SUBFRACTIONATION OF RAT LIVER MICROSOMES: EFFECTS OF PHENOBARBITAL AND 3-METHYL CHOLANTHRENE

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Subfractionation of rat liver microsomes by the use of rate-zonal centrifugation has been carried out using both normal and induced rats. Phenobarbital and methyl cholanthrene were found to have very distinctive effects on the distribution of cytochrome P-450 within the microsomal vesicles. The altered distribution observed with methyl cholanthrene is inhibited by thioacetamide and ethionine. These results suggest that the P-450 produced after treatment with methyl cholanthrene is preferentially formed in, or bound to, a vesicle distinct from that which contains the P-450 in the normal or phenobarbital-induced animal.

Phenobarbital and methyl cholanthrene represent prototypes of the two classes of microsomal mixed function oxidase inducers (1). Previous studies have indicated that these two compounds cause the production of spectrally different forms of cytochrome P-450 (2), the terminal oxidase of the mono-oxygenase system. Furthermore the oxidase activities which are effected by each compound utilize distinct classes of substrates. The work to be described represents results of experiments designed to separate, by physical means, the enzymes and related components effected by these two classes of inducers. To this end we have been applying the method of rate-zonal centrifugation for sub-fractionation of rat liver microsomes. In the course of these studies it has been found that pretreatment of the animals with inducers of the mixed-function oxidases has a pronounced effect on the distribution of those oxidases. These studies indicate clearly that phenobarbital and methyl cholanthrene can exert their effects at distinct sites within the endoplasmic reticulum.

METHODS

A. Preparation of 10,000 xg supernatant

Four male rats (125-150 g) were killed by decapitation. The livers were perfused in situ with 0.15 M KCl and then excised and homogenized in 0.25 M sucrose (1 g tissue/5 ml) with a Teflon pestle and glass mortar. The combined homogenates were spun at 1000 xg for 10 minutes. The supernatant was drawn off and re-centrifuged at 10,000 xg for 10 minutes. The supernatant from this spin was then re-centrifuged at 10,000 xg for 10 minutes. The final supernatant was used for density gradient centrifugation.

B. Rate-Zonal Centrifugation

Rate-zonal centrifugation was performed using a titanium B-XV rotor[1] (3). A gradient generator designed by Anderson and Rutenberg (4) using a 600 ml mixing flask was used to produce the sucrose gradient. The sucrose concentration of the light solution was 10% while the heavy gradient solution had a sucrose concentration of 40%. The sucrose solutions were buffered with 0.05 M PO_4, pH 7.0-7.1. After the 1200 ml gradient was in the rotor, the remaining rotor volume was filled with 55% sucrose. A sample of 40 to 45 of homogenate was pumped into the center of the rotor followed by 240 ml of buffer which served as an overlay. The rotor was then accelerated to 27,000 rpm and centrifugation was continued for 150 minutes (w^2 t = 7.1 x 10^{10}). The rotor was decelerated to 3,500 rpm and the gradient was displaced through the center of the rotor. Fractions of 40 ml were collected at a rate of about 18 ml/minute.

C. Analyses

Gradient shape was determined by measuring the refractive index of the fractions using a Bausch and Lomb Abbe refractometer. Protein was determined by the Biuret method as described by Layne (5). Carbon monoxide and ethyl isocyanide difference spectra were measured by the method of Omura and Sato (6). P-450 concentration was determined by using an extinction coefficient of 91 $cm^{-1}mM^{-1}$ (6).

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[1]The B-XV rotor was fabricated by the Oak Ridge Gaseous Diffusion Plant and supplied to us under a cooperative agreement with the Oak Ridge Molecular Anatomy Program directed by Dr. N. G. Anderson.

Benzpyrene hydroxylase activity was determined by the method of Nebert and Gelboin (7). RNA was determined using the method described by Schneider (8).

