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# Induction of liver microsomal mixed-function oxidases by volatile hydrocarbons*

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Within the past decade, numerous reports have been concerned with environmental factors which modify drug activity and toxicity in experimental animals. For example, the insecticides DDT and chlordane have an inductive effect on the microsomal drug-metabolizing enzymes, leading to an enhanced oxidation of various drugs in the rat [1, 2]. Ferguson [3] reported a decrease in the hexobarbital and pentobarbital sleeping times in mice housed in red cedar-chip bedding. One year later, Vesell [4] observed a 2- to 3-fold induction of drug-metabolizing enzymes in mice and rats placed on softwood bedding (white pine, red cedar or ponderosa pine); this inductive effect was reversed when the animals were switched to hardwood bedding (maple, birch, beech), or when the red cedar bedding was extracted with hexane. Extraction of cedarwood with diethyl ether resulted in the isolation of cedrol and cedrene, which were the agents responsible for enhanced drug metabolism [5]. Jori *et al.* [6] found that eucalyptol, a component of essential oils, increased *N*-demethylation of aminopyrine, *O*-demethylation of *p*-nitroanisol and *para*-hydroxylation of aniline in female rats.

Since our laboratory is concerned with mixed-function oxidation reactions and since a new animal room deodorizing agent (TRAIL†) is becoming widely used and is being considered in our animal care facilities, studies were designed to determine whether the constituents of this agent had any inductive effects on the liver microsomal mixed-function oxidase system of rats and rabbits, thereby subsequently altering drug metabolism and modifying drug activity and toxicity.

*Animals.* Male ARS Sprague–Dawley rats (125–150 g) from Madison, Wis. and male New Zealand white rabbits (1·5 to 2·0 kg) from Ancare, Long Island were used in the experiments. All animals had free access to Purina laboratory chow and tap water at all times. The animals were housed in stainless steel suspended wire-bottom cages; underneath each cage was a waste product collection tray. In the experimental groups, 4 oz. of the deodorizing agent concentrate, TRAIL, was diluted with 1 gallon of water and placed into each collection tray to a depth of one-half inch, as suggested by the manufacturer. The diluted concentration of TRAIL was 50 per cent of the manufacturer's recommended usage. The control groups were housed in different animal rooms in the absence of the deodorizing agent. The solution was changed every other day throughout the experimental period. Ventilation in all rooms resulted in 14 complete air changes per hr. Exposure of the experimental animals to the deodorizing agent was for periods of 1, 2, 4, 7 and 14 days. No deaths were observed throughout the periods of exposure. At the end of each period, both control and experimental rabbits and rats were sacrificed by decapitation.

Rats were also given intraperitoneally various quantities of α-terpineol or isobornyl acetate, two volatile components of TRAIL. These two volatile hydrocarbons were generous gifts of Pharmacal Research Laboratories. Control groups were injected with equivalent volumes of warmed isotonic NaCl.

In Tables 1 and 2, the control groups consisted of a total of 15 rats, 3 from each of the days of treatment. Since the values of the control rats used for days 1, 2, 4, 7 and 14 did not differ significantly from one another, they were combined and represent the means for 15 rats.

*Preparation of microsomes.* After decapitation, livers were removed and treated as described previously [7]. The livers were homogenized in 5 vol. of cold 0·25 M sucrose with a glass–Teflon homogenizer; microsomal fractions were prepared by the $CA^{++}$-sedimentation procedure described previously [8]. Identical results were obtained from microsomes prepared by the classical differential ultracentrifugation method [9].

*Enzyme assays.* Each drug-metabolizing reaction mixture contained the following components: Tris–HCl buffer (0·2 M), pH 7·5, $NADP^+$ (0·4 mM), isocitric acid (8·0 mM), $MgCl_2$ (5·0 mM), isocitric dehydrogenase (15 μg/ml, Sigma Type IV), microsomal protein (1 mg/ml) and either ethylmorphine or aminopyrine (8·0 mM) in a final volume of 3·0 ml. Incubations were carried out aerobically with shaking at 37° for 8 min in a Dubnoff metabolic shaker.

