MOLECULAR PHARMACOLOGY, 7, 26–32

Effects of Extraction with Isooctane upon the Properties of Liver Microsomes

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(Received August 5, 1970)

SUMMARY

When rat liver microsomes were extracted with isooctane (2,2,4-trimethylpentane), the resultant preparation was essentially devoid of the ability to produce a type I difference spectrum upon addition of hexobarbital. At high concentrations of hexobarbital, a type II spectral change was obtained with the extracted microsomes. The type II interaction of aniline with isooctane-extracted microsomes was not enhanced in the presence of hexobarbital, as it was in the case of untreated microsomes. Hexobarbital binding to cytochrome P-450 of untreated microsomes was inhibited in the presence of isooctane, but the amount of residual isooctane in the extracted microsomes was not sufficient to explain the loss of hexobarbital-binding activity. The isooctane extracts contained phosphatidylcholine and phosphatidylethanolamine. The results are discussed in terms of a model in which the type I binding form of cytochrome P-450 involves an association of hemoprotein with a phospholipid, or with some other microsomal constituent in an interaction involving phosphatides.

INTRODUCTION

In recent years a number of studies have shed light on the lipid requirements of microsomal mixed-function oxidase and related enzyme systems. Thus, a phospholipid requirement has been demonstrated for the NADH–cytochrome *c* reductase (1) and the stearyl coenzyme A desaturase (2) of avian liver microsomes and the UDP-glucuronyltransferase of guinea pig liver microsomes (3). A number of microsomal reductases and dehydrogenases were found to be inactivated following enzymatic hydrolysis of microsomal phospholipids (4). The "solubilized" liver microsomal hydroxylating system of Coon and Lu has been shown to require an unidentified lipid fraction for fatty acid ω-hydroxylation (5) or for drug oxidations (6). Shoeman *et al.* (7) demonstrated that after digestion of rat liver microsomes with steapsin, much of the hexobarbital-binding activity of the microsomes was lost whereas the aniline-binding activity was increased. Similar results were seen after treatment with phospholipase C, which hydrolyzed much of the phosphatidylcholine and phosphatidylethanolamine of the liver microsomes (8).

In an attempt to solubilize the mixed-function oxidase systems of liver microsomes,

This work was supported in part by United States Public Health Service Grants GM-16488 (R. W. E.) and EC-00225 (K. C. L.) A brief preliminary report of some of the data has appeared [*Pharmacologist* 11, 250 (1969)].

[1] Much of this work was carried out during the tenure of a University of Florida Faculty Development Grant and United States Public Health Service Special Fellowship GM-15266.

Tagg and Mitoma (9) extracted microsomes with isooctane. Although they were unsuccessful in achieving solubilization of active enzyme systems, these workers found that the extracted microsomes were deficient in demethylating activity toward aminopyrine and in hydroxylating activity for hexobarbital. The aromatic hydroxylating activity of these extracted microsomes, however, was the same as or higher than that of the untreated preparations.

It was interesting to note that those substrates (hexobarbital and aminopyrine) toward which the oxidizing activities of the liver microsomes were decreased after extraction with isooctane are those which produce a characteristic type I difference spectrum (10, 11) upon addition to microsomes. Those substrates (aniline and acetanilide), on the other hand, whose hydroxylation was unchanged or enhanced by isooctane extraction are those which yield type II difference spectra. We therefore decided to determine what effect extraction with isooctane would have upon the binding of substrates of these types to microsomal hemoprotein, as measured spectrophotometrically.

MATERIALS AND METHODS

Male Holtzman rats (200–250 g, body weight) that had been treated by injection with sodium phenobarbital (50 mg/kg/day) for 3 days were used as sources of liver microsomes. Animals were killed 24 hr after the final dose of phenobarbital and 12 hr after withdrawal of food.

Microsomes, prepared as previously described (10), were suspended in a buffer mixture containing 50 mM Tris-chloride, pH 7.4, in 0.25 M sucrose to give a protein concentration of 30 mg/ml. Batches were stirred for 20 min in an ice bath with ⅔ volume of isooctane (2,2,4-trimethylpentane). The resulting emulsion was centrifuged at 80,000 × *g* for 30 min. The supernatant isooctane layer, interface material, and aqueous layer were removed, and the sediment was resuspended in half the original volume of Tris–sucrose buffer.