RESULTS

Rate-zonal centrifugation of the 10,000 xg supernatant from normal 24-hour fasted rats resulted in the protein distribution indicated in Figure 1. In this experiment, as well as in the others reported below, the distribution patterns were consistently reproducible under the specified conditions. Fractions 6-9 represent the soluble proteins. RNA analysis (not indicated) showed that fractions 10-12 contain predominantly RNA rich particles which are presumably free ribosomes. Fractions 14-36 contain the protein fractions which exhibit activities characteristic of microsomal enzymes. The levels of P-450 have been taken as representative of the mixed-function oxidase system. The distribution of P-450 across the gradient from normal microsomes is also shown in Figure 1. While P-450 is found to be spread across the entire microsomal region it is somewhat more concentrated in the earlier fractions (20-24).

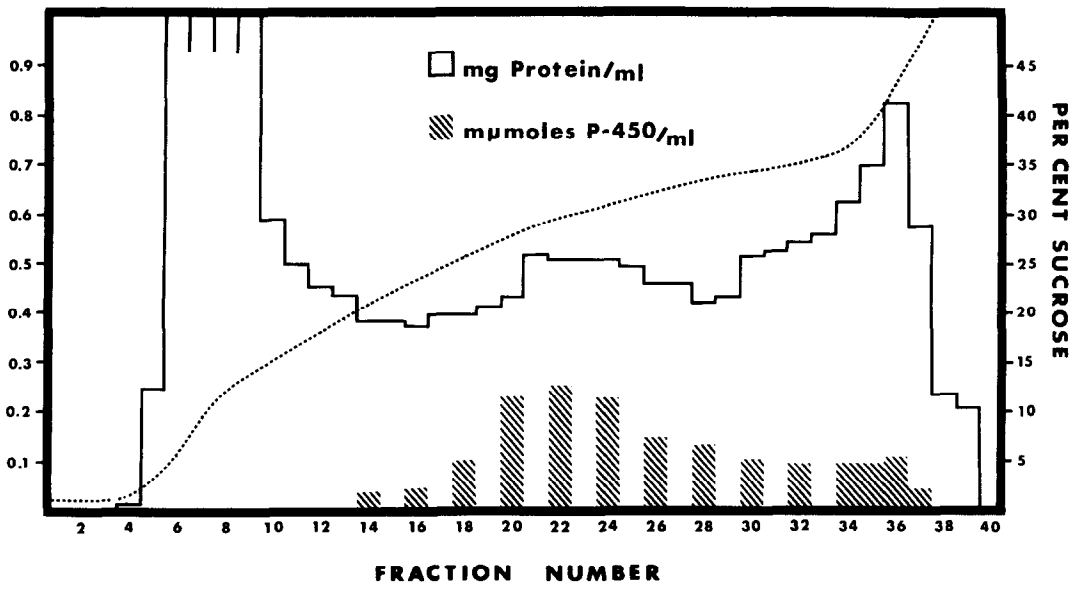

Figure 1. Rate-zonal centrifugation of the 10,000 xg supernatant from the livers of normal 24 hr fasted rats.

When the rats are pretreated with phenobarbital for 5 days prior to sacrifice the distribution of P-450 across the gradient is markedly altered (Figure 2A). When this distribution is plotted as percent of control (Figure 3A) it is readily seen that the major increase in activity is found in the lighter portion of the gradient (Fractions 14-18). These particles presumably correspond to the smooth endoplasmic reticulum which is known to increase under these conditions.

