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EFFECT OF PHENOBARBITAL AND 3-METHYLCHOLANTHRENE ADMINISTRATION *IN VIVO* AND ISOOCTANE EXTRACTION *IN VITRO* ON METYRAPONE BINDING TO REDUCED CYTOCHROME P-450*

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Abstract—The changes of metyrapone binding to reduced cytochrome P-450 during phenobarbital and methylcholanthrene pretreatment were compared with the effect of the inducers on cytochrome P-450 content of rat liver microsomes. The maximal absorbance change at 446 nm caused by addition of metyrapone to reduced microsomal preparations was increased under the influence of both inducers. The apparent spectral dissociation constant K_S was decreased by phenobarbital pretreatment but increased by methylcholanthrene pretreatment. These changes of metyrapone binding also occurred if the increase in cytochrome P-450 content was suppressed by concomitant pretreatment with the inhibitor of protein synthesis cycloheximide. During methylcholanthrene pretreatment the increase of the maximal absorbance at 446 nm which appeared after addition of metyrapone began rapidly and reached a maximum 36 hr after a single dose of the inducer. At this time no increase of cytochrome P-450 content was detected. The changes of metyrapone binding observed after methylcholanthrene pretreatment could be imitated by extraction of the microsomes with isooctane. The inducer mediated modifications of the binding characteristics of metyrapone are discussed in relation to the lipid environment of cytochrome P-450 and to the synthesis of an inducer specific form of the hemoprotein.

CYTOCHROME P-450 content and drug metabolizing activity of liver microsomes can be enhanced by pretreatment of the animals with two broad classes of substances one of them consisting of a large number of chemically unrelated compounds of which phenobarbital (PB) is a protagonist, the other consisting of carcinogenic polycyclic hydrocarbons among which 3-methylcholanthrene (MC) has been widely investigated. Evidence has accumulated that the hydrocarbon-induced cytochrome P-450 which has been named cytochrome P_1-450[3] or P-448[4] differs in many respects including enzymic activity as well as optical and magnetic parameters from the hemoprotein originally present in untreated animals and from that induced by PB. Thus, substrate specificity is rather different in control and PB-stimulated microsomes on one hand and in MC-stimulated microsomes on the other hand.[5,6] The absorption maximum of the carbon monoxide complex of the reduced cytochrome P-450 is shifted from 450 to 448 nm after pretreatment with MC.[3] The cytochromes P-420 derived from the active hemoprotein exhibit different properties depending on the pretreatment to which the animals were subjected.[7] Differences were also

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observed in ligand binding to oxidized and reduced cytochrome P-450. Thus, addition of hexobarbital to oxidized microsomal preparations and to partially purified cytochrome P-450 leads to aberrant difference spectra in MC-stimulated microsomes when compared to the difference spectra observed in control and PB-stimulated microsomes.[8,9] The ratio of the two Soret bands in the difference spectrum caused by ethyl isocyanide in reduced microsomes and in partially purified cytochrome P-450 preparations depends on the inducing agent which was applied.[3,10] The binding of metyrapone (MP) to reduced cytochrome P-450 is increased by PB pretreatment but decreased by MC pretreatment.[11] The level of high spin cytochrome P-450 as judged from EPR data was found to be considerably higher in MC-stimulated microsomes and in partially purified preparations derived from them than in the respective preparations from untreated and PB-pretreated animals.[12,13]

In this paper the influence of pretreatment with PB and MC on MP binding to reduced microsomes is described. The ability of concomitant administration of cycloheximide to prevent the effect of the inducers on MP binding was also investigated.

METHODS

Pretreatment of the animals. Male Sprague–Dawley rats of about 150 g body wt were used. For pretreatment with PB the animals received 3 × 80 mg/kg sodium PB i.p. at 24 hr intervals. For pretreatment with MC 3 × 20 mg/kg MC dissolved in commercial salad oil were given i.p. at 12 hr intervals. To study the time course of induction single doses of the inducers were given. In some experiments 1 mg/kg cycloheximide was given i.p. 4 hr before each application of PB or MC, respectively. Some animals received 3 × 1 mg/kg cycloheximide at 24-hr intervals without any PB or MC. Control animals were treated with saline or salad oil i.p. The animals were sacrificed 24 hr after the last injection of PB or 36 hr after the last injection of MC and 12 hr after withdrawal of food. In time course experiments the animals were sacrificed 6, 12 or 36 hr after PB or MC administration.