Spectrophotometric studies were performed as previously described (8, 9) with an Aminco-Chance double-beam recording spectrophotometer. Lineweaver-Burk diagrams were constructed by plotting the reciprocal of the sum of the amplitudes of the peak and trough of the difference spectra (ΔA) against the concentration of the substrate. When the effect of one substance, called the modifier, upon the binding of another substance, called the substrate, was to be determined, the modifier was placed in both reference and sample cuvettes with the microsomes and buffer, and, after recording of a baseline, successive increments of the substrate were added to the sample cuvette.

Cytochromes b_5 and P-450 were determined as described by Omura and Sato (12). Protein was estimated by a biuret procedure (13). Acetanilide hydroxylase and aminopyrine demethylase activities were measured as previously described (14).

The concentration of residual isooctane in extracted microsomes was determined by gas chromatography in ether extracts on 3% SE-30 columns at 35°, using a hydrogen flame ionization detector at 100°. Similar extracts of untreated microsomes, to which known amounts of isooctane had been added, provided the reference samples. The chromatographic peaks were symmetrical, and occurred in an area that was devoid of peaks in chromatograms of extracts of untreated microsomes to which no isooctane had been added.

RESULTS

Some of the properties of the untreated and isooctane-extracted microsomes are shown in Table 1. About 80% of the protein was lost in the extraction operation. In this experiment, approximately equal proportions of cytochromes b_5 and P-450 of the microsomes were also lost, so that the relative contents of these hemoproteins per milligram of protein were not changed. The K_s and ΔA_{max} values for aniline were calculated from the Lineweaver-Burk plots constructed from spectrophotometric titration data. The apparent dissociation constant was the same in untreated and extracted microsomes, but the maximal absorbance change at infinite aniline concentration was greater with extracted microsomes than with untreated microsomes.

TABLE 1

Characteristics of untreated and isooctane-extracted microsomes

The microsomal suspension (20 ml, 30 mg of protein per milliliter) was extracted as described in MATERIALS AND METHODS.

Microsomes	Protein	Cytochrome b_5		Cytochrome P-450		Aniline binding		
		Amount	Activity	Amount	Activity	K_s	ΔA_{max}/mg protein	ΔA_{max}/nmole cytochrome P-450
	mg	*nmoles*	*nmole/mg protein*	*nmoles*	*nmoles/mg protein*	*mM*		
Untreated	600	342	0.57	1280	2.13	1.25	0.027	0.013
Extracted	113	59	0.52	247	2.19	1.25	0.044	0.020

The relationship of hexobarbital binding in the two preparations, however, was quite different. Figure 1 shows the typical type I difference spectrum produced by hexobarbital in untreated microsomes, with a peak at about 390 nm and a trough at about 425 nm. With the isooctane-extracted preparations, however, a concentration of hexobarbital that produced an appreciable spectral change in the untreated microsomes yielded only a barely detectable type I difference spectrum. As hexobarbital concentrations were increased, the difference spectrum changed in character, and eventually, when hexobarbital concentrations were quite high (12.5 mM), the spectral change was similar to those called type II spectra.

We have indicated (15) that when a type I compound is used as a modifier, there is an enhancement in the binding affinity of type II substrates for microsomal cytochrome P-450. As shown in Fig. 2, the amplitudes of the difference spectra produced when suboptimal concentrations of aniline were added to untreated microsomes were progressively increased in the presence of increasing concentrations of hexobarbital, up to a certain point. Maximal stimulation of aniline binding occurred at about 2 mM hexobarbital, after which higher concentrations of hexobarbital began to inhibit aniline binding. Figure 3 shows that in isooctane-extracted microsomes the binding of aniline was, if anything, slightly inhibited in the presence of concentrations of hexobarbital that caused marked enhancement in unextracted microsomes. In neither preparation was the maximum binding capacity for aniline altered, but the apparent value of K_s was changed when hexobarbital was present.

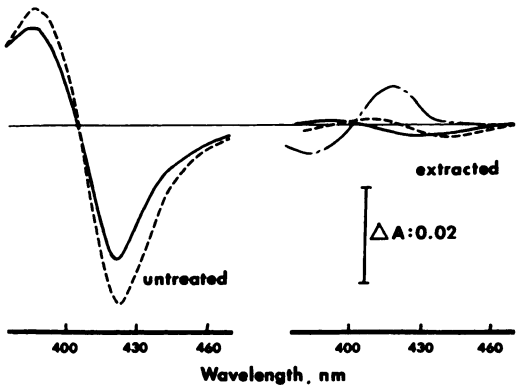

FIG. 1. *Difference spectra recorded after addition of hexobarbital to untreated and isooctane-extracted microsomes*

Microsomes from phenobarbital-treated rats were suspended (1 mg of protein per milliliter) in a medium 50 mM with respect to Tris buffer, pH 7.4, and 15 mM with respect to KCl. Hexobarbital concentrations were 0.8 mM (——), 4.9 mM (- - -), and 12.5 mM (-·-·).