Aminopyrine and ethylmorphine *N*-demethylation were determined from formaldehyde production according to Schenkman *et al.* [10]. Formaldehyde standard was carried throughout the procedures with each assay and permitted quantification of the enzymic activities.

Cytochromes $b_5$ and P-450 were measured with the Aminco DW-2 dual wavelength recording spectrophotometer as described by Omura and Sato [11]. Spectral changes produced by the addition of substances to suspensions of liver microsomes were also recorded on the same spectrophotometer. NADPH cytochrome *c* reductase activity was measured by the method of Phillips and Langdon [12], whereas NADPH cytochrome P-450 reductase activity was measured as described previously [13]. Protein concentrations were determined by the method of Lowry *et al.* [14]. Lineweaver–Burk kinetic plots [15] were determined by the method of least squares.

$NADP^+$, trisodium isocitrate, isocitric dehydrogenase and cytochrome *c* were purchased from Sigma Chemical Co. Aminopyrine was obtained from Aldrich Chemical Co. and ethylmorphine from Mallinckrodt Chemical Works.

Based on earlier reports that the drug-metabolizing enzymes in liver microsomes of mice and rats were induced by softwood bedding [4, 5], we determined whether the mixed-function oxidase system of rats and rabbits housed in animal quarters treated with a new deodorizing agent (TRAIL) was affected by this agent. Male rats were placed in quarters which contained the deodorizing agent for a period ranging from 1 day to a maximum of 14 days. A number of hepatic microsomal parameters were studied after 1, 2, 4, 7 and 14 days of treatment. As seen in Table 1, a statistically significant increase in the content of both cytochromes P-450 and $b_5$ occurred after 4 days of exposure to the volatile agent. The cytochrome P-450 content was increased to 0·56 nmole/mg of microsomal protein (33 per cent stimulation); similarly, the cytochrome $b_5$ content was stimulated 34 per cent after 4 days. After 14 days of treatment, a further increase in the content of both hemo-proteins (P-450 and $b_5$) was observed, reaching 95 and 57 per cent respectively. Interestingly, the electron-transferring flavoprotein, NADPH cytochrome c reductase, did not show a significant increase in activity until the rats were exposed to the agent for 7 days. Similarly, both aminopyrine and ethylmorphine demethylation activities were enhanced only after 7 days of exposure. Furthermore, the percentage increase in drug-metabolizing enzyme activities paralleled the flavoprotein reductase activity

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† TRAIL is the trade name of Pharmacal Research Laboratories, Greenwich, Conn.

Table 1. Effect of treatment of animal care rooms with a volatile hydrocarbon-containing disinfectant agent on some hepatic microsomal parameters in male rats*

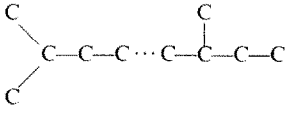
| Parameters | Controls | Days of treatment 1 | 2 | 4 | 7 | 14 |
|---|---|---|---|---|---|---|
| Cytochrome $b_5$ content† | 0·35 ± 0·02 | 0·40 ± 0·04 | 0·45 ± 0·03 | 0·47 ± 0·02‡ | 0·52 ± 0·02‡ | 0·55 ± 0·02§ |
| Cytochrome P-450 content† | 0·42 ± 0·04 | 0·46 ± 0·03 | 0·51 ± 0·04 | 0·56 ± 0·02‡ | 0·76 ± 0·03§ | 0·82 ± 0·03¶ |
| NADPH cytochrome *c* reductase• | 49 ± 2 | 48 ± 2 | 55 ± 3 | 52 ± 4 | 74 ± 3§ | 79 ± 4§ |
| Aminopyrine *N*-demethylase** | 3·4 ± 0·5 | 3·7 ± 0·3 | 3·3 ± 0·5 | 4·2 ± 0·4 | 4·9 ± 0·2‡ | 5·6 ± 0·1§ |
| Ethylmorphine *N*-demethylase** | 3·5 ± 0·3 | 3·4 ± 0·4 | 3·6 ± 0·5 | 4·5 ± 0·2 | 5·4 ± 0·2§ | 5·7 ± 0·1¶ |