Preparation of microsomes. The livers were perfused *in situ* with ice cold saline and then homogenized in 3 vol. of 0·25 M sucrose soln containing 0·02 M Tris–HCl buffer pH 7·4 and 0·005 M EDTA.[14] Nuclear fragments and mitochondria were sedimented by centrifugation at 1600 *g* for 15 min and at 9000 *g* for 30 min. The microsomal pellet was obtained by centrifugation of the 9000 *g* supernatant at 105,000 *g* for 1 hr. The sediment was washed once. For extraction experiments PB-stimulated microsomes containing about 30 mg protein/ml were mixed with 2/3 vol. of isooctane. The mixture was gently homogenized in an ice bath for 30 min by a glass homogenizer.[15] The emulsion was then centrifuged at 105,000 *g* for 1 hr. Interface material and sediment were resuspended together in sucrose soln. Protein content was determined according to Lowry *et al.*[16] For spectral recordings of MP binding the microsomal pool was divided into portions containing 5 mg protein which were thawed immediately before the experiments to avoid variation of the aging processes.[11] It was established that the results obtained with these samples were consistent with those obtainable with fresh microsomes.

Spectrophotometric measurements. Spectrophotometric studies were performed in an Aminco Chance split beam spectrophotometer at 30°. The total heme content of the microsomal preparations was determined by the pyridine hemochromogen method as described by Omura and Sato[17] using an extinction coefficient o

32·4 cm^{-1} mM^{-1} for the extinction difference between 557 and 575 nm in the dithionite reduced minus oxidized difference spectrum. Cytochrome b_5 was determined by the extinction difference between 424 and 450 nm after reduction of the sample cuvette with NADH using an extinction coefficient of 105 cm^{-1} mM^{-1}.[18] Hemoglobin was not present in the microsomal suspensions to a measurable degree as verified by running difference spectra after saturation of the sample cuvette with carbon monoxide. Extinction coefficients for the cytochrome P-450 carbon monoxide complex were estimated in fresh preparations by calculating the difference between the total heme content and the cytochrome b_5 content. This calculation is based on the assumption that cytochrome P-450 and cytochrome b_5 are the only hemoproteins present in liver microsomes. It is obvious that only approximate extinction coefficients can be obtained by this procedure.*

For analysis of difference spectra of MP in reduced microsomes 5 ml samples containing 1 mg protein/ml were reduced with 1 mg sodium dithionite/ml and the pH was then readjusted to 7·8 since this is the pH optimum of the MP dependent absorbance change at 446 nm.[11] The reduced sample was divided into two parts and a base line was established. MP was added in 10 and 100 μl quantities while the same volume of buffer was added to the reference cuvette.

RESULTS

A number of spectral properties of reduced rat liver microsomes were influenced by pretreatment of the animals with PB and MC (Table 1). The extinction coefficient of the carbon monoxide complex of reduced cytochrome P-450 which was 82 cm^{-1} mM^{-1} in control preparations was increased to 107 cm^{-1} mM^{-1} after MC pretreatment while it was not significantly changed in PB-stimulated microsomes. The cytochrome P-450 content was increased by both MC and PB to an extent which is in agreement with the data that have been published before. The binding of MP to reduced cytochrome P-450 was influenced profoundly by both pretreatment procedures. In MC-stimulated microsomes the absorption maximum at 446 nm was shifted to 445 nm. The maximal absorbance change in the Soret region was increased by a factor of about 2 as related to the cytochrome P-450 content after both induction processes. The apparent spectral dissociation constant K_S for this absorbance was, however, influenced contrarily by both inducers. After PB pretreatment a marked decrease of K_S was noted. In contrast a considerable increase in the K_S value by a factor of about 20 was observed in MC-stimulated microsomes indicating that the high affinity of the microsomal preparation for MP is lost during MC pretreatment.

Comparable changes of MP binding could not be observed when PB or MC were added to *in vitro* incubations of reduced microsomes. This was also true if the inducer had been preincubated with microsomal suspensions in the presence of NADPH and air. Thus, it seems unlikely that metabolites of PB or MC which may be formed via oxidative pathways during the induction phase are able to cause the modifications of MP binding described above *in vitro*. It must be assumed that these modifications are in some way related to the induction process itself.

* Since individual ϵ values were not determined for special preparations (Figs. 1–5), an ϵ of 91 cm^{-1} mM^{-1} according to Omura and Sato[17] was used if not stated otherwise.