Figure 4 shows that isooctane produces a difference spectrum of type I when added to liver microsomes. The positions of the peak and trough were 387 and 422 nm, respectively. When isooctane was used as a modifier and hexobarbital as substrate, it was found that isooctane affected the binding of hexobarbital to microsomal cytochrome P-450 in a manner analogous to that of a noncompetitive inhibitor of an enzyme-substrate reaction (Fig. 5). The concentration that caused 50% inhibition of hexobarbital binding was 2.6 mM.

The effect of addition of isooctane *in vitro* upon the activity of drug-oxidizing enzyme systems of liver microsomes was tested. As shown in Table 2, isooctane caused no inhibition, under these circumstances, of either aminopyrine demethylase or acetanilide hydroxylase, when added at concentrations up to 10 mM.

The results of thin-layer chromatography of the isooctane extracts are presented in

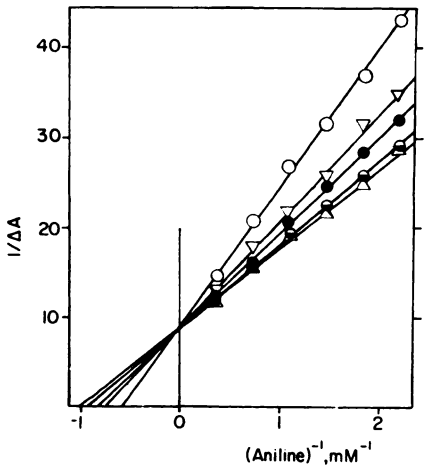

FIG. 2. *Effect of hexobarbital upon binding of aniline to hemoprotein of untreated microsomes*

Microsomes (1 mg of protein per milliliter) from phenobarbital-treated rats were suspended in Tris-KCl buffer as described in Fig. 1. Hexobarbital concentrations were: ○, none; ▽, 0.2 mM; ◒, 0.8 mM; △, 2.1 mM; ●, 4.2 mM.

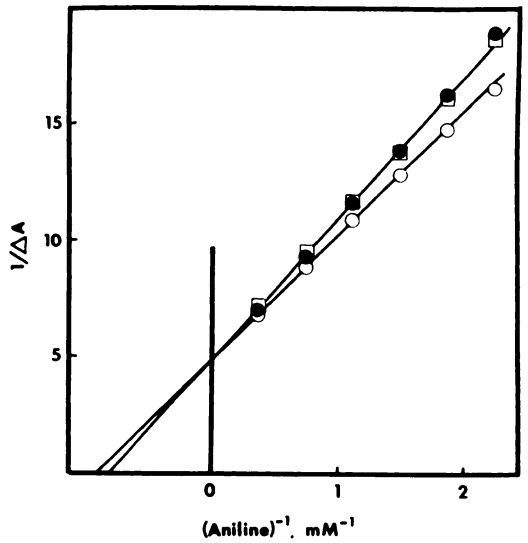

FIG. 3. *Effect of hexobarbital upon binding of aniline to hemoprotein of isooctane-extracted microsomes*

The cuvettes contained 0.67 mg of protein per milliliter in Tris-KCl buffer, as in Fig. 1. Hexobarbital concentrations were: ○, none; ●, 0.2 mM; □, 0.8 mM.