* Procedures and assays were as described in Methods. Values represent means ± S. E. for eight animals; control values are for 15 rats (three per day of treatment; no significant differences were found between control groups).
† nmoles per mg of microsomal protein.
‡ $P < 0·05$.
§ $P < 0·01$.
¶ $P < 0·001$.
• nmoles cytochrome *c* reduced per mg of microsomal protein per min.
** nmoles formaldehyde formed per mg of microsomal protein per min.

through 14 days of treatment. The NADPH cytochrome P-450 reductase activity measured in two control rats was 4·4 and 5·1 nmoles P-450 reduced/mg of microsomal protein/min (three measurements per rat), increasing to 6·8 and 5·9 nmoles/mg/min in two rats exposed to the agent for 4 days. Averaging the two values in each group showed a 34 per cent increase in reductase activity similar to that observed with cytochrome P-450. After 14 days of treatment, the reductase activity had increased to 84 per cent above the control activity (8·1 nmoles/mg/min).

When rabbits were similarly treated with the same agent (Table 2), none of the hepatic microsomal parameters measured differed significantly from those of the controls. Even after 14 days of treatment, neither cytochrome P-450 content nor that of its reductase differed from control values. Surprisingly, rabbit hepatic microsomal ethylmorphine *N*-demethylase activity was only one-fourth the aminopyrine demethylation activity. Bend *et al.* [16] have reported a 2-fold difference between *N*-demthylation of aminopyrine (6·6 nmoles/mg/min) and ethylmorphine (3·5 nmoles/mg/min); however, when the absolute activities reported by Bend *et al.* [16] were compared with our values, their aminopyrine and ethylmorphine demethylation activities were 3- and 7-fold higher respectively. Gillette and Gram [17] reported, in male rabbits, a microsomal ethylmorphine *N*-demethylase activity of 4·0 nmoles/min/mg. However, Holtzman *et al.* [18] did obtain ethylmorphine demethylase values (0·85 nmole/min/mg of microsomal protein) similar to ours, using smooth microsomes from adult male rabbits. Comparing NADPH cytochrome *c* reductase activity in control rabbits, we found only 33 nmoles cytochrome *c* reduced per min per mg of microsomal protein, whereas Bend *et al.* [16] and Gillette and Gram [17] observed approximately five times higher activity. However, the cytochrome P-450 content reported [16, 17] was not different from our value of about 1·2 nmoles/mg of protein. Hence the demethylase activity appears to parallel the reductase activity.

The agent (TRAIL) which was exposed to the rats and rabbits was found to contain three volatile hydrocarbons, two of which belong to a class of organic compounds designated "terpenes," a term referring strictly to hydrocarbons of the composition $C_{10}H_{16}$. The two terpenes (Fig. 1), α-terpineol and isobornyl acetate, are monocyclic and bicyclic compounds, respectively, whose carbon skeletons are constructed of two isoprene units, joined in the following way:



Both compounds apparently interact with the hepatic microsomal mixed-function oxidase system, as evidenced by the type I spectral change obtained upon addition of each terpene to a microsomal suspension (Fig. 1). This was not surprising, since camphor, a terpene closely related to isobornyl acetate, is a well known substrate of the bacterial (*Pseudomonas putida*) cytochrome P-450 ($P\text{-}450_{cam}$) enzyme system [19, 20].

If indeed both α-terpineol and isobornyl acetate are substrates of the hepatic microsomal mixed-function oxidase system, then a competitive inhibition should be observed when two substrates of the enzyme system are simultaneously present. Such an inhibition is depicted in Figs. 2 and 3. Both isobornyl acetate and α-terpineol increase the apparent $K_m$ without affecting the $V_{max}$(4·8 nmoles/min/mg of microsomal protein). In the absence of either terpene, the apparent $K_m$ for aminopyrine *N*-demethylation was 0·29 mM; in the presence of 100 μM isobornyl acetate, the apparent $K_m$ increased to 0·63 mM (Fig. 2), while in the presence of 110 μM α-terpineol a 5-fold increase in

