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IN VITRO EFFECTS OF STRAIGHT-CHAIN ALKANES (*n*-HEXANE THROUGH *n*-DODECANE) ON RAT LIVER AND LUNG CYTOCHROME P-450

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To evaluate the effect of straight-chain alkanes on normal detoxication reactions, we studied the in vitro effect of the homologous series n-*hexane through* n-*dodecane on two cytochrome P-450 (EC 1.14.14.1) enzyme activities. Benzo[a]pyrene hydroxylase (BaPOHase) and 7-ethoxycoumarin deethylase activities were measured in liver and lung microsomes of control and* β-*naphthoflavone-treated rats. In the presence of 2 m*M n-*hexane through* n-*dodecane, liver BaPOHase activity decreased from 67% of control with* n-*dodecane to 21% of control with octane. Lung benzo[a]pyrene hydroxylase was insensitive to all tested alkanes at 2 m*M. *In the presence of 2 m*M *alkanes, liver 7-ethoxycoumarin deethylase activity decreased from 73% of control with* n-*octane to 28% with* n-*octane. Lung 7-ethoxycoumarin deethylase was also sensitive to the alkane series. In the presence of 2 m*M *alkane the greatest effect was obtained with* n-*octane and represented a 56% loss in activity. Alkane concentration-dependence measurements showed 0.02–0.20 m*M *as the sensitive region of the curve for* n-*octane with maximal loss of activity achieved at 0.20 m*M. *Liver ethoxycoumarin deethylase activity from* β-*naphthoflavone-treated rats was less sensitive towards the reactive alkane,* n-*octane, than the activity from control rats. Double-reciprocal-plot analysis revealed the maximal velocity (V_{max}) was decreased in the presence of 0.2 m*M n-*octane. Hence this hydrocarbon did not exert its effect solely as an alternate substrate. The data show the* n-*alkanes,* n-*hexane through* n-*dodecane, interfered with a normal detoxication pathway in a manner that was chainlength-dependent, tissue-specific, and dependent on the preexposure history of the animal.*

INTRODUCTION

Straight-chain alkanes are a continuing source of pollution in environmental and occupational settings. They constitute a large component of petroleum refining products (National Institute for Occupational Safety and Health, 1977a,b) and are found in emissions from coke ovens (Richards et al., 1979) and diesel light-duty vehicles (Cuddihy et al., 1980). Straight-chained alkanes are also used as solvents in the manufacture of glue, cement, and ink (National Institute for Occu-

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pational Safety and Health, 1977a; Couri et al., 1978). Exposure to the lower-chain alkanes results in neurotoxicity and dermal sensitivity (National Institute for Occupational Safety and Health, 1977a), while higher-chain alkanes exhibit comutagenic and cocarcinogenic properties (Sice, 1966; Bingham and Falk, 1969; Van Duuren and Goldschmidt, 1976; Lankas et al., 1978).

n-Alkanes are also biochemically active xenobiotics that are metabolized by the cytochrome P-450 enzyme system (Frommer et al., 1972; Toftgard and Nilsen, 1982), and such metabolism has been the focus of studies on the mechanism of toxicity. We have approached the issue of potential health effects of *n*-alkanes in a different way—by evaluating directly their effect on normal metabolic detoxication reactions for other compounds. Regardless of the metabolic fate of the *n*-alkanes—what are the consequences of the interaction(s) between the hydrocarbons and components of a detoxication pathway? The question is important because it recognizes the complexity of exposure conditions.

To approach this question, we investigated the *in vitro* effect of a homologous series of *n*-alkanes (from *n*-hexane to *n*-dodecane, abbreviated *n*C6–*n*C12) on the microsomal cytochrome P-450-mediated metabolism of two polyaromatic compounds. The cytochrome P-450-dependent monooxygenase system pathway is a major detoxication pathway in animals (Pelkonen and Vähäkangas, 1980). Interference with its catalytic activity by aliphatic hydrocarbons may have consequences for the metabolic detoxication of aromatic compounds. We will show that such cytochrome P-450-related (P-450-related) metabolism is sensitive to alkanes in a manner that is chainlength-dependent and tissue-specific. Furthermore, we will demonstrate the sensitivity is dependent on the preexposure history of the animal.