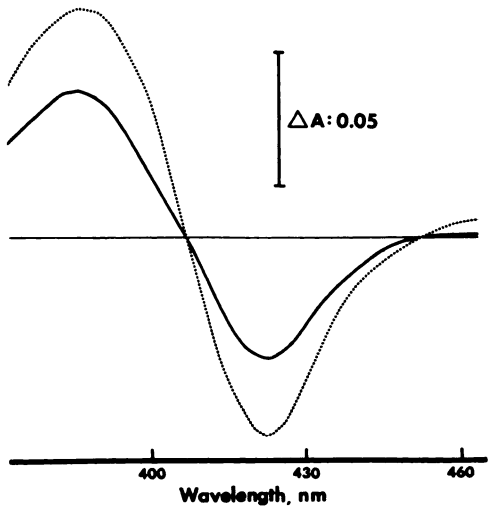

FIG. 4. *Difference spectra recorded after addition of isooctane to rat liver microsomes*

The protein concentration was 2 mg/ml, and the buffer was Tris-KCl as in Fig. 1. Isooctane was added in a maximum of 8 μl of absolute ethanol; equivalent volumes of ethanol were added to the reference cuvette. Final isooctane concentrations: ——, 1 mM; ····, 8 mM.

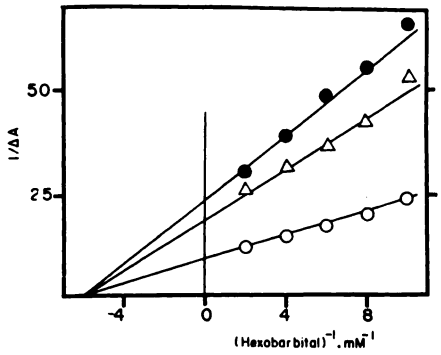

FIG. 5. *Effect of isooctane upon binding of hexobarbital by hemoprotein of unextracted microsomes*

The microsomal protein concentration was 2 mg/ml, and the buffer was Tris-KCl as in Fig. 1. Isooctane was added to reference and experimental cuvettes in up to 5 μl of absolute ethanol. Isooctane concentrations: ○, none; △, 2.5 mM; ●, 5 mM.

TABLE 2

Effect of isooctane upon microsomal hydroxylations

The enzyme source was the 9000 × *g* supernatant fraction of liver homogenates from phenobarbital-treated rats. Isooctane was introduced in 20 μl of absolute ethanol. A similar volume of ethanol was added to the control flasks.

Isooctane concentration	Aminopyrine *N*-demethylation	Acetanilide hydroxylation
M	*% control*	*% control*
10^{-7}	100	105
10^{-6}	97	97
10^{-5}	97	100
10^{-4}	97	103
10^{-3}	88	111
10^{-2}	107	121

TABLE 3

Thin-layer chromatography of isooctane extract of liver microsomes

Chromatography was performed on silica gel with a neutral solvent system (chloroform–methanol–water, 65:26:4 by volume) or a basic system (chloroform–methanol–40% aqueous methylamine, 65:26:8 by volume). After drying, the neutral chromatogram was sprayed with 0.1% ninhydrin in 50% aqueous acetone and heated for 5 min at 100°. Both chromatograms were then sprayed with the modified Zinzadze reagent for phospholipids (16), and finally with 50% sulfuric acid. All spots not otherwise indicated were visualized with the Zinzadze phospholipid reagent.

Phospholipid	Neutral system		Basic system	
	Reference	Extract	Reference	Extract
	R_F	R_F	R_F	R_F
		0.95[a]		0.97[a]
Phosphatidylethanolamine	0.68[b]	0.57[b]	0.82	0.80
Phosphatidylcholine	0.28	0.29	0.63	0.63
		0.19		0.30
		0.04[a]		0.02[a]
Lysophosphatidylcholine	0.07		0.34	
Lysophosphatidylethanolamine	0.21[b]		0.49	
Phosphatidylserine	0.23[b]		0.40	
Sphingomyelin	0.18		0.47	

[a] H_2SO_4-positive.

[b] Ninhydrin-positive.

Table 3. After development with neutral or alkaline solvent systems, spots were found corresponding to phosphatidylcholine and phosphatidylethanolamine. No indication of the presence of a number of other phospholipids was found. Some unidentified material was also present in the extract. The greatest amount of this was in a spot just behind the solvent front, which did not react as phospholipid but gave intense colors upon heating with concentrated sulfuric acid, and was probably steroidal.

Attempts to restore the capacity of type I substrates for binding to the extracted microsomes were not successful. Dried isooctane extracts, either dissolved in ethanol or suspended by sonication, did not restore the difference spectrum characteristic of hexobarbital interaction with cytochrome P-450. In like manner, the addition of solutions or suspensions of phosphatidylcholine or of cholesterol to isooctane-extracted microsomes did not allow the formation of a type I spectrum when hexobarbital was added.

The concentration of residual isooctane in two different preparations of extracted microsomes was found by gas chromatographic analysis to be 0.27 and 0.68 μmole/mg of protein.