TABLE 1. SPECTRAL PROPERTIES OF REDUCED LIVER MICROSOMES FROM UNTREATED, PB PRETREATED, AND MC PRETREATED RATS

	ϵ (cm^{-1} mM^{-1})	Cyt. P-450 content† (nmoles/mg protein)	ΔA_{max}(446–490 nm)‡ (per mg protein/ml)	(per nmole cyt. P-450/mg protein)	K_S§ (μM)
Control	81·9 ± 5·6	1·17 ± 0·12	0·017 ± 0·002	0·015 ± 0·001	16·1 ± 2·4
	(n = 9)	(n = 10)	(n = 10)	(n = 10)	(n = 3)
PB	76·3 ± 6·9	2·19 ± 0·16***	0·079 ± 0·005***	0·037 ± 0·003***	2·4 ± 0·2***
	(n = 6)	(n = 7)	(n = 7)	(n = 7)	(n = 8)
MC	107·0 ± 8·2*	1·63 ± 0·13**	0·050 ± 0·005***	0·031 ± 0·003***	387·4 ± 75·3**
	(n = 5)	(n = 9)	(n = 9)	(n = 9)	(n = 5)

Values are means ± S.E.M. *$P < 0·05$, **$P < 0·01$, ***$P < 0·001$.
† Calculated from the individual ϵ values for each pretreatment procedure.
‡ MP concn. 2·5 mM.
§ These data are taken from a previous publication.[11]

In addition to these findings, experiments were performed to test the influence of concomitant cycloheximide application *in vivo* and of isooctane extraction *in vitro* on MP binding in stimulated microsomes. Moreover, time course and dose dependency of the inducer mediated changes of MP binding were studied. In these experiments no complete K_S determinations like those in Table 1 were performed. Instead of complete titration two test concentrations of MP, derived from the K_S determinations in Table 1, were used, one being relatively low (0·04 mM) and one relatively high (2·5 mM). In preparations with high affinity for MP (low K_S values), e.g. control microsomes and PB-stimulated microsomes, even the low concentration of 0·04 mM will cause almost maximal absorbance at 446 nm. Increasing the concentration to 2·5 mM will not markedly enhance this absorbance. In contrast, preparations with low affinity for MP (high K_S values), e.g. MC-stimulated microsomes, will exert only poor absorbance at 446 nm with the low concentration of 0·04 mM. Increasing the concentration to 2·5 mM will then lead to maximal absorbance.

From these considerations it is obvious that the use of the two test concentrations of MP will give information on the affinity of a microsomal preparation for MP and of its alteration under the experimental conditions of Figs. 1–5.

The relationship between modification of MP binding and synthesis of cytochrome P-450 or P-448 was studied in the presence of an inhibitor of protein synthesis (Figs. 1 and 2). It can be seen from the left part of Fig. 1 that the increase of cytochrome P-450 content due to PB pretreatment was blocked by concomitant application of cycloheximide. In contrast, the increase in the absorbance change at 446 nm with both 0·04 and 2·5 mM MP observed after PB pretreatment was not prevented by concomitant cycloheximide pretreatment as can be derived from the right part of Fig. 1. Cycloheximide application by itself did not influence MP binding.

Figure 2 shows an effective blockade of the increase in cytochrome P-450 content due to MC induction when cycloheximide was added concomitantly. On the other hand, no effect of cycloheximide application on the changes of MP binding observed in MC stimulated microsomes was detected. In control microsomes the absorbance change at 446 nm was nearly maximal at a ligand concentration of 0·04 mM. This

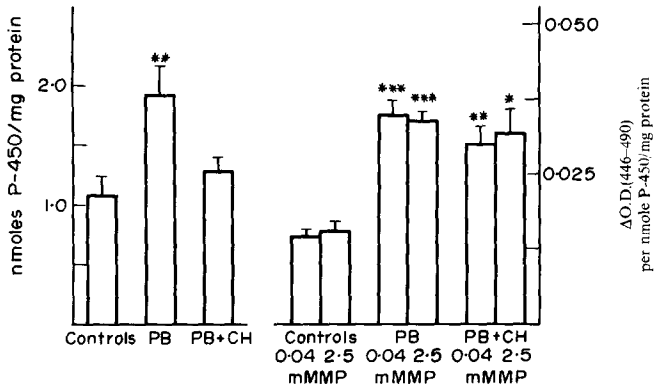

FIG. 1. Influence of concomitant cycloheximide pretreatment on the effect of PB pretreatment on MP binding to reduced cytochrome P-450 in rat liver microsomes. Control animals received saline, PB animals received 3 × 80 mg PB/kg at 24 hr intervals, PB + CH animals were treated as PB animals, but received 1 mg cycloheximide/kg 4 hr prior to each PB treatment. Values are means ± S.E.M. from 4–5 independent microsomal preparations. *P < 0·05, **P < 0·01, ***P < 0·001.

