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INDUCTION OF DRUG METABOLISM—VI.

EFFECTS OF PHENOBARBITAL AND 3-METHYLCHOLANTHRENE ADMINISTRATION ON *N*-DEMETHYLATING ENZYME SYSTEMS OF ROUGH AND SMOOTH HEPATIC MICROSOMES*

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Abstract—The induction of the microsomal hemoproteins, cytochromes P-450 and P_1-450, and of *N*-demethylase activities in hepatic microsomal subfractions from rats were studied at various times after administration of phenobarbital or 3-methylcholanthrene. After a single dose of phenobarbital, *N*-demethylase activity and cytochrome P-450 levels increased initially only in rough microsomes (RER) whereas a single dose of 3-methylcholanthrene caused almost simultaneous increases of the two enzymes in both RER and smooth microsomes (SER). The increases in *N*-demethylase activities during this early period of induction by 3-methylcholanthrene were paralleled by a change in P-450 hemoprotein from cytochrome P-450 to cytochrome P_1-450 in both microsomal subfractions, but the total amount of P-450 hemoprotein remained essentially unchanged. These results add to existing evidence that phenobarbital and 3-methylcholanthrene produce their inductive effects by different mechanisms and raise the possibility that cytochrome P_1-450 may be synthesized in both RER and SER.

EARLY STUDIES by Fouts[1] suggested that hepatic drug-metabolizing enzymes are concentrated predominantly in the smooth endoplasmic reticulum (SER),§ but subsequent studies have shown that some drugs are metabolized as readily by rough endoplasmic reticulum (RER) as by SER.[2–4] The distribution of enzyme activity between SER and RER depends not only upon the substrate, but upon the species from which the microsomes are obtained and the method of separating SER and RER.[2,4] Inducing agents cause temporal changes in the distribution of enzymes between the two microsomal fractions. Three to six hr after rats received phenobarbital, Orrenius[5] observed increases in microsomal aminopyrine *N*-demethylase, NADPH-cytochrome *c* reductase and cytochrome P-450 which were accountable almost entirely by increased concentrations in RER; by 24 hr, concentrations in the SER had increased until distribution of enzymes was about equal in the RER and SER. Gram *et al.*[6] showed that different inducing agents may cause different distributions of enzymes in the microsomal

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§ While recognizing that microsomes are artifacts of the endoplasmic reticulum (ER), rough and smooth microsomes are designated RER and SER, respectively, throughout the text.

subfractions. Depending upon the substrate, phenobarbital administration caused either no change in microsomal drug-metabolizing enzyme activity or it increased activity. Increases occurred in both RER and SER. Rates of metabolism of some substrates were increased and others were decreased after the administration of 3-methylcholanthrene, but a given change was seen only in one microsomal subfraction or the other, not in both. In view of the generally accepted concept that phenobarbital and 3-methylcholanthrene produce their inductive effects by different mechanisms,[7] it is not too surprising that they should also produce different distribution patterns of increased enzyme activities in RER and SER.

Administration of 3-methylcholanthrene and other polycyclic hydrocarbons to rats causes induction of a microsomal P-450 hemoprotein differing in certain of its physical and biochemical characteristics from the cytochrome P-450 found in hepatic microsomes from untreated rats and rats treated with phenobarbital.[7–17] This hemoprotein was named cytochrome P_1-450[8] to designate a previously unrecognized species of cytochrome P-450; it is also frequently referred to as cytochrome P-448 because when reduced and complexed with carbon monoxide, it gives a maximum absorption peak at 448 mμ rather than at 450 mμ.[9] Cytochrome P_1-450 is most readily distinguished from cytochrome P-450 by the absorption spectra produced when the hemoproteins are reduced and liganded with ethyl isocyanide; the ratio of the heights of the absorption maxima seen at 455 mμ and 430 mμ[18] is greater with cytochrome P_1-450 than with cytochrome P-450.[8] This enables early recognition of 3-methylcholanthrene-induced synthesis of P-450 hemoprotein in the presence of constitual P-450 hemoprotein, an opportunity not afforded when phenobarbital is used as the inducing agent, because the *de novo* P-450 hemoprotein caused to be produced in this manner is not readily distinguishable from that already existing in the membranes. Imai and Siekevitz[19] studied changes in the ethyl isocyanide difference spectra of P-450 hemoprotein in microsomes at sequential time intervals after 3-methylcholanthrene administration, but did not investigate these changes in RER and SER.

The purpose of the current investigation was to determine what differences might exist in the distribution between RER and SER of the enhanced concentrations of P-450 hemoproteins and *N*-demethylating activities caused by phenobarbital and 3-methylcholanthrene administration to rats. Two *N*-demethylases were studied: 3-methyl-4-methylaminoazobenzene (3-MMAB) *N*-demethylase, which is induced by both phenobarbital and 3-methylcholanthrene, and ethylmorphine *N*-demethylase, which is induced by phenobarbital, but not by 3-methylcholanthrene.