METHODS

Animal Pretreatment

Sprague-Dawley rats (male, 250–300 g) were injected ip with 1 dose of β-naphthoflavone (βNF) in corn oil at 80 mg/kg rat, 24 h before sacrifice. Rats so treated are referred to as βNF rats. Control animals received corn oil and are referred to as CTL rats. The βNF regimen resulted in increased activities of two P-450-related enzyme activities in each organ (Table 1). Only liver 7-ethoxycoumarin deethylase (ECase) and lung benzo[a]pyrene hydroxylase (BaPOHase), however, were increased more than twofold.

Microsome Preparation

Animals were sacrificed by decapitation. Livers and lungs were removed and combined such that each sample contained three livers or

TABLE 1. *Effect of βNF Pretreatment[a] on Rat Lung and Liver Microsomal BaPOHase and ECase Activities[b]*

	Specific activity			
	Lung		Liver	
Activity	CTL	βNF	CTL	βNF
BaPOHase (sp. act.)	8.2 ± 0.97	61.0 ± 11.0[c]	1.6 ± 0.16	2.5 ± 0.27[c]
ECase (sp. act.)	0.15 ± 0.029	0.25 ± 0.048[c]	0.66 ± 0.10	3.0 ± 0.76[c]
Protein (mg/g tissue)	5.2 ± 0.27	4.9 ± 1.0	12.0 ± 2.5	12.0 ± 2.2

[a] Details of βNF pretreatment, preparation of microsomal fractions, enzyme assays, and definitions of specific activity (sp. act.) are in methods section.

[b] Data are averages of four experiments in which each group (CTL or βNF) contained the organs (lung or liver) of three animals.

[c] Data were analyzed by Student's *t*-test, significant at $p \leq 0.05$.

three lungs. Microsomes were prepared by methods previously described (Danner-Rabovsky and Groseclose, 1982). Briefly, washed microsomes were prepared by differential centrifugation of the homogenates and stored at −80°C in 0.05 *M* Tris, pH 7.4, plus 0.25 *M* sucrose until used.

Enzyme Assays

Two P-450 enzyme activities were measured. BaPOHase was assayed by the appearance of 3-hydroxybenzo[a]pyrene (3HOBaP) (Nebert, 1978; Weibel et al., 1977) and ECase by its product, 7-hydroxycoumarin (7HOC) (Greenlee and Poland, 1978). Liver assays were carried out in 0.03 *M* HEPES, pH 7.6, and lung assays in 0.03 *M* HEPES, pH 8.0. The NADPH required in the assay was providing by a generating system that consisted of 2 units glucose-6-phosphate dehydrogenase, 10 μmol each glucose 6-phosphate and $MgCl_2$, and 5 μmol NADP per milliliter of 0.03 *M* HEPES buffer. The reaction mixture was incubated for 10 min at 37°C and then used in the P-450 enzyme assays so that the final NADPH concentration was 3–4 m*M*. Further details of the procedures have been described (Danner-Rabovsky and Groseclose, 1982). NADPH-cytochrome *c* reductase was assayed by the method of Strobel and Dignam (1978).

To accommodate the water-insolubility of the P-450 substrates as well as the alkanes, CH_3OH was used as a carrier solvent in all assays. The sensitivities of BaPOHase and ECase, as well as the differential sensitivities of induced and noninduced activities to organic solvents, have been reported (Weibel et al, 1971; Kawalek and Andrews, 1980). To provide continuity within our study and permit the direct comparison of data, substrates and alkanes were dissolved in CH_3OH and, when

necessary, additional CH_3OH was added to control tubes without alkane. Under these conditions, CH_3OH concentration reached 4–6% (v/v) 0.98–1.5 *M*). In general, the effect of the extra CH_3OH was to decrease the specific activities of liver BaPOHase and ECase and lung ECase by 20–25% and lung BaPOHase by 0–20%.