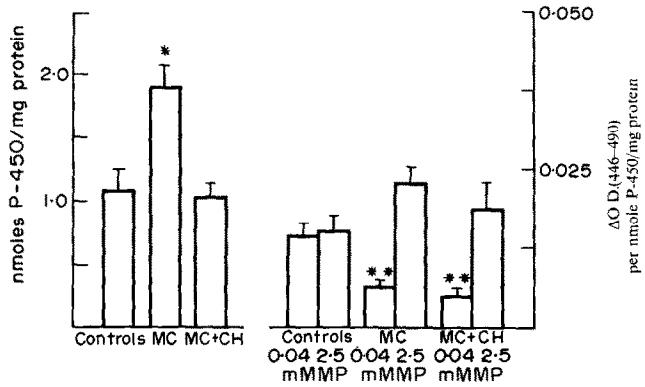

FIG. 2. Influence of concomitant cycloheximide pretreatment on the effect of MC pretreatment on MP binding to reduced cytochrome P-450 in rat liver microsomes. Control animals received oil, MC animals received 3 × 20 mg MC/kg at 12 hr intervals, MC + CH animals were treated as MC animals but received 1 mg cycloheximide/kg 4 hr prior to each MC treatment. Values are means ± S.E.M. from 5 to 6 independent microsomal preparations. *P < 0·01, **P < 0·001.

was not true for MC-stimulated microsomes in which a smaller Soret band was seen with 0·04 mM MP than in control microsomes. However this peak was considerably enhanced when the final ligand concentration was increased to 2·5 mM. The same titration pattern was obtained in microsomes from animals which received MC + cycloheximide. Thus, cycloheximide administration will not prevent the MC mediated decrease of affinity for MP.

Figure 3 illustrates the increase of cytochrome P-450 content and of the maximal absorbance change at 446 nm due to MP binding during the course of MC induction. It can be seen from the diagram that both processes do not take place in parallel. Maximal absorbance at 446 nm increased rather rapidly during the first hours after

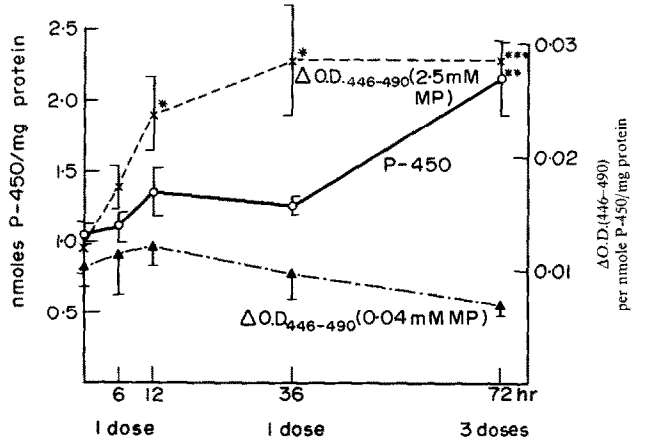

FIG. 3. Time course and dose dependency of the effect of MC pretreatment on the maximal absorbance change at 446 nm occurring after MP addition to reduced rat liver microsomes. The animals received 1 × 20 mg MC/kg or 3 × 20 mg MC/kg, respectively. On the abscissa the hours after the first administration of MC are marked. Values are means ± S.E.M. from three independent microsomal preparations. *P < 0·05, **P < 0·02, ***P < 0·01. In the curve obtained with 0·04 mM MP S.E.M. is only drawn in one direction.