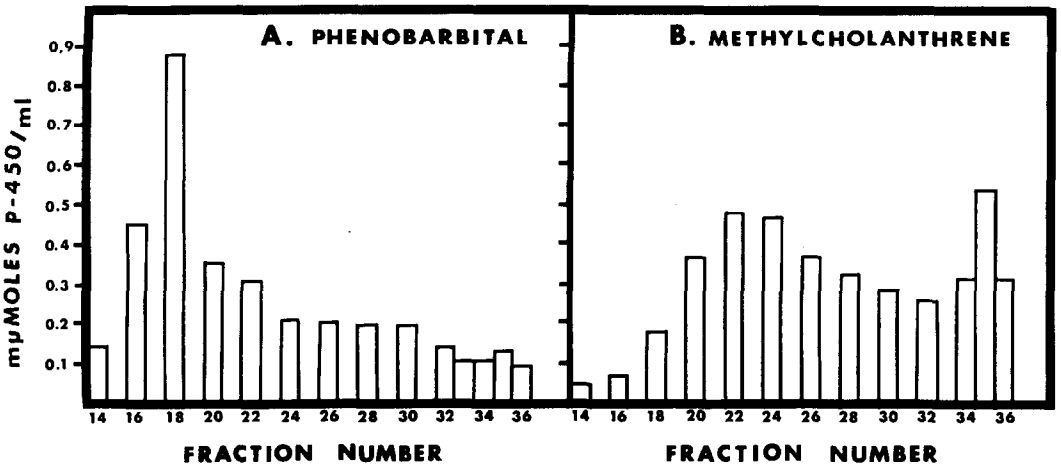

Figure 2. Distribution of P-450 after rate-zonal centrifugation of the 10,000 xg supernatant from livers of treated rats. A. Phenobarbital - 50 mg/kilo/day in saline, 5 days. B. Methyl cholanthrene - 20 mg/kilo/day in PEG_{200}, 3 days.

When methyl cholanthrene is administered to the rats prior to sacrifice, the distribution of cytochrome P-450 (Figure 2B, 3B) is substantially different from that seen with either normal or phenobarbital-treated animals. In this case there is a pronounced increase in the P-450 content of the particles having higher S values. It thus seems that the newly induced P-450 is preferentially formed in, or bound to, a vesicle distinct from that carrying the phenobarbital-induced P-450. Another possibility is that the particles which normally contain P-450 in untreated and phenobarbital-induced animals are converted by MC to particles with an increased sedimentation rate. If this latter hypothesis is true then induction with both agents simultaneously should result in a distribution pattern similar to