Total P-450 was analyzed as the dithionite reduced carbon monoxide adduct (Estabrook et al., 1972). Protein was assayed by the method of Lowry et al. (1951) with bovine serum albumin as standard.

Units of activity have been defined as nmoles product formed per minute for liver and lung ECase and liver BaPOHase, and picomoles per minute for lung BaPOHase. Specific activity is defined as units per milligram microsomal protein.

Kinetic Studies

Lineweaver–Burke plots were constructed from substrate concentration-dependence curves (Segal, 1976). We chose to use the same constant conditions throughout the study, because interpretion of kinetic data obtained from microsomal P-450 activities is complicated by membrane concentration (Perry et al., 1976; Backes and Canady, 1981) and solvent (Weibel et al., 1971; Kawalek and Andrews, 1980; Backes and Canady, 1981) effects.

Stock substrate solutions in CH_3OH were prepared, from which equal volumes were used for each experimental substrate concentration. Also, when the effect of alkane on the substrate dependence was measured, an equivalent volume of CH_3OH was added to the tubes where alkane was absent. In this way the concentration of CH_3OH was held constant. Also, to compare kinetic parameters between CTL and βNF liver microsomes, the same concentration of microsomal protein was used.

Materials and Equipment

Chemicals were purchased from the following sources: Tris, HEPES, bovine serum albumin, glucose- 6-phosphate (G6P), and G6P dehydrogenase (Torula yeast, type XII) from Sigma Chemical Co., St. Louis, Mo.; 7-ethoxycoumarin (7EC), 7HOC, benzo[a]pyrene (BaP) (gold label), βNF, and *n*C6, *n*C7, *n*C8, *n*C9, *n*C10, *n*C11, and *n*C12 from Aldrich Chemical Co., Milwaukee, Wis.

3HOBaP was received from the National Cancer Institute's Chemical Carcinogen Reference Standard Respository, a function of the Division of Cancer Cause and Prevention. All other chemicals were reagent grade. Deionized; glass-distilled water was used for all experiments.

A Beckman L3-50 preparative ultracentrifuge with a 60Ti rotor was used for high-speed centrifugation. Routine absorbance measurements were made on a Gilford 2400 spectrophotometer. Fluorescence measurements were made on a Farrand Mark II spectrofluorometer.

Carbon monoxide–dithionite difference spectra of cytochrome P-450 were recorded on a Carey 17D recording spectrophotometer.

RESULTS

Effect of *n*-Alkanes on BaPOHase

When 2 m*M* *n*C6–*n*C12 each were present in the reaction mixtures, the conversion of BaP to 3HOBaP by CTL rat liver microsomes was decreased. The data in Fig. 1 show that the decrease was maximal between seven and nine carbons. The *n*C6 was less effective than *n*C7–*n*C9 and there was a progressive decrease in inhibitory activity from *n*C9 to *n*C12.

The effect of *n*-alkanes on rat liver BaPOHase was further studied by measuring the recovery of 3HOBaP as a function of alkane concentration for *n*C8 and *n*C12. The data in Fig. 2 were obtained with two microsome preparations. In agreement with Fig. 1, *n*C8 effectively prevented the formation of 3HOBaP while *n*C12 did not. With each preparation, a 50% decrease in 3HOBaP production was effected within the 20–200 μ*M* *n*C8 range.

The response of CTL rat lung microsomal BaPOHase to 2 m*M* *n*C6–*n*C12 was much less compared to the liver activity (Fig. 1). The absolute decrease in specific activity was negligible, and no clear trend was observed. When βNF lung microsomes with a sevenfold increase in BaPOHase activity were used, sensitivity to the alkanes was not increased.

