed by incubation of blots with ^{125}I-labeled protein A (5×10^4 cpm/ml) and cutting out protein bands of interest, as described previously [14].

Calculations. Apparent kinetic parameters of *n*-hexane hydroxylation in liver and lung microsomes were calculated by nonlinear least-squares regression analysis as described by McManus *et al.* [16].

RESULTS

Assay of n-hexane hydroxylation in liver and lung microsomes. Initial experiments showed that the formation of 1-, 2-, and 3-hexanol was linear up to a 12-min incubation period with incubations containing maximally 2 mg microsomal protein from either liver or lung microsomes. The pH-optimum was broad, 6.8 to 7.7, and similar for microsomes from both tissues. The apparent kinetic parameters for *n*-hexane hydroxylation by liver and lung microsomes from untreated rats were estimated (Fig. 1 and Table 1).

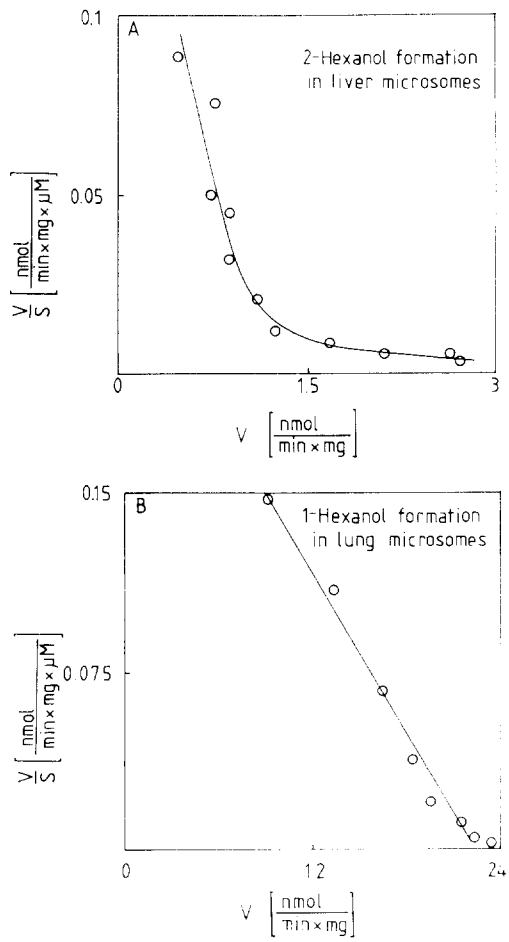

Fig. 1. Kinetics of *n*-hexane hydroxylation. Eadie–Scatchard plots are shown for hydroxylation of *n*-hexane to 2-hexanol in liver microsomes (A) and to 1-hexanol in lung microsomes (B) prepared from untreated rats. Similar results were obtained in two separate experiments.

Table 1. Apparent kinetic parameters for *n*-hexane hydroxylation in rat liver and lung microsomes

Metabolite	Liver				Lung	
	K_{m_1} (μM)	V_{max_1} (nmoles/mg/min)	K_{m_2} (μM)	V_{max_2} (nmoles/mg/min)	K_m (μM)	V_{max} (nmoles/min/mg)
1-Hexanol	0.4	0.09	300	1.2	9	2.2
2-Hexanol	6.0	1.0	1100	4.6	50	1.3
3-Hexanol	*	*	290	0.5	65	0.2

Liver and lung microsomes were prepared from untreated rats. The substrate concentration was varied from 0.05 to 1 mM. Estimates of kinetic parameters were obtained using nonlinear least-squares regression analysis assuming the existence of a two-enzyme system in liver microsomes and a one-enzyme system in lung microsomes.

* No estimates are given due to the limited number of data points that could be obtained at low substrate concentrations.

As shown in Fig. 1, the shape of the Eadie–Scatchard plot for 2-hexanol formation in liver microsomes indicates the presence of a two-enzyme system. Plots of similar shapes were obtained for 1-hexanol and 3-hexanol formation. In contrast, plots for formation of all three hydroxylated metabolites in lung microsomes were linear, suggesting a one-enzyme system for each metabolite. Values for K_m and V_{max} calculated using nonlinear least-squares regression analysis are given in Table 1. It can be seen that the apparent K_m values for the low-affinity enzyme in liver microsomes were similar for 1- and 3-hexanol, whereas the corresponding value for 2-hexanol was substantially higher. However, 2-hexanol was the metabolite preferentially formed. In lung microsomes, the formation of 1-hexanol shows the lowest K_m and the highest V_{max}. The involvement of cytochrome P-450 in the hydroxylation of *n*-hexane in lung microsomes is strongly indicated by the absolute dependence on NADPH (metabolite formation reduced to <5% for all metabolites) as well as the inhibition of the formation of all metabolites by carbon monoxide. Under a $CO:O_2$ atmosphere of 80:20, 90% inhibition of 2- and 3-hexanol formation and >95% inhibition of 1-hexanol formation were obtained.

