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CHOLESTASIS AS AN *IN VIVO* MODEL FOR ANALYSIS OF THE INDUCTION OF LIVER MICROSOMAL MONOOXYGENASES BY SODIUM PHENOBARBITAL AND 3-METHYLCHOLANTHRENE

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Abstract—Cholestasis was used as a model of sharp decrease in the amounts of substrate-binding and catalytic centres of cytochrome P-450 for sodium phenobarbital and 3-methylcholanthrene. Based on this model, the induction effects of sodium phenobarbital and 3-methylcholanthrene on rat liver microsomal monooxygenases were analyzed. Under conditions excluding the primary binding and metabolism of the inducer by monooxygenases, sodium phenobarbital retains its capacity for induction. By contrast, 3-methylcholanthrene exerted no inducing effects under the same conditions as confirmed by the lack of increase of cytochrome P-448 content. From the data obtained it is suggested that in mechanism of sodium phenobarbital induction of liver microsomal monooxygenases the activation of protein synthesis is affected by the inducer itself. As for 3-methylcholanthrene, it is assumed that the synthesis of specific protein (cytochrome P-448) could be initiated by the microsomal metabolites of this inducer.

In the last years increased activity of the liver microsomal monooxygenase systems after xenobiotics administration to animals have been described with particular emphasis on differences between the specific effects of the such inducers as 3-methylcholanthrene and sodium phenobarbital [1, 2]. These differences, as well as the additive effects of these inducers on the activity of the microsomal monooxygenase system [3, 4], suggest the different mechanisms of the induction achieved by 3-methylcholanthrene and phenobarbital. Because the intracellular receptor(s) for these inducers is (are) unknown, it is difficult to study any definite mechanism of their action. It should be borne in mind that some current theories of induced enzyme formation assign the key role to the initial step of the enzyme-inducer complex formation; incidentally the role of the inducer consists in either enzyme stabilization or in the stimulation of the *de novo* synthesis of specific protein by the inducer or by its primary metabolic products [5, 6]. Since phenobarbital and 3-methylcholanthrene are type I substrates for microsomal cytochrome P-450 [7], it seems reasonable to assume that the active centre of cytochrome P-450 may serve as receptor for these inducers.

It has been reported earlier [8, 9] that changes in the amount of binding sites for phenobarbital and 3-methylcholanthrene in the cytochrome P-450 molecule affect differently their inducing effects. These observations led us to the supposition that the inducer itself is involved in the activation of the genome and subsequent protein synthesis induced by phenobarbital while the products of the primary metabolism of the inducer in microsomes are responsible for the inducing effect of 3-methylcholanthrene. To verify this supposition, the inducibility of microsomal monooxygenases in cholestatic liver by phenobarbital and 3-methylcholanthrene was studied, in view of the fact that cholestasis development is associated with considerable decrease in the binding and metabolism of type I substrates [10, 11].

MATERIALS AND METHODS

Male albino Wistar rats (160–180 g) were used in all experiments. Bile duct ligation was carried out as described by Hutterer *et al.* [12]. The rats with ligated bile duct were treated with 3-methylcholanthrene (50 mg per kg body weight) diluted in a minimum volume of olive oil or with phenobarbital (100 mg per kg body weight) in 0.9% NaCl on fifth or fourth days after operation, respectively. Above mentioned inducers were injected intraperitoneally (i.p.) once daily during two or three days, respectively. The other rats with ligated bile duct were injected with corresponding amounts of olive oil or 0.9% NaCl, respectively. The sham-operated rats treated with phenobarbital or 3-methylcholanthrene were used as controls. The animals were starved overnight before every treatment with inducer and also before being killed. All rats were decapitated on seventh day after bile duct ligation.

The liver was perfused *in situ* with ice-cold isolation medium: 1.15% (w/v) KCl/0.02 M-Tris–HCl, pH 7.4. All procedures for the preparation of the microsomal fraction from rat livers were as previously described [8, 9, 13].

The amount of cytochromes P-450, P-448 and P-420 were determined from CO-difference spectra of $Na_2S_2O_4$-treated microsomal fraction as described by Omura and Sato [14] by using $\varepsilon = 91$ litre/mmole/cm for cytochromes P-450 and P-448, and $\varepsilon = 111$ litre/mmole/cm for cytochrome P-420.