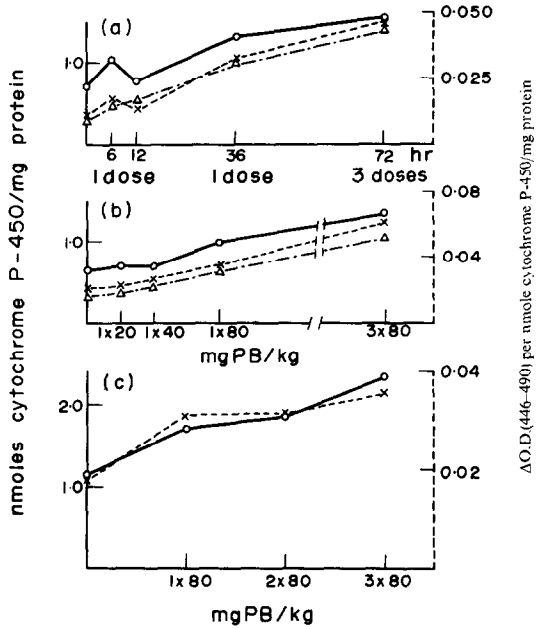

FIG. 4. Time course and dose dependency of the effect of PB pretreatment on the maximal absorbance change at 446 nm occurring after addition of MP to reduced rat liver microsomes. Data from three single experiments are plotted. In Fig. 4(a) the abscissa gives the hours after the first administration of PB (80 mg/kg). For repeated PB applications intervals were 24 hr. In Fig. 4(b) and (c) the animals were sacrificed 24 hr after the single, respectively, the last PB application. (○) P-450, (△) $\Delta O.D._{446-490}$(0·04 mM MP), (×) $\Delta O.D._{446-490}$(2·5 mM MP).

the application of a single dose of MC reaching a maximum between 12 and 36 hr. At this time no significant induction effect on cytochrome P-450 content was observed. This becomes prominent only after three successive doses of MC and 72 hr after the administration of the first dose. In contrast, no further increase of the maximal absorbance at 446 nm as measured 36 hr after a single MC application was

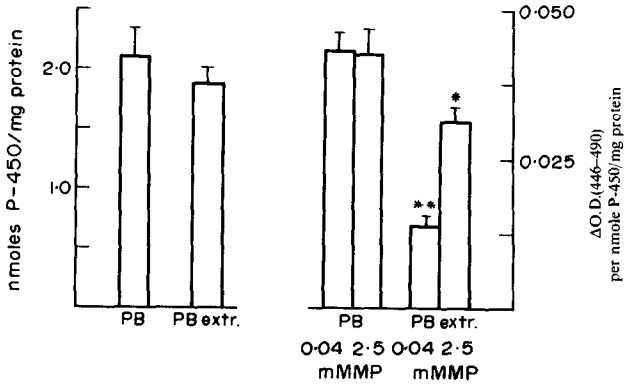

FIG. 5. Influence of isooctane extraction on MP binding to reduced cytochrome P-450 in PB-stimulated rat liver microsomes. The animals received 3 × 80 mg PB/kg. The extraction of the microsomal preparations was performed as described in Methods. Values are means ± S.E.M. from five extractions performed with five independent microsomal preparations. *$P < 0.01$, **$P < 0.001$.

obtained by increasing the number of MC applications and the induction time. No comparable increase was seen with the low MP concentration of 0·04 mM. The divergent time course of the spectral effects exhibited by the two MP concn means that the affinity of the preparations for MP was decreased.

Different results were obtaind during PB pretreatment. In Fig. 4 the data of three single experiments, each of which was performed with a different pattern of PB application, are plotted. The diagrams show that increase of cytochrome P-450 content and increase of the absorbance change at 446 nm appear in parallel with both MP concn under all conditions tested.

The effect of isooctane extraction of PB-stimulated microsomes on the affinity for MP is summarized in Fig. 5. MP binding in extracted PB-stimulated microsomes was similar to MP binding in unextracted MC-stimulated microsomes (see Fig. 2), as the maximal absorbance change at 446 nm which was somewhat smaller than in unextracted preparations was not achieved with the low MP concn of 0·04 mM but only with the high MP concn of 2·5 mM. This indicates that an affinity loss for MP took place during the extraction procedure. These changes were not accompanied by a significant decrease in cytochrome P-450 content as can be seen from the left part of Fig. 5. The effect could not be obtained if the extraction procedure was simulated with sucrose soln instead of isooctane.