Effect of *n*-Alkanes on ECase

At 2 m*M*, *n*C6–*n*C12 caused reduced recovery of the product, 7HOC, when each was present in the reaction with liver microsomes

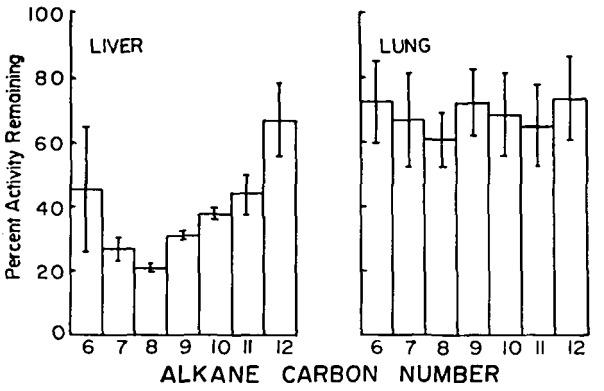

FIGURE 1. Effect of *n*C6–*n*C12 on CTL rat liver and lung microsomal BaPOHase. Alkanes were added at a final concentration of 2 m*M*; 100% activity is the recovery of 3HOBaP in the absence of alkane. Bars are SEM from duplicate experiments. 100% Specific activity: liver, 0.73, 0.95; lung, 9.2, 7.6.

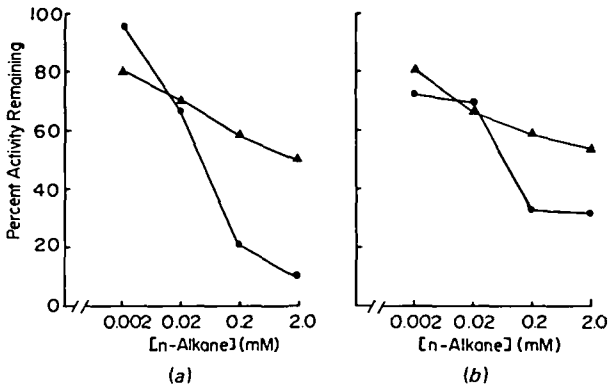

FIGURE 2. Effect of varying alkane concentrations on CTL liver microsomal BaPOHase: ●, *n*C8; ▲, *n*C12. 100% Specific activity refers to the activity in the absence of alkane. (*a*) 100% Specific activity 1.5 nmol/min · mg protein; 21 μg protein in the reaction. (*b*) 100% Specific activity 0.91 nmol/min · mg protein; 20 μg protein in the reaction.

from CTL rats (Fig. 3). Similar to the observation with liver BaPOHase, maximal inhibition appeared with *n*C7–*n*C9. The decreases in activity of ECase as a function of *n*C6, *n*C8, and *n*C12 concentration are shown in Fig. 4. Again, *n*C8 was the most effective alkane compared to *n*C6 or *n*C12, and the sensitive portion of the concentration range occurred between 20 and 200 μ*M*.

To determine the effect of preexposure to βNF on the sensitivity of rat liver ECase to *n*-alkanes, enzyme activity was measured in the presence of 2 m*M* *n*C8 (Table 2). The data show that the presence of 2 m*M*

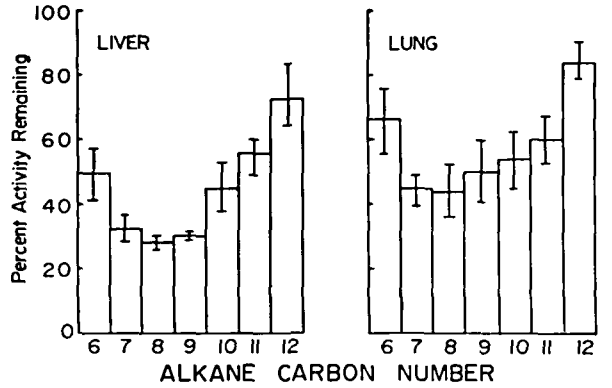

FIGURE 3. Effect of *n*C6–*n*C12 on control (CTL) rat liver and lung microsomal ECase. Alkanes were added at a final concentration of 2 m*M*; 100% activity is the recovery of 7HOC in the absence of alkane. Bars are SEM from triplicate experiments. 100% Specific activity: liver, 0.39, 0.31, 0.52; lung, 0.10, 0.15, 0.14.