Maximal binding of a type I substrates (aminopyrine, phenobarbital and 3,4-benzpyrene) and type II

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substrate (aniline) to cytochrome P-450(P-448), designated as the constant ΔA_{max}, was estimated as described in the work of Kato *et al.* [15]. The results were expressed in $\Delta A_{421(430)-500\,nm} \cdot 10^3$ per mg of protein. As for 3,4-benzpyrene, the results were expressed in $\Delta A_{388-398\,nm} \cdot 10^3$ per mg of protein.

Difference spectra were recorded in a Hitachi model 356 two-wavelength double beam spectrophotometer, in a split-beam mode.

The activity of NADPH-cytochrome *c* reductase was determined kinetically under conditions similar to those of Phillips and Langdon [16]. Results were expressed as nmoles of cytochrome reduced/min per mg of protein. ε was taken as 19.1 litre/mmole/cm [17].

The activity of NADPH-cytochrome P-450(P-448) reductase was measured as described by Gnosspelius *et al.* [18]. The results were expressed in nmoles of cytochrome reduced/min per mg of protein. NADPH oxidation and the stimulation of NADPH oxidation by addition of 1.6 mM phenobarbital were determined in a Hitachi-356 apparatus (in a double beam mode) by following the changes of absorbance at 340 nm versus 374 nm [19]; ε for NADPH was 6.22 litre/mmole/cm.

Aminopyrine *N*-demethylase activity was determined essentially as described by Smuckler *et al.* [20]. The incubation medium contained microsomal fraction (5–6 mg of protein), 0.1 M-Na_2HPO_4/KH_2PO_4 buffer (pH 7.5), 5 mM-aminopyrine, 0.3 mM-$NADP^+$ and a NADPH-generating system consisted of 7 mM-glucose-6-phosphate, 10 mM-$MgCl_2$ and 3 units of glucose-6-phosphate dehydrogenase (EC 1.1.1.49). Total volume of the incubation medium was 3 ml. The amount of formaldehyde formed was measured by the procedure of Nash [21].

Aniline *p*-hydroxylase activity was measured by the method of Imai *et al.* [22]. The incubation medium contained in final volume of 2 ml: 0.1 M-Tris–acetic acid buffer (pH 8.0), microsomal fraction (6 mg of protein), 8 mM-aniline, 0.32 mM-$NADP^+$ and NADPH-generating system as described above. The amount of *p*-aminophenol formed was measured at 630 nm, by using *p*-aminophenol as standard.

3,4-Benzpyrene hydroxylase activity was assayed by the method of Nebert and Gelboin [23] with slight modifications. The incubation time was 4 min and started from the moment excess NADPH (0.5 mmole) was added. The reaction was stopped by addition of 1.0 ml acetone, and the mixture was shaken with 3.0 ml of hexane for 10 min. An aliquot (1.0 ml) of the organic layer was extracted with 1.0 ml of 1 N NaOH, and the fluorescence of this extract was measured immediately at 398-nm excitation and 522-nm emission in a Hitachi model MPF-4 fluorescence spectrophotometer and compared to the fluorescence of a standard 3-hydroxybenzpyrene (kindly provided by Dr. H. Gelboin, National Cancer Institute, NIH, Bethesda, Md).

Enzymic lipid peroxidation activity was estimated by determining the amount of malondialdehyde (MDA) formed with the aid of thiobarbituric acid reaction [24]. The incubation medium of the NADPH-dependent lipid peroxidation system was identical with the one used by Hochstein *et al.* [25]. The incubation time course started from the moment of excess NADPH (0.8–1.0 mmole) was added, whereas all the parameters enumerated were evaluated before adding NADPH as indicators of "zero time". The temperature of the shaking water bath was 37° and incubation time, 10 min. The reaction was stopped by the adding of 0.2 mM EDTA. Measurement of oxygen uptake was followed as described previously [26] in a LP-7 polarograph with a Clark-type electrode.

The protein content was determined by the biuret method [27]. Presented results are the average ± SD from 3–4 microsomal preparations. Each preparation consisted of liver microsomes from 7–8 rats.

RESULTS AND DISCUSSION

Characteristics of the model. In a previous paper [11] it has been shown that the microsomal enzymic hydroxylation systems which are sensitive to any damage of the phospholipid component of microsomal membrane [13, 28], considerably decrease their activity during cholestasis development. Thus, the structural and functional changes in the microsomal fraction have been suggested to be due to the accumulation in hepatocytes of dihydroxy cholates exerting detergent-like effects on the endoplasmic reticulum membranes [11].