Metabolism of n*-hexane by purified cytochrome P-450 isozymes.* Table 2 gives the rates of formation of 1-, 2-, and 3-hexanol in reconstituted monooxygenase systems containing isozymes PB-B, PB-D, or BNF-B. The two PB-inducible isozymes exhibited similar regioselectivities and preferentially hydroxylated *n*-hexane in the 2-position. The metabolite profiles for these two enzymes were very similar to those obtained with intact PB microsomes [4, 6], which is consistent with a major role for isozymes PB-B and PB-D in *n*-hexane metabolism catalyzed by such microsomes. Isozyme BNF-B preferentially hydroxylated *n*-hexane in the 3-position, which is also the case with intact BNF-microsomes [4].

Inhibition of microsomal n*-hexane hydroxylation by cytochrome P-450 antibodies.* To further investigate the role of specific isozymes in the liver and lung microsomal metabolism of *n*-hexane, we used antibodies to isozymes PB-B and BNF-B. As can be seen in Fig. 2A, anti-PB-B inhibited 2- and 3-hexanol formation in lung microsomes from untreated rats, whereas the formation of 1-hexanol was unaffected. Similar results were obtained with anti-PB-B and lung microsomes from PB- or BNF-treated animals (data not shown). As seen in Fig. 2B, anti-BNF-B showed no inhibitory effects on the formation of any metabolite by control lung microsomes. In lung microsomes from BNF-treated rats, only the formation of 3-hexanol was inhibited to any significant degree (35%) by anti-BNF-B (18 mg/nmole P-450) (data not shown).

In control liver microsomes, anti-PB-B showed no inhibitory effects, whereas when liver microsomes from PB-treated rats were used, these antibodies efficiently inhibited the formation of all three hexanols (Fig. 2C). It is interesting to note that the residual rate of metabolite formation in the presence of the highest concentration of anti-PB-B used was

Table 2. Metabolism of *n*-hexane by purified cytochrome P-450 isozymes

P-450 isozyme	Turnover number		
	1-Hexanol	2-Hexanol	3-Hexanol
	(nmoles metabolite/nmole P-450/min)		
P-450-PB-B	1.4	33	9.4
P-450-PB-D	1.0	18	7.7
P-450-BNF-B	0.5	4.6	11

Assays were performed as described under Materials and Methods. The data represent the means of replicate determinations (N = 2–3) from two separate experiments.

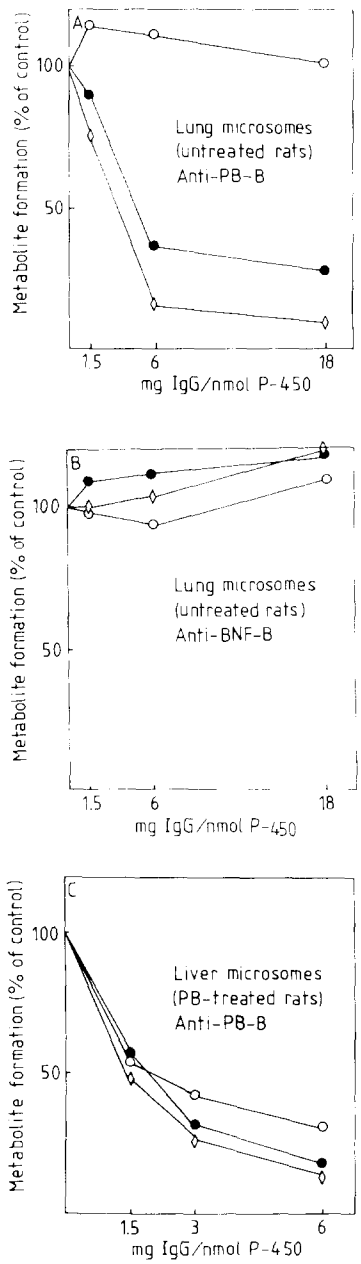

Fig. 2. Effect of antibodies on the formation of (○) 1-hexanol, (◇) 2-hexanol, and (●) 3-hexanol from *n*-hexane in lung and liver microsomes. Anti-PB-B and anti-BNF-B denote antibodies to the major cytochrome P-450 isozymes induced in rat liver by phenobarbital (PB) and β-naphthoflavone (BNF) respectively. The data are expressed as a percentage of the metabolite formation in appropriate incubations containing preimmune IgG. Similar results were obtained in two separate experiments. The 100% values in nmoles/min/mg protein were: 2.0 (1-hexanol), 1.8 (2-hexanol), and 0.4 (3-hexanol) for control lung and 0.7 (1-hexanol), 15 (2-hexanol), and 2.8 (3-hexanol) for PB-liver microsomes