Table 2. Effect of treatment of animal care rooms with a volatile hydrocarbon-containing disinfectant agent on some hepatic microsomal parameters in rabbits*

| Parameters | Controls | Days of treatment 1 | 2 | 4 | 7 | 14 |
|---|---|---|---|---|---|---|
| Cytochrome $b_5$ content† | 0·66 ± 0·03 | 0·69 ± 0·04 | 0·62 ± 0·05 | 0·73 ± 0·06 | 0·59 ± 0·06 | 0·57 ± 0·06 |
| Cytochrome P-450 content† | 1·17 ± 0·13 | 1·48 ± 0·15 | 1·02 ± 0·09 | 1·42 ± 0·16 | 0·93 ± 0·18 | 1·49 ± 0·18 |
| NADPH cytochrome *c* reductase‡ | 33 ± 4 | 25 ± 5 | 28 ± 4 | 36 ± 6 | 25 ± 6 | 35 ± 5 |
| Aminopyrine *N*-demethylase§ | 1·9 ± 0·2 | 1·9 ± 0·2 | 2·0 ± 0·2 | 1·4 ± 0·4 | 1·6 ± 0·3 | 1·7 ± 0·3 |
| Ethylmorphine *N*-demethylase§ | 0·52 ± 0·03 | 0·55 ± 0·01 | 0·47 ± 0·04 | 0·51 ± 0·03 | 0·45 ± 0·02 | 0·52 ± 0·03 |

* Procedures and assays were as described in Methods. Values represent means ± S. E. for six animals; control values are for 15 animals (three per day of treatment; no significant differences were found between control groups).
† nmoles per mg of microsomal protein.
‡ nmoles cytochrome *c* reduced per mg of microsomal protein per min.
§ nmoles formaldehyde formed per mg of microsomal protein per min.

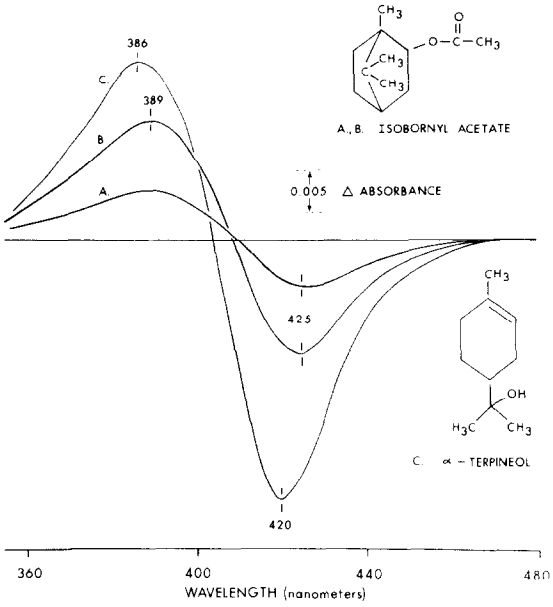



Fig. 1. Isobornyl acetate- and α-terpineol-induced type I spectral changes in control rat liver microsomes. Liver microsomes from adult male rats were suspended to 2·7 mg protein/ml in 0·1 M Tris–HCl (pH 7·4) and were distributed between two cuvettes; the cytochrome P-450 concentration was 1·13 μM. Curve A: 10 μl isobornyl acetate (1·7 mM, final concentration) was added to the sample cuvette, while an equivalent volume of Tris buffer was added to the reference; curve B: 20 μl isobornyl acetate (3·4 mM, final concentration); curve C: addition of 10 μl α-terpineol (1·7 mM, final concentration) to a new suspension of microsomes containing 2·7 mg protein/ml. Both terpene compounds are similar in structure in that, excluding the acetate moiety, α-terpineol is an isomer of isoborneol.

the $K_m$ (1·4 mM) was observed. These data suggest that α-terpineol has a greater affinity for the microsomal enzyme system than does isobornyl acetate. This is also suggested by the data in Fig. 1 in which curve A represents the type I spectral change that follows the addition of 1·7 mM isobornyl acetate and curve C represents the same spectral change obtained with the same concentration of α-terpineol.