Our choice of the time interval for phenobarbital and 3-methylcholanthrene administration against the background of developing cholestasis was guided by two considerations. First, during days 4–7 after bile duct ligation there occurs a plateau in the cytochrome P-450 content and the activity of NADPH-cytochrome *c* reductase as well as a plateau in the binding of aniline, a substrate using the ligand of cytochrome P-450 heme

Table 1. Effect of cholestasis on the maximal binding of phenobarbital by liver microsomes and the activity of NADPH-oxidase

Experimental conditions	ΔA_{max}	Activity of NADPH-oxidase without substrate	Activity of NADPH-oxidase in the presence of 1.6 mM phenobarbital
1. Control group (sham-operated rats)	5.9	11.8	14.3
2. 1. + phenobarbital added during 3 days	3.9	21.8	26.1
3. Cholestasis during 7 days	0.1	5.1	5.1
4. 3. + phenobarbital administration starting from day 4 of cholestasis development	3.4	7.7	12.1

Maximal binding of phenobarbital (ΔA_{max}) was expressed in $\Delta A_{421-500\,nm} \cdot 10^3$ per mg of protein. Activity of NADPH-oxidase was expressed in nmoles NADPH/min per mg of protein.

Table 2. Effect of 3-methylcholanthrene treatment on the spectral and kinetic constants of liver microsomal monooxygenase system from control (sham-operated) and cholestatic rats

Animal groups	Cytochrome P-450(P-448) nmoles/mg of protein	%	Rate of NADPH-dependent reduction of "hemoprotein–benzpyrene" complex nmoles hemoprotein reduced/min per mg of protein	%	Maximal binding of 3,4-benzpyrene $\Delta A_{388-500\,nm} \cdot 10^3$ per mg of protein	%	3,4-Benzpyrene metabolism pmoles 3-OH benzpyrene formed/min per mg of protein	%
1. Control group	0.64 ± 0.04		4.84 ± 0.38		185 ± 12		182 ± 10	
2. 1. + 2 days of 3-MC treatment	1.15 ± 0.12 $P_{1,2} < 0.001$	182	14.60 ± 0.86 $P_{1,2} < 0.001$	302	740 ± 59 $P_{1,2} < 0.001$	400	1152 ± 90 $P_{1,2} < 0.001$	633
3. Cholestasis during 4 days	0.46 ± 0.03 $P_{1,3} < 0.001$	72	2.60 ± 0.11 $P_{1,3} < 0.001$	53	65 ± 5 $P_{1,3} < 0.001$	35	72 ± 5 $P_{1,3} < 0.001$	40
4. Cholestasis during 7 days	0.49 ± 0.04 $P_{1,4} < 0.01$	77	1.20 ± 0.10 $P_{1,4} < 0.001$	25	37 ± 2 $P_{1,4} < 0.001$	20	47 ± 4 $P_{1,4} < 0.001$	26
5. 3. + 2 days of 3-MC treatment	0.49 ± 0.05 $P_{1,5} < 0.05$ $P_{4,5} > 0.05$	77	1.22 ± 0.04 $P_{1,5} < 0.001$ $P_{4,5} > 0.05$	26	43 ± 4 $P_{1,5} < 0.001$ $P_{4,5} > 0.05$	23	224 ± 18 $P_{1,5} > 0.05$ $P_{4,5} > 0.001$	123
6. 5./4. (%)	100		102		116		486	