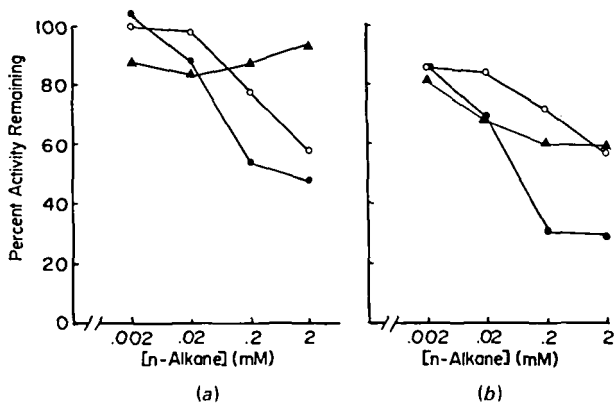

FIGURE 4. Effect of varying alkane concentrations on control liver microsomal ECase: ○, C6; ●, C8; ▲, C12. 100% Specific activity refers to the activity in the absence of alkane. (*a*) 100% Specific activity 1.04 nmol/min · mg protein; 21 μg protein in the reaction. (*b*) 100% Specific activity 0.52 nmol/min · mg protein; 25 μg protein in the reaction.

*n*C8 resulted in less reduction of specific activity when microsomes from βNF-treated rats, with a fourfold increase in specific activity, were used than when microsomes from CTL rats were the source of ECase.

Lung ECase activity was also sensitive to the alkanes (Fig. 3). Although the extent of inhibition of product release was not as great as with liver microsomes, maximum sensitivity occurred with *n*C7–*n*C8. When the alkanes *n*C6, *n*C8, and *n*C12 were tested over a large concentration range, inhibition of 7HOC release was most affected by *n*C8 and the sensitive region occurred between 20 and 200 μ*M* (Fig. 5).

Effect of *n*-Alkanes on Total P-450 Content

Using CTL liver microsomes, total P-450 content was determined by spectral analysis (Estabrook et al., 1972) in the presence and absence of 2 m*M* *n*C8. The presence of *n*C8, the most reactive alkane, did not cause formation of P-420 or reduced P-450 concentration.

TABLE 2. Effect of 2 m*M* *n*C8 on Liver Microsomal ECase Activities in CTL and βNF Rats

	Specific activity			
	CTL		βNF	
Addition	Experiment 1	Experiment 2	Experiment 1	Experiment 2
None	0.50 (100)[a]	0.50 (100)	2.3 (100)	1.3 (100)
2 m*M* *n*C8	0.14 (28)	0.14 (28)	1.7 (73.9)	0.98 (75.4)

[a] Numbers in parentheses are percent activity remaining.

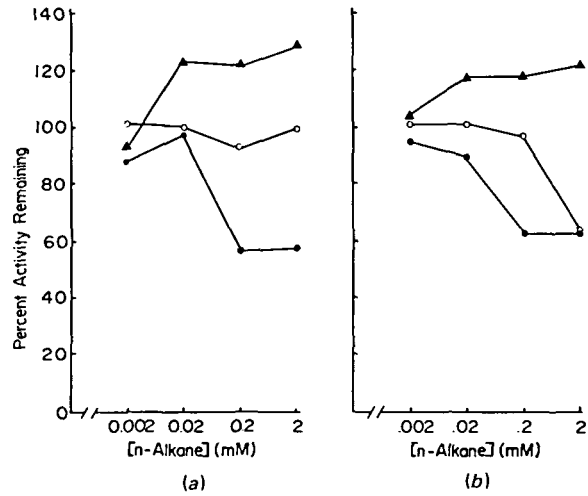

FIGURE 5. Effect of varying alkane concentrations on control lung microsomal ECase: ○, C6; ●, C8; ▲, C12. 100% Specific activity refers to the activity in the absence of alkane. (*a*) 100% Specific activity 0.12 nmol/min · mg protein; 62 μg protein in the reaction. (*b*) 100% Specific activity 0.097 nmol/min · mg protein; 98 μg protein in the reaction.