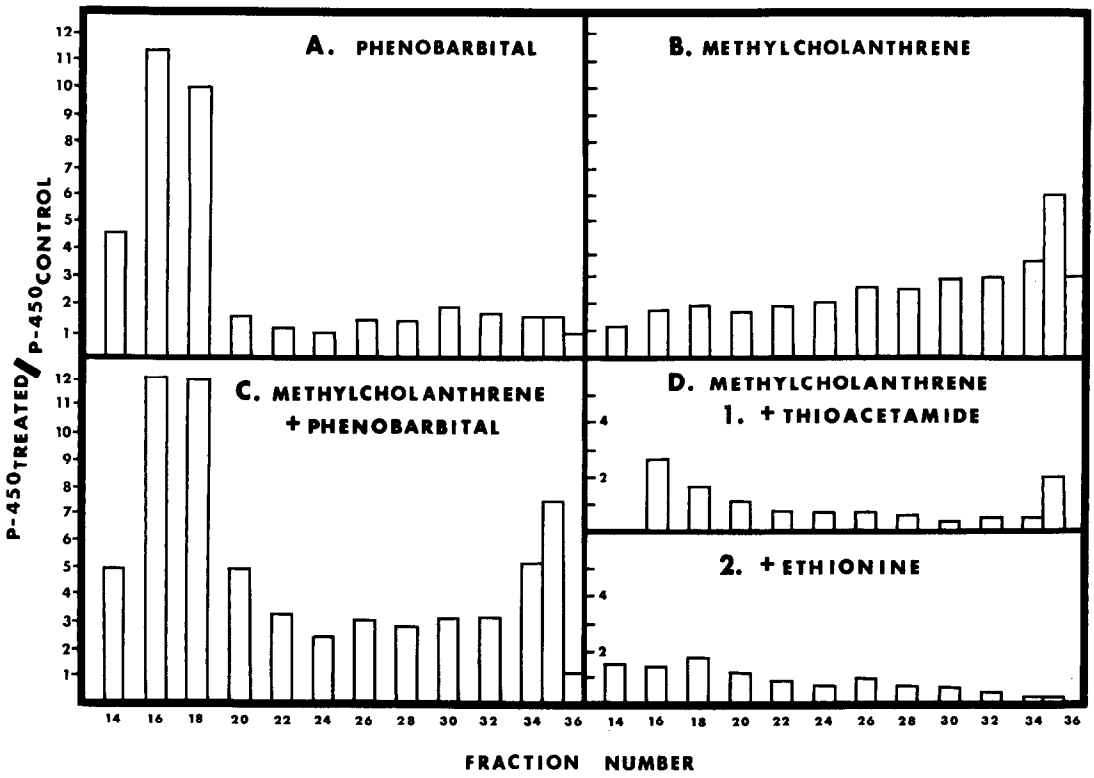

Figure 3. Distribution of P-450 after rate-zonal centrifugation of the 10,000 xg supernatant from livers of treated rats expressed as a ratio to control values. A. Phenobarbital treated. B. Methyl cholanthrene treated. C. Phenobarbital - 50 mg/kilo/day in saline, 5 days, Methyl cholanthrene - 20 mg/kilo/day in PEG_{200} for the 3 days prior to sacrifice. D. Methyl cholanthrene plus inhibitor: 1. Methyl cholanthrene - 20 mg/kilo/day in PEG_{200}, 5 days, Thioacetamide - 50 mg/kilo/day in saline, 5 days. 2. Methyl cholanthrene - 20 mg/kilo/24 hrs prior to sacrifice, Ethionine (d,l) - 100 mg/kilo, 60 and 30 minutes prior to the administration of methyl cholanthrene.

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that obtained with MC alone. When rats were treated with both phenobarbital and MC the distribution shown in Figure 3C was obtained. It is readily apparent that in this case both regions of the gradient show an increase in the levels of P-450. This would seem to indicate that MC does not cause an alteration of the slower sedimenting particles by a direct chemical of physical interaction but rather, causes a de novo synthesis of the component(s) responsible for the observed distribution. Further indications of the validity of this hypothesis were obtained by carrying out experiments using the metabolic inhibitors ethionine and thio-

acetamide. In the presence of ethionine, methyl cholanthrene produced no significant alteration of the gradient profile (Figure 3D). There was no increase in benzpyrene hydroxylase activity[2] nor was there any production of spectrally altered P-450[2]. Thioacetamide, on the other hand, had a mixed effect. It completely inhibited the increase in the levels of P-450 in the faster sedimenting particles (Figure 3D), but did not prevent the increase in benzpyrene hydroxylase activity[2] nor the production of altered P-450[2]. These latter observations were in agreement with a recent report by Sladek and Mannering (9) that thioacetamide has little effect on MC induction as measured by either the 3-methyl-4-methylaminoazobenzene-N-demethylase activity or by the production of a spectrally altered P-450.

DISCUSSION

It has been well established that treatment of rats with phenobarbital results in a proliferation of the smooth endoplasmic reticulum of the livers (10,11), an increase in mono-oxygenase activity (1) and an increase in the level of cytochrome P-450, the terminal oxidase of the mono-oxygenase system (10,12). Methyl cholanthrene also causes an increase in P-450 levels but in this case the cytochrome has spectral characteristics which are significantly different from those of normal and phenobarbital-induced microsomes (2). The effect of methyl cholanthrene on the morphology of the liver cell is not clear. Fouts and Rogers (11), using benzpyrene and methyl cholanthrene, reported only a slight increase in smooth endoplasmic reticulum as observed by electron microscopy of liver sections. However, in a later report from the same laboratory, Gram et al. (13) reported that methyl cholanthrene had pronounced effects on the morphology of fractionated microsomes.

In the studies reported above we have found that the effect of 3-methylcholanthrene can be readily distinguished from that of phenobarbital by the application of rate-zonal centrifugation to the sub-fractionation of rat liver microsomes. The increase in the levels of P-450 caused by methyl cholanthrene is in a particle which is physically distinct from that produced by phenobarbital

induction. It has also been found that the increase in benzpyrene hydroxylase[2] and spectral alteration of P-450[2] can occur in the absence of these physical changes. These results suggest two quite distinct modes of action for methyl cholanthrene; (a) an increase in the levels of P-450, and (b) alteration of the properties of the oxidase system.

The nature of the membrane fraction affected by methyl cholanthrene treatment is currently being investigated by electron microscopy. It is hoped that these investigations will lead to a better understanding of the morphological effects of methyl cholanthrene not only as a mono-oxygenase inducer, but also as a carcinogenic agent.

Acknowledgements

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[2]These results will be reported fully in a publication currently in preparation.

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