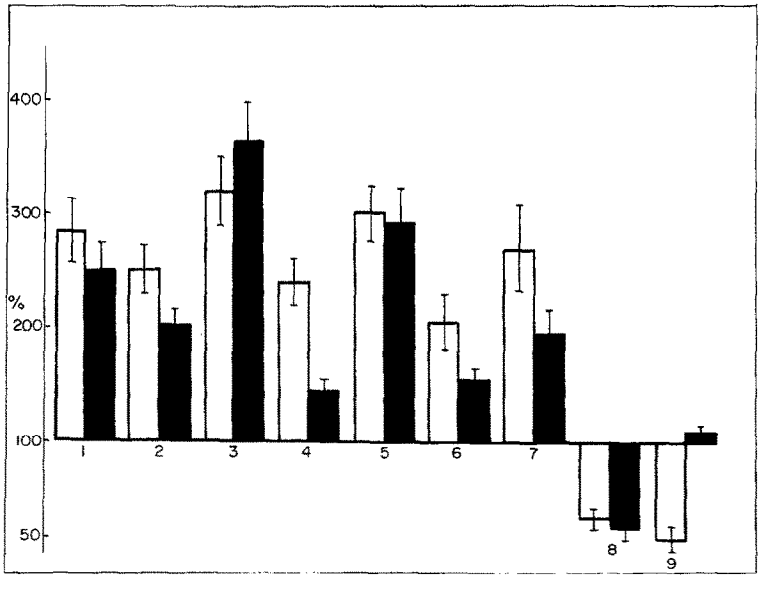

Fig. 1. Effects of induction by phenobarbital on the amount and enzymic activity of the components of microsomal monooxygenase system from control (light columns) and cholestatic (dark columns) rat liver. Abscissa: 1—cytochrome P-450 content; 2—activity of NADPH-cytochrome *c* reductase; 3—activity of NADPH-cytochrome P-450 reductase; 4—maximal binding of aminopyrine; 5—activity of aminopyrine *N*-demethylase; 6—maximal binding of aniline; 7—activity of aniline P-hydroxylation; 8—activity of NADPH-dependent lipid peroxidation reaction; 9—activity of ascorbate-dependent lipid peroxidation reaction.

Ordinate: induction effect of phenobarbital treatment of control and experimental rats (sham-operated rats and rats after 4 days of cholestasis, respectively). In the latter case all the experimental parameters were done in % to ones observed in microsomes on day 7 cholestasis and expressed as 100% level.

iron [7]. Second, the amount of binding sites for type I substrates, estimated by the value of ΔA_{max} [29], decreases significantly for aminopyrine [11] and 3,4-benzpyrene and practically drops out for phenobarbital (see Tables 1 and 2).

The data of Table 2 indicate that the rate of 3,4-benzpyrene hydroxylation by liver microsomal fraction decreases by almost 75 per cent 7 days after bile duct ligation. In addition, phenobarbital has no stimulating effect on the activity of NADPH-oxidase (Table 1), which is in accordance with the data of Martin [30] and indicates that an oxidative metabolism of this type I substrate is almost abolished. Hence, the capacity of phenobarbital and 3-methylcholanthrene for the induction of microsomal monooxygenase system may be assessed under conditions when the initial binding and metabolism of the inducer by the enzyme are practically absent.

Phenobarbital-caused induction. The results in Fig. 1 indicate that phenobarbital treatment of animals of the control and experimental group results in almost equal increase in the content and enzymic activity of the components of the microsomal NADPH-linked electron-transport chain. Thus the administration of phenobarbital produces an increase in the amount of cytochrome P-450 up to 250–280 per cent of the control value in both groups examined. The activity of NADPH-cytochrome *c* reductase and NADPH-cytochrome P-450 reductase increases to approximately 250 per cent and 320–350 per cent, respectively. Aminopyrine *N*-demethylase activity is induced by phenobarbital nearly 3-fold in the comparable groups.

The somewhat smaller increase of maximal binding of aminopyrine may be explained by the competition of this substrate with hydroxylated cholesterol derivatives, which are also type I substrates and have high affinity for cytochrome P-450 [13, 31]. This interpretation is supported by the larger stimulation by aminopyrine of the initial rate of NADPH-cytochrome P-450 reductase in liver microsomes of control rats, than that in experimental microsomes.

It may especially be noted that maximal binding of type II substrate aniline, and the velocity of its hydroxylation in the sample microsomal preparations, are both much less pronounced when compared with the control phenobarbital-pretreated preparations (Fig. 1).

Effects of 3-methylcholanthrene treatment. On the other hand, the administration of 3-methylcholanthrene to rats does not lead to any changes in the content of CO-binding hemoprotein in microsomes (Table 2), although the peak in the CO-spectra of its $Na_2S_2O_4$-reduced form is at 448 nM. There is no appreciable increase in the binding of 3,4-benzpyrene by sample cytochrome P-448, while induction with 3-methylcholanthrene of control rats is associated with an almost 4-fold increase in the binding of the substrate. However, there occurs a surprisingly high level in the activity of 3,4-benzpyrene hydroxylase after 3-methylcholanthrene administration to cholestatic rats (Table 2).

Similarly, Spencer and Fischer [32] have observed that treatment of partially hepatectomyzed rats with 1,2-benzanthracene results in a 24-fold increase in the activity of 3,4-benzpyrene hydroxylase, whereas induction in control animals is accompanied by only a 4-fold increase in the enzyme activity. No unambiguous explanation can be offered for this impressive increase in 3,4-benzpyrene metabolism after surgical partial hepatectomy nor after "functional partial hepatectomy" to which cholestasis may be referred.