approximately similar to the rate observed with control liver microsomes. In liver microsomes from both control and BNF-treated rats, anti-BNF-B at 6 mg/nmole P-450 inhibited the formation of 2- and 3-hexanol (25 and 40% respectively) but had no effect on the formation of 1-hexanol.

Table 3. Metabolism of 2-hexanol in rat liver microsomes

Treatment	Formation of 2,5-hexanediol (nmoles/min/mg)
Control	2.9
PB	2.5
PB + anti-PB-B*	2.6 (104)
BNF	2.5
BNF + anti-BNF-B*	1.4 (56)

Data represent the mean of duplicate determinations. Liver microsomes from five rats in each treatment group were pooled.

* Six mg IgG per nmole P-450 was used, and metabolite formation as a percentage of the formation without antibody added is given in parentheses.

Further metabolism of 2-hexanol. 2-Hexanol is the major metabolite of *n*-hexane in liver microsomes and is a precursor of the neurotoxic metabolite 2,5-hexanedione. The formation of this metabolite requires the introduction of a second hydroxyl group in a symmetrical position to the first hydroxyl group. To investigate which cytochrome P-450 isozymes might be responsible for the second hydroxylation step, the formation of 2,5-hexanediol from 2-hexanol was assayed. As seen in Table 3, the rate of formation of 2,5-hexanediol was very similar in liver microsomes from the variously treated rats. In addition anti-PB-B in a concentration that markedly inhibited the formation of 2-hexanol from *n*-hexane failed to inhibit the formation of 2,5-hexanediol from 2-hexanol in liver microsomes from PB-treated animals, whereas anti-BNF-B caused a 50% inhibition in liver microsomes from BNF-treated rats. In lung microsomes the formation of 2,5-hexanediol was less than 0.08 nmole/min/mg irrespective of the pretreatment.

The three hexanols and 2,5-hexanediol were also tested as substrates for cytosolic alcohol dehydrogenase. 2- and 3-Hexanol were the best substrates for liver alcohol dehydrogenase (4.0 and 4.4 nmoles NADH formed/min/mg respectively) followed by 1-hexanol and 2,5-hexanediol (2.7 and 1.6 nmoles NADH formed/min/mg). No activity was detectable with NADP as the cofactor or with lung cytosol as the enzyme source.

Immunoquantitation of cytochrome P-450 isozymes in rat lung microsomes. As a more direct way of assessing the presence of specific cytochrome P-450 isozymes in rat lung microsomes, we used SDS-polyacrylamide gel electrophoresis coupled with transfer to nitrocellulose and immunological detection [13–15, 17]. Under the electrophoretic conditions used, isozymes PB-B and PB-D did not separate, and since both are recognized by anti-PB-B, only the sum of the two isozymes was measured. In accordance with results of Guengerich *et al.* [17], the levels of PB-B plus PB-D were approximately equal to the spectrally determined content of P-450 in control lung microsomes, and were not affected by PB or BNF treatment. In contrast, levels of BNF-B

were induced approximately 25-fold by BNF (data not shown).

DISCUSSION

In the present study we have obtained evidence for the involvement of several different cytochrome P-450 isozymes in the primary metabolism of *n*-hexane in rat lung and liver microsomes. Cytochrome P-450 isozymes immunochemically related to cytochrome PB-B were found to be a major component in untreated rat lung as judged by both antibody inhibition studies and immunoblotting. This confirms similar findings by others in both rat and rabbit [17, 18]. It is apparent that these enzymes play a major role in the primary metabolism of *n*-hexane in the untreated rat lung as well as in liver from phenobarbital-treated animals. At present we cannot with certainty ascribe the microsomal activity to isozyme PB-B as opposed to PB-D, since both proteins are recognized by the antibody used in this study and have similar activities with *n*-hexane as the substrate. However, in this context it is of interest that Philpot *et al.* [18] have reported that only the isozyme corresponding to PB-B is present in rat lung.