In an attempt to determine which volatile component of the commercial product was enhancing the content of cytochrome P-450 and the activities of NADPH cytochrome *c* reductase and *N*-demethylase, various concentrations of each terpenoid compound were injected intraperitoneally for 3 days (Table 3). Surprisingly, rats treated with α-terpineol did not have higher aminopyrine or ethylmorphine *N*-demethylase activities; instead, there was a tendency toward lower cytochrome $b_5$ and cytochrome P-450 content and decreased *N*-demethylase activities. Increasing the administered dose of α-terpineol above 80 mg/100 g of body weight resulted in the death of the animal. On the other hand, a 100-mg dose of isobornyl acetate per 100 g of body weight caused a 2·5-fold increase in cytochrome P-450 content, a 2·6-fold increase in both *N*-demethylase activities and a 2·0-fold increase in NADPH cytochrome c reductase activity. Further evidence suggesting that isobornyl acetate is responsible for the induction of the microsomal mixed-function oxidase system was provided when a solution of TRAIL, with isobornyl acetate left out, was injected intraperitoneally for 3 days at concentrations ten times the manufacturer's recommended use; both the mixed-function oxidase activities and the cytochrome P-450 content did not differ from those seen in untreated rats.

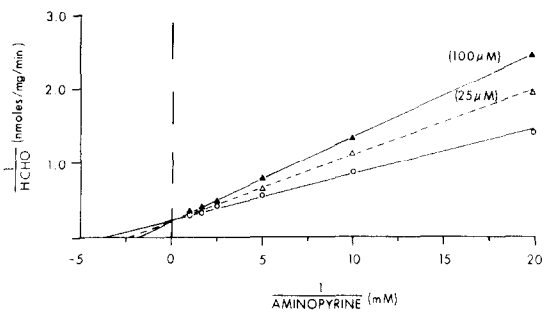



Fig. 2. Effect of isobornyl acetate addition on aminopyrine *N*-demethylation. Lineweaver–Burk plot of the enzyme activity in the presence of 25 μM, 100 μM or no isobornyl acetate. Both the inhibitor and substrate were present in the assay medium, which was preincubated for 5 min at 37° to generate NADPH from 0·5 mM $NADP^+$. The reaction was initiated by addition of microsomes (1 mg/ml) to the medium and the incubation was continued for 8 min at 37°. Activity is expressed as nmoles HCHO per min per mg of microsomal protein. The $K_m$ (mM) values in the presence of 0, 25 and 100 μM isobornyl acetate were 0·29, 0·48 and 0·63, respectively, as determined by the method of least squares. The $V_{max}$ was 4·8 nmoles/mg/min.

The third volatile hydrocarbon component of TRAIL, isopropanol, had no inductive effects when administerd intraperitoneally at concentrations ranging from 10–200 mM; when isopropanol was omitted from the TRAIL solution, induction still occurred. The concentration of isopropanol in TRAIL after dilution was 25 mM.

The results reported here suggest that at least one of the volatile hydrocarbons, isobornyl acetate, induces the hepatic microsomal mixed-function oxidase system. This is not surprising, since Vesell [4] had suggested the possibility of enzyme induction by terpenoid compounds. Wade *et al.* [5] showed that two volatile hydrocarbon constituents of cedarwood, cedrene and cedrol, were effective inducers of microsomal enzymes via the inhalation route of administration. The fact that α-terpineol did not induce the mixed-function oxidase system is, however, rather surprising, since α-terpineol is simply an isomer of isoborneol, the latter obtained by cleavage of the acetate ester. Furthermore, Jori *et al.* [6] reported that eucalyptol (cineole), another isomer of α-terpineol, administered via the inhalation route was found to increase the metabolism of amino-