Thus, analysis of the rate-limiting steps of the metabolism of 3,4-benzpyrene in the sample microsomal fractions excluded cytochrome P-448 content, the amount of binding sites for 3,4-benzpyrene and, finally, the NADPH-dependent reduction of the cytochrome-3,4-benzpyrene complex as determinants of the hydroxylation activity for this polycyclic hydrocarbon during 3-methylcholanthrene treatment of cholestatic rats. The exclusion of these factors casts doubt on the inducing character of this phenomenon. It may be that, in the presence of detergent-like cholates, cytochrome P-448 acquires additional catalytic properties as other membrane-bound enzymes do after treatment of microsomal fraction with surface-active agents [33, 34]. Under the given experimental conditions one cannot rule out the inhibition of epoxide hydratase coupled with cytochrome P-448 [35], which results in impairment of the usual redistribution of 3,4-benzpyrene metabolic products with predominant yield of phenol, 3-hydroxybenzpyrene.

When assessing the absence of cytochrome P-448 increase in microsomal fraction from livers of 3-methylcholanthrene-treated cholestatic rats, one has to take into account the possible decrease of CO-peak of $Na_2S_2O_4$-reduced form of the hemoprotein as a result of the damage effect of agents characteristically operative during cholestasis. Agents of this kind are primarily di- and trihydroxy bile acids which accumulate during cholestasis [36] and possess detergent properties. The most pronounced effect on microsomal membranes among other derivatives is that of deoxycholate which, at low concentrations, is capable of converting cytochrome P-450 into P-420, which is accompanied by decrease of P-450-catalyzed hydroxylation reactions [13]. Secondly, the lipid peroxidation of microsomal phospholipids activated in the presence of bile acids and their salts [37, 38] is accompanied by the oxidative degradation of cytochrome P-450 [39].

Effects of deoxycholate and lipid peroxidation upon control and induced microsomal cytochromes. With all this in view, experiments were performed to detect the *in vitro* effects of deoxycholate treatment (Fig. 2) and the activation of the enzymic and ascorbate-dependent lipid-peroxidation reactions (Fig. 3) on the spectral characteristics of cytochromes from the liver of control and phenobarbital- or 3-methylcholanthrene-induced rats.

A 30 min incubation of the microsomal fraction from liver of control rats, in the presence of excessive amounts of NADPH and ADP–$FeCl_3$ complex, decreased markedly P-450 content (Fig. 3a). Under the same incubation conditions, the phenobarbital-induced cytochrome P-450 and 3-methylcholanthrene-induced cytochrome P-448 are very much less sensitive to the lipoperoxidative reactions (Fig. 3b, c). The amount of malondialdehyde formed, as well as of oxygen consumption rate during lipid peroxidation in both microsomal samples, is negligibly small, when compared with controls (Table 3). The data obtained indicate that the microsomes from the liver of induced rats are much more resistant to both enzymic and nonenzymic lipid peroxidation.

Increasing sodium deoxycholate concentrations

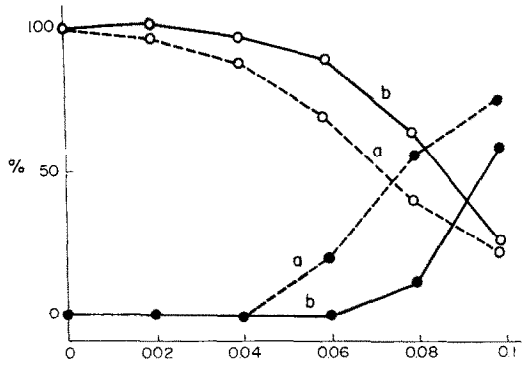

Fig. 2. Effect of *in vitro* added sodium deoxycholate on the conversion of microsomal cytochromes P-450 and P-448 to cytochrome P-420.
(a) Control microsomal preparations; (b) microsomal preparations after induction by 3-methylcholanthrene; ○—cytochrome P-450(P-448); ●—cytochrome P-420. Initial amounts of microsomal CO-binding proteins were (expressed as 100% level): (a) 0.83 nmoles/mg protein, (b) 1.56 nmoles/mg protein. The contents of cytochromes P-420 are shown by using ε mM 111 cm^{-1}.