Although the immunoquantitation of cytochromes PB-B + PB-D in control lung microsomes indicated isozyme levels similar to the total cytochrome P-450 content determined spectrally, this should not be interpreted to mean that no other major isozymes are present in these microsomes. As reported by Guengerich *et al.*, the sum of the levels of eight individual cytochrome P-450 isozymes in liver microsomes determined by an immunochemical method similar to ours yielded values in excess of the spectral content of cytochrome P-450, possibly due to the presence of apo-cytochrome P-450 [13]. In fact, four lines of evidence suggest that control lung microsomes contain a major cytochrome P-450 isozyme distinct from cytochromes P-450 PB-B or PB-D. First, lung microsomes produce equal amounts of 1-hexanol and 2-hexanol from *n*-hexane, whereas intact liver microsomes from PB-treated rats as well as reconstituted monooxygenase systems containing PB-B or PB-D produce 1-hexanol and 2-hexanol in a ratio of 1:20. Second, antibodies to PB-B inhibit the formation of 1-hexanol, 2-hexanol, and 3-hexanol by rat liver microsomes from PB-treated animals, but inhibit only 2-hexanol and 3-hexanol formation by lung microsomes, suggesting that the contributions of PB-B and PB-D to 1-hexanol formation by lung microsomes are minimal. Third, the existence of a separate isozyme responsible for 1-hexanol formation in lung microsomes is supported by the kinetic analysis of metabolite formation, which showed a markedly lower K_m value for 1-hexanol formation compared to the formation of 2- and 3-hexanol. Fourth, we have shown previously that the suicide substrate chloramphenicol inhibits the formation of 2- and 3-hexanol but not of 1-hexanol from *n*-hexane in rat lung microsomes [6]. It should be noted that the cytochrome P-450 isozyme responsible for 1-hydroxylation of *n*-hexane in lung microsomes is also distinct from isozyme BNF-B, as evidenced by the lack of inhibition by anti-BNF-B as well as the lack of inducibility by BNF.

Whereas the metabolism of *n*-hexane by control lung microsomes can be mainly attributed to cytochromes P-450 PB-B and/or PB-D plus a constitutive 1-hydroxylase, the situation is more complicated for control liver. Kinetic analysis suggests the involvement of at least four enzymes in *n*-hexane metabolism by control liver microsomes since a two-enzyme system was indicated for the formation of each of the three hexanols and since the two K_m values for 2-hexanol formation were clearly different from the corresponding K_m values for the formation of 1-hexanol. The identity of these isozymes is presently unknown. None is inhibited by antibodies to PB-B, whereas at least one isozyme may be related to BNF-B, as judged by the partial inhibition by anti-BNF-B of 2-hexanol and 3-hexanol formation.

From the standpoint of the bioactivation of *n*-hexane, our finding that conversion of 2-hexanol to 2,5-hexanediol was not catalyzed by cytochrome P-450-PB-B or P-450-PB-D has important implications with regard to the different steps in the conversion of inhaled *n*-hexane to 2,5-hexanedione. First, whereas the primary hydroxylation to 2-hexanol can take place in the lung or the liver, only the liver is capable of catalyzing the second hydroxylation to 2,5-hexanediol. Second, the enzymatic basis for the increased serum concentration of 2,5-hexanedione observed after *n*-hexane exposure of rats pretreated with phenobarbital or xylene [5] appears to be enhanced formation of 2-hexanol catalyzed by cytochrome P-450-PB-B or PB-D, rather than enhancement of some later step. It is known that phenobarbital does not induce liver alcohol dehydrogenase [10]. The failure to detect any significant alcohol dehydrogenase activity in lung cytosol with hexanols as substrates further emphasizes the crucial role of liver enzymes with regard to the formation of 2,5-hexanedione.

In summary, this study has shown that cytochrome P-450-PB-B and/or PB-D appear to be of major importance with regard to the primary hydroxylation of *n*-hexane in lung microsomes from untreated rats and in liver microsomes from phenobarbital-treated rats. A separate cytochrome P-450 isozyme, present only in liver, catalyzes the formation of 2,5-hexanediol from 2-hexanol. As a consequence, metabolism of *n*-hexane in the liver may be necessary for the formation of the neurotoxic metabolite, 2,5-hexanedione.

Acknowledgements—We thank Elisabeth Sjöholm and Ingvar Betnér for their expert assistance. This study was supported by grants from the Swedish Work Environment Fund and the Swedish Medical Research Council. J. H. is the recipient of a Faculty Development Award in Pharmacology from the Pharmaceutical Manufacturers Association Foundation and of a Research Career Development Award from the National Institute of Environmental Health Sciences (ES 00151).

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