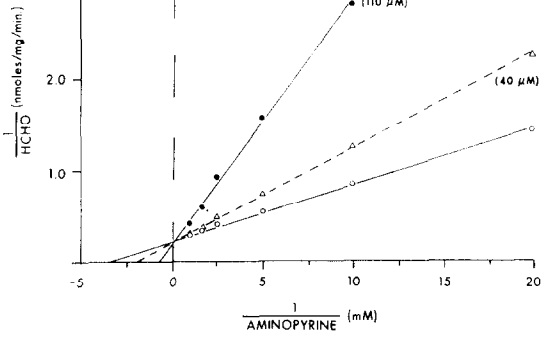



Fig. 3. Effect of α-terpineol addition on aminopyrine *N*-demethylation. Lineweaver–Burk plot of the enzyme activity in the presence of 40 μM, 110 μM or no α-terpineol. Conditions were (and activity was expressed) as in Fig. 2. The $K_m$ (mM) values in the presence of 0, 40 and 110 μM α-terpineol were 0·29, 0·36 and 1·42 mM, respectively, as determined by the method of least squares. The $V_{max}$ was 4·8 nmoles/mg/min.

Table 3. Effects of intraperitoneal administration of α-terpineol and isobornyl acetate on the induction of the liver mixed-function oxidase system in male rats*

| Parameters | Control | α-Terpineol | | Isobornyl acetate | |
|---|---|---|---|---|---|
| | | A | B | C | D |
| Cytochrome $b_5$ content† | 0·31 ± 0·03 | 0·16 ± 0·04 | 0·15 ± 0·04 | 0·58 ± 0·03‡ | 0·58 ± 0·04‡ |
| Cytochrome P-450 content† | 0·40 ± 0·04 | 0·42 ± 0·05 | 0·29 ± 0·03 | 0·67 ± 0·04‡ | 0·97 ± 0·05§ |
| Aminopyrine *N*-demethylase | 4·1 ± 0·4 | 3·0 ± 0·5 | 2·6 ± 0·6 | 6·6 ± 0·3§ | 10·7 ± 0·5§ |
| Ethylmorphine *N*-demethylase‖ | 4·5 ± 0·3 | 3·8 ± 0·3 | 3·2 ± 0·3 | 7·1 ± 0·5§ | 11·5 ± 0·6§ |
| NADPH cytochrome *c* reductase¶ | 43 ± 2 | 42 ± 2 | 46 ± 3 | 61 ± 3** | 86 ± 3§ |

* Each group of four male rats was injected with various concentrations of α-terpineol or isobornyl acetate for 3 days. A and B 40 and 80 mg α-terpineol/100 g of body weight respectively; C and D, 50 and 100 mg isobornyl acetate/100 g of body weight. Values represent mean ± S. E.

† nmoles per mg of microsomal protein.

‡ $P < 0{\cdot}01$.

§ $P < 0{\cdot}001$.

‖ nmoles formaldehyde formed per mg of microsomal protein per min.

¶ nmoles cytochrome *c* reduced per mg of microsomal protein per min.

** $P < 0{\cdot}05$.

pyrine, *p*-nitroanisol and aniline *in vitro*. Although, α-terpineol does not induce the mixed-function oxidase system, it interacts with the latter, as evidenced by the intense type I spectral change and the competitive inhibition of aminopyrine *N*-demethylation.

Based on these results and others, once again, one cannot over-emphasize the importance of the controlled chemical-free environment in which experimental animals are housed. Without such controls, data obtained from studies concerning kinetics of drug metabolism, induction of the enzyme system by various xenobiotics, the mechanism of induction, and the purification and reconstitution of the drug-metabolizing enzyme system would be meaningless.

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## Perifused fat cells—Kinetic analysis of epinephrine-stimulated lipolysis

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Many reports have appeared demonstrating that epinephrine increases the rate of hydrolysis of triglyceride in adipose tissue, presumably by activation of triglyceride lipase [1, 2]. Recently, a perifused fat cell system has been described which allows for continuous monitoring of the changes in lipolytic activity in isolated fat cells and provides a technique by which the kinetics of these changes can be observed [3]. Based on results from this system, a kinetic model has been constructed which describes the increase in lipolytic rates after the addition of epinephrine ($10^{-5}$ M final concentration) and the decrease in lipolytic rates after cessation of the hormone.

It was assumed that sufficient triglyceride exists within the cell to completely saturate the lipase enzymes. Based on this assumption, the rate of glycerol production was taken to be proportional to the amount of activated lipase present in the fat cells. One unit of lipase enzyme was defined as that amount which produced 1 nmole glycerol/