Kinetic Parameters

To determine if the mechanism of *n*C8 inhibition of P-450-catalyzed formation of 7HOC could be ascertained under the conditions of our experiments, Lineweaver–Burke plots were constructed from substrate concentration curves. Data from ECase were used rather than BaPOHase, because a substantial effect was observed with lung as well as liver and the BaP reaction is complicated by inhibitory properties of some of the reaction products (Keller et al., 1982). We chose *n*C8 because it was the most reactive alkane toward ECase activity.

Examples of data obtained with CTL lung and CTL liver microsomes are given in Fig. 6, and estimated K_m(app) and V_{max} values are given in Table 3. To ensure sufficient activity with lung microsomes, more protein was used in the assay (32.8 μg) compared to the 9 μg used when liver was analyzed. The data show that maximal velocity in the presence of 0.2 m*M* *n*C8 did not return to the value estimated when the alkane was absent. Apparent K_m(app) values remained constant for CTL and βNF liver, whereas the K_m(app) increased in the case of lung microsomes.

Effect of *n*-Alkanes on NADPH Synthesis and Metabolism

Full expression of microsomal P-450 enzyme activity requires the flow of electrons from NADPH to the P-450–substrate complex through cytochrome P-450 reductase (Gillette et al., 1972). When each of the

n-alkanes at 2 m*M* was included in the NADPH generation system, no decreases in NADPH formation were observed under the conditions outlined in the methods section. To determine if the alkanes affected P-450 reductase activity, the enzyme was assayed with the substrate, cytochrome c, in CTL liver and lung. CTL liver was chosen because it was more sensitive toward the alkanes than was βNF liver. Neither the liver nor lung activity was affected by any of the alkanes at 2 m*M*. In the absence of *n*C6–*n*C12, the specific activities were 177 nmol/min · mg protein for CTL liver and 158 nmol/min · mg protein for CTL lung.

DISCUSSION

Effect of *n*-Alkanes

We have shown the alkanes *n*C6–*n*C12 each interfered with P-450-catalyzed product formation in a manner dependent on chainlength. Similar observations have been made by others who studied complex compounds containing sidechains of varying lengths. Control liver microsomes were most active with phenoxazone substrates having short

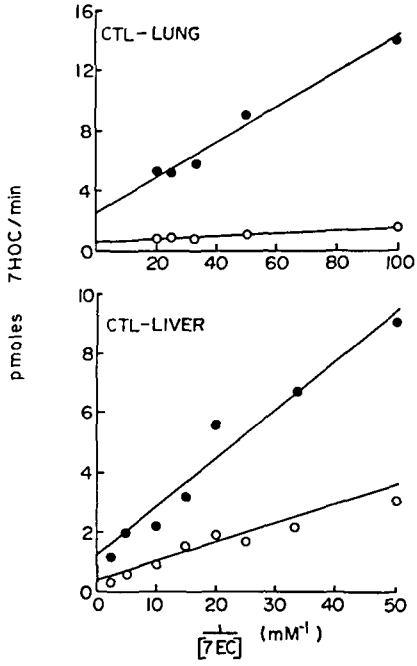

FIGURE 6. Effect of *n*C8 on apparent K_m and V_{max} values: ○, no additions; ●, in the presence of 0.2 m*M* *n*C8. The 0.5-ml reaction contained 32.8 μg CTL lung microsomal protein or 9 μg CTL liver microsomal protein.