DISCUSSION

In the experiments presented here the effect of pretreatment on cytochrome P-450 content is compared with the effect of pretreatment on MP binding to reduced cytochrome P-450. Cytochrome P-450 content increases depending on the amount of the inducer applied. This increase can be suppressed by concomitant inhibition of protein synthesis due to cycloheximide treatment of the animals. The extinction coefficient of the carbon monoxide complex is not changed after PB pretreatment but enhanced by about 30 per cent after MC pretreatment which is in agreement with previous findings in both microsomes and partially purified preparations of cytochrome P-450.[19,10]

The spectral changes at 446 nm occurring upon addition of MP to reduced microsomes are modified by PB and MC pretreatment of the animals. These inducer effects do not occur *in vitro* as judged from incubation experiments performed in the presence of the inducers. Thus it may be assumed that they are due to events which are related to the induction process. From our results it is, however, evident that the changes of MP binding to reduced cytochrome P-450 do not appear in parallel with the increase of cytochrome P-450 content. By concomitant application of cycloheximide the effect of the inducers on cytochrome P-450 content was blocked while their effect on MP binding was fully developed even under these conditions.

A similar discrepancy has been observed between the cycloheximide effect on inducer mediated increase in cytochrome P-450 content and on the concomitant change in the ratio of the 430 and 455 nm Soret bands of the ethyl isocyanide difference spectrum in reduced microsomes.[20] This finding led the authors to the conclusion that the change of the ethyl isocyanide spectrum after MC pretreatment was not dependent on the *de novo* synthesis of a new molecular species of cytochrome P-450 being formed under the influence of MC. This conclusion might also apply to our findings on MP binding to reduced cytochrome P-450 but it should be noted

that decisive evidence for the hypothesis cannot be derived from the data available since the presence of inducer specific protein cannot be excluded even if the total cytochrome P-450 content is not increased. The ratio of genuine and inducer specific protein may be about the same in inducer-treated and inducer + cycloheximide-treated animals leading to comparable affinity for the ligand in both preparations.

From these considerations further evidence is needed before assuming that the inducer mediated changes of MP binding are not related to the *de novo* synthesis of cytochrome P-450. The assumption is somewhat supported at least in the case of MC induction by the experiments which show a different dose dependency and time course of the increase in cytochrome P-450 content and of the decrease in MP binding during MC induction. Moreover, it was possible to imitate the effect of MC pretreatment on MP binding by extraction of PB-stimulated microsomes with isooctane. This extraction procedure has formerly been used to abolish type I substrate binding in microsomes by disturbing the lipid environment of the heme moiety within the microsomal membranes. It has been reported that phosphatidylethanolamine and phosphatidylcholine are removed from the membranes by this treatment,[15] although there is no quantitative data on the isooctane effect.

Our results with isooctane treated microsomes suggest that the changes of MP binding which are observed after MC pretreatment and which are very similar to those caused by the extraction procedure may be due to modifications of the lipid environment of the cytochrome caused by the inducer rather than to the appearance of an inducer specific form of the cytochrome. The essential role of a lipid component in substrate binding and enzymic activity has been demonstrated by means of phospholipase treatment,[21] extraction procedures,[15] and dietary studies.[22] Recently it has been shown that drug metabolizing activity can be reconstituted after partial purification of the components of the microsomal monooxygenase system by combining cytochrome P-450, NADPH–cytochrome P-450 reductase and phosphatidylcholine.[23]

Schulze and Staudinger reported that the increase in total phospholipid content of microsomes is different after PB pretreatment and MC pretreatment amounting to 90 per cent in PB stimulated microsomes but to only 20 per cent in MC stimulated microsomes.[24] No striking modification of the phospholipid pattern was found in these studies by either pretreatment procedure. Thus, no persuasive explanation for the effect of MC pretreatment on MP binding concerning definite structural changes of the microsomal membranes can be derived from the finding of these authors. However, it cannot be excluded that some modification of lipid arrangement within the microsomal membranes will profoundly influence the access of MP to the reduced hemoprotein.

The results presented here suggest that the induction process is composed of a number of non-uniform events which can be differently influenced and which, in the case of MC induction, exhibit a different time course and dose dependency. Further insight into the processes which influence MP binding to reduced cytochrome P-450 may be obtained with two experimental procedures which have become available recently: (1) It has been shown that the inducibility of aryl hydrocarbon hydroxylase activity by hydrocarbons is determined genetically in mice.[25] A comparison of the effects of MC pretreatment in genetically responsive animals and genetically non-responsive animals might offer an explanation for the findings described in this paper.

(2) The partial purification of cytochrome P-450 has been considerably improved during the last two years.[10,23,26] The investigation of MP binding to the partially purified hemoprotein after it is detached from its genuine lipid environment may show if the changes of MP binding after the application of an inducer are due to the synthesis of an inducer specific form of the cytochrome or to modifications of its arrangement within the microsomal membranes.

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