DISCUSSION

The data presented here show that after isooctane extraction rat liver microsomes no longer have an appreciable ability to bind hexobarbital with the production of a type I difference spectrum. At quite high concentrations of hexobarbital, however, a difference spectrum is obtained that falls into the range of the type II patterns. In this regard, the isooctane-extracted microsomes are very similar to those formed during treatment of rats with 3-methylcholanthrene.

Because isooctane itself produces a type I difference spectrum upon addition to liver microsomes, it might be asked whether the loss of hexobarbital-binding capacity resulted from inhibition of hexobarbital binding by residual isooctane in the microsomes. The amount of the latter was found to be such that the concentration of isooctane in the cuvettes during the spectrophotometric titrations was less than 0.3 mM. The concen-

tration of isooctane required for 50% inhibition of the binding of hexobarbital is 2.6 mM. The inhibition of binding of hexobarbital by isooctane is noncompetitive; the fractional inhibition at any given concentration would therefore be equal to the ratio of the experimental concentration I to the quantity $(I + I_{50})$. The inhibition of hexobarbital binding which would be caused by the residual isooctane in these experiments was therefore 10–20%. The loss of hexobarbital-binding capacity found after isooctane extraction of rat liver microsomes was, however, over 90%; for this to have been due solely to inhibition of hexobarbital binding, the isooctane concentration would have had to have been greater than 20 mM. It is therefore concluded that this loss was not in large measure caused by inhibition of binding by residual isooctane.

Much of the loss of type I binding capacity would therefore appear to be due to some change in the microsomes attendant upon extraction with isooctane. The changes in affinities noted here are quite similar to those which occur upon enzymatic hydrolysis of microsomal phosphatides (7, 8). Thin-layer chromatography of the isooctane extract showed that it contained phosphatidylcholine and phosphatidylethanolamine. It is possible, therefore, that the form of the microsomal cytochrome P-450 which reacts with type I substrates requires phospholipid for activity. It is interesting to note the work of Hart *et al.* (17), who studied the interaction of cytochrome *c* and phospholipids in aqueous media, and who found that complexes of cytochrome *c* with phosphatidylserine and phosphatidylcholine were soluble in isooctane.

The results of the present work may be explained on the basis of a scheme (Fig. 6) similar to that which we have previously proposed from other evidence (15). Here, two related forms of cytochrome P-450 are envisioned, represented by **E** and **EX**, which differ by the association of one of the forms with some endogenous microsomal component or metabolite, designated by **X**. Each form in this scheme is capable of reacting with one of the two general types of substrate. The resulting complexes are shown

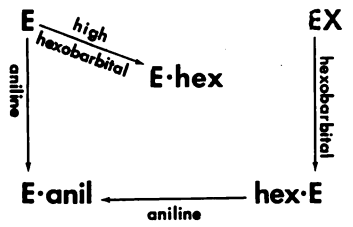

FIG. 6. *Scheme for interaction of hexobarbital and aniline with two related forms of cytochrome P-450*

here as **E·anil** and **hex·E**; the latter can, according to this model, react with aniline to form **E·anil**. The symbols for the complexing drugs, **anil** and **hex**, are shown on different sides of the symbol **E**, to indicate that the reaction with these two types of substrate may involve different reaction sites. According to this scheme, hexobarbital could convert part of the cytochrome P-450 of untreated microsomes to a form that can more readily react with aniline, thus enhancing the binding of aniline. At higher concentrations, hexobarbital may also react with the aniline binding site, resulting in eventual inhibition of aniline binding.

If, upon extraction with isooctane, the normal hexobarbital-binding form of the hemoprotein were selectively destroyed or modified, hexobarbital could no longer react with the cytochrome in its normal manner, and therefore the usual type I difference spectrum would not be produced. At higher concentrations, however, hexobarbital could react with the type II binding site, with the resulting development of a type II difference spectrum. Another consequence of the loss of the specific hexobarbital-binding site in the isooctane-extracted microsomes is that hexobarbital could no longer convert **EX** to a complex that readily reacts with aniline, and so there would no longer be any enhancement of aniline binding in the presence of hexobarbital.

It would appear that **X** might be a lipoprotein portion of the microsomal membrane, or that phospholipid might be involved in the complex, labeled **EX**, of cytochrome P-450 with some endogenous component of microsomes.

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