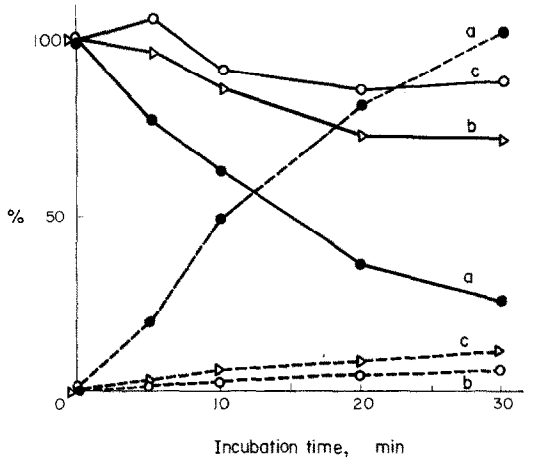

Fig. 3. Effect of NADPH-dependent lipid peroxidation on the content of CO-binding hemoproteins in control and experimental microsomal preparations.
(a) Control preparations; (b) preparations from the livers of phenobarbital-treated rats; (c) preparations from the livers of 3-methylcholanthrene-treated rats; solid lines—cytochrome P-450(P-448) content; dotted lines—malondialdehyde accumulation. Initial amounts of CO-binding hemoproteins were (expressed as 100% level): (a) 0.83 nmoles/mg protein; (b) 2.03 nmoles/mg protein; (c) 1.56 nmoles/mg protein. Final amounts of malondialdehyde accumulated after 30 min incubation were: (a) 50 nmoles/mg protein; (b) 3 nmoles/mg protein; (c) 6 nmoles/mg protein.

demonstrated clearcut differences in the resistance of control cytochrome P-450, phenobarbital-induced cytochrome P-450 and cytochrome P-448 to damage effects of the detergent (Fig. 2). Thus, it was found (Fig. 2b) that, at concentrations of sodium deoxycholate lower than 0.1%, less cytochrome P-448 is converted into its enzymically-inactive form (cytochrome P-420), as compared with the control cytochrome P-450 (Fig. 2a). These data are consistant with those of Imai and Siekevitz [40] according to which the cytochrome P-448 treated *in vitro* with chaotropic agents and organic solvents is more stable to "converting" attacks than cytochrome P-450.

Therefore, the presence of detergents and the lipid peroxidation reactions cannot be implicated as causes of unchanged level of liver microsomal cytochrome P-448 content in cholestatic rats treated with 3-methylcholanthrene. Taken together, all the data concerning the substrate-binding and electron-accepting capacities of experimental cytochrome P-448 make the very occurence of induction in the given experiments doubtful.

CONCLUSIONS

Cholestasis, as an experimental model of sharp decrease of the amount of catalytic centres in cytochrome P-450 for type I substrates, including phenobarbital and 3-methylcholanthrene, demonstrated that the first one, unlike 3-methylcholanthrene, is capable of inducing monooxygenase system under conditions which exclude the step of primary binding and metabolism of this inducer in microsomes. According to our earlier observation, the absence of binding sites for phenobarbital in the cytochrome P-448 molecule does not prevent the induction of the monooxygenase system to proceed in a manner of the phenobarbital-type [9]. On the contrary, when the binding sites for 3-methylcholanthrene are increased in phenobarbital-induced cyto-

Table 3. Effect of lipid peroxidation reactions on the oxygen uptake and malondialdehyde (MDA) accumulation in microsomes from rats induced by phenobarbital (PB-microsomes) and 3-methylcholanthrene (MC-microsomes)

Microsomal preparations	Rate of oxygen uptake (natoms O_2/min per mg of protein) NADPH-dependent lipid peroxidation reaction	ascorbate-dependent lipid peroxidation reaction	Rate of MDA accumulation (nmoles/min per mg of protein)
Control preparations	194	145	2.50
PB-microsomes	69	39	0.10
MC-microsomes	120	53	0.36

The control rats (sham-operated animals) were injected with corresponding amounts of olive oil or 0.9% NaCl. These solvents had no effect on the activity of lipid peroxidation reactions in liver microsomes.

chrome P-450 molecule, an inducing effect of 3-methylcholanthrene on microsomal monooxygenase system is very much enhanced [8].

Integrating the present data with those obtained previously, it may be suggested that the substrate molecule itself is involved in the mechanism of phenobarbital-caused activation of the genome and the subsequent protein synthesis; in the case of induction by 3-methylcholanthrene, the active agents seem to be the products of the primary metabolism of the polycyclic hydrocarbon in microsomes.

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