TABLE 3. Effect of *n*C8 on K_m(app) and V_{max} of Rat Lung and Liver Microsomal ECase Activity

Tissue	Experiment	K_m(app) (m*M*)		V_{max} (pmol/min)	
		—	+0.2 m*M* *n*C8	—	+0.2 m*M* *n*C8
Lung, CTL[a]	1	0.016	0.047	1.6	0.39
	2	0.011	0.078	2.4	0.91
Liver, CTL[b]	1	0.13	0.15	2.3	0.85
	2	0.15	0.16	2.5	0.99
Liver, βNF[b]	1	0.013	0.016	19.4	13.5
	2	0.012	0.018	6.9	4.2

[a] With 32.8 μg microsomal protein in the assay.
[b] With 9 μg microsomal protein in the assay.

(one to three carbons) alkyl ether sidechains (Burke and Mayer, 1983). Binding parameters obtained with alkylamines (Jefcoate et al., 1969) and alkylbenzimidazoles (Dickens and Bridges, 1982) showed a trend in which maximum binding occurred with a chain length of seven to eight carbons. In our study, we used alkanes of varying chainlengths that were not part of another structure. By using simple alkanes, we were able to avoid potential complexities provided by the nonalkyl moieties that could influence sidechain effects. Our concern was directed at the *n*-alkanes because they are present in contaminated environments.

The effects we and others have observed cannot be a function only of the properties of the alkanes. The chainlength dependence of reactivity toward the alkoxyl resorufins was altered when liver microsomes were obtained from phenobarbitone- or methylcholanthrene-induced mice (Burke and Mayer, 1983). Our data showed the inhibition of rat liver ECase by *n*C8 was reduced in microsomes from rats pretreated with βNF. The inhibition data suggest the liver microsomal ECase activity from βNF-treated rats may reside in an enzyme form different from that found in liver microsomes from control rats. The conclusion is supported by our kinetic data, which showed ECase activity in βNF-liver microsomes could be differentiated from the CTL liver activity by the lower K_m(app) of the former. Using 3-methycholanthrene-treated rats, Guengerich (1978) also found a low K_m(app) for ECase in liver microsomes. The presence of different P-450 forms in animals subjected to a prior exposure may have implications for the health effects of later exposures.

CTL lung microsomal ECase activity was sensitive to *n*-alkanes, as was the CTL liver activity. The similarity, however, disappeared when the K_m(app) values were compared. Although different protein concentrations were used, we believe the conclusion is valid because dissociation constants for binding to P-450 decrease with decreasing protein

concentration (Backes and Canady, 1981). Estimated K_m(app) values for CTL lung and βNF liver microsomal 7ECase activities were similar; however, the enzyme forms were differentiated by their sensitivities towards *n*C8.

Our experiments were based on the decreased recovery of P-450-catalyzed formation of 3HOBaP or 7HOC when each alkane was present in the reaction. Such decreases may be due to a direct effect on enzyme activity or membrane structure or the function of alkanes as alternate substrates. The function of *n*-alkanes as P-450-substrates has been documented (Frommer et al., 1972; Toftgard and Nilsen, 1982), and an alternate substrate would be expected to behave as a competitive inhibitor (Testa and Jenner, 1976). Our data, however, suggest that in the case of *n*C8 inhibition of ECase activity, mechanisms other than *simple* competitive inhibition may be operative. Such mechanisms may involve nonsubstrate binding sites, membrane effects or a non-Michealis enzyme intermediate (Krupka and Laidler, 1961), but the data presented herein do not permit us to distinguish between the possibilities.

In summary, our study has demonstrated that the alkanes *n*C6–*n*C12 (environmental and occupational contaminants) directly affected P-450 activity toward other substrates. Hence not only may the alkanes exert a health effect due to their respective metabolic fates, they may also interfere with the normal metabolic reactions of the tissue. We wish to emphasize the latter point because the pollutants to which people are exposed do not occur as single entities. They occur as components of complex mixtures, and their ability to interfere with normal detoxication reactions should be considered in order to more clearly understand their potential health effects.

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