METHODS

Animals. Male Holtzman strain rats (100–120 g) were employed in two general types of studies. In the first, sodium phenobarbital and 3-methylcholanthrene were administered intraperitoneally in daily doses of 40 mg/kg in saline and 20 mg/kg in corn oil, respectively, for 4 days. The animals were starved for 24 hr, sacrificed, and their livers removed. In the second type of study, rats received single intraperitoneal injections of sodium phenobarbital, 100 mg/kg, or 3-methylcholanthrene, 20 mg/kg, and their livers were removed 3, 6, 9, 12, 24 and 48 hr later. These animals were starved for 12 hr before sacrifice.

Preparation of microsomal subfractions. Livers were perfused with a cold solution

of isotonic (1·15%) KCl to remove hemoglobin, and a 25% homogenate in cold 0·25 M sucrose was prepared. All subsequent tissue manipulations were carried out in a cold room maintained at 0–4°. The homogenate was centrifuged at 10,000 *g* for 20 min in a refrigerated Lourdes centrifuge, model LRA (rotor No. 9 RA). The 10,000 *g* supernatant fraction was diluted with 0·25 M sucrose solution so that each milliliter contained the equivalent of 250 mg of liver tissue. Subfractionation of the supernatant fraction was carried out essentially by the method of Dallner[20] using density gradient centrifugation in the presence of Cs^+. To 9·85 ml of the supernatant fraction, 0·15 ml of 1·0 M CsCl was added to give a final Cs concentration of 15 mM. Seven and one-half ml of this mixture were carefully layered over 4·5 ml of 1·30 M sucrose–0·015 M CsCl solution. The tubes were centrifuged for 90 min in a Spinco ultracentrifuge, model L2-50, using a Ti 50 rotor at a speed of 50,000 rev/min (r_{av} 165,000 *g*). The upper 8 ml of the supernatant fraction, including the fluffy double layer around the gradient boundary, was removed by aspiration and diluted to 10 ml with 0·25 M sucrose solution. This was centrifuged at 100,000 *g* for 60 min to obtain the SER pellet, which was rinsed with 1·15% KCl solution and resuspended in the same solution to a volume such that each milliliter contained SER equivalent to 1 g of wet liver. The pellet obtained from the centrifugation at 165,000 *g*, which contained RER, was suspended in 1·15% KCl solution and centrifuged at 100,000 *g* for 60 min. The pellet was resuspended in a volume of 1·15% KCl solution so that each milliliter contained RER equivalent to 500 mg of wet liver. Electron micrographs* of these pellets from phenobarbital- and 3-methylcholanthrene-treated animals were similar to those reported by Gram *et al.*[6] The protein contents of the subfractions were determined by the method of Lowry *et al.*[21]

Enzyme assays. Ethylmorphine and 3-methyl-4-methylaminoazobenzene (3-MMAB) *N*-demethylase activities were determined using an incubation mixture described previously.[22] Incubation times were 15 min for ethylmorphine and 7·5 min for 3-MMAB, during which times reaction rates were linear. Ethylmorphine and 3-MMAB were employed in saturating substrate concentrations of 2×10^{-3} M and 2×10^{-4} M, respectively. When ethylmorphine was the substrate, HCHO formed was determined by a modified procedure of Nash as described previously.[23] When 3-MMAB was the substrate, HCHO formed was determined by the chromatropic acid procedure described previously.[24] Carbon monoxide and ethyl isocyanide difference spectra were determined using a recording Beckman model DB dual beam spectrophotometer as described previously[8] except that a final concentration of ethyl isocyanide of 4·5 mM rather than of 3·45 mM was used. Each cuvette contained RER or SER suspensions equivalent, respectively, to 0·5 or 1 g of wet liver/ml of 1·0 M KH_2PO_4 buffer, pH 7·4.

RESULTS

Effects of 4 *days of administration of phenobarbital and* 3*-methylcholanthrene on the* N*-demethylase activities and P*-450 *hemoprotein concentrations in RER and SER.* Specific activities of ethylmorphine *N*-demethylase, cytochrome P-450 content and the 455 and 430 mμ peak heights of the ethyl isocyanide difference spectra of RER and

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TABLE 1. EFFECTS OF PHENOBARBITAL (PB) AND 3-METHYLCHOLANTHRENE (MC) TREATMENTS ON P-450 HEMOPROTEIN CONTENTS AND ETHYLMORPHINE *N*-DEMETHYLASE ACTIVITIES OF HEPATIC MICROSOMES FROM RATS*

Measurement	Treatment	RER	SER
Ethylmorphine *N*-demethylase activity‡	Control	0·19 ± 0·01†	0·22 ± 0·02
	PB	0·76 ± 0·03	0·56 ± 0·03
	MC	0·28 ± 0·01	0·18 ± 0·05
Cytochrome P-450§	Control	0·018 ± 0·001	0·011 ± 0·004
	PB	0·053 ± 0·001	0·040 ± 0·003
	MC	0·046 ± 0·003	0·014 ± 0·004
455 mμ Peak‖	Control	0·009 ± 0·001	0·005 ± 0·000
	PB	0·030 ± 0·001	0·021 ± 0·002
	MC	0·034 ± 0·001	0·011 ± 0·002
430 mμ Peak¶	Control	0·014 ± 0·002	0·008 ± 0·000
	PB	0·033 ± 0·001	0·028 ± 0·002
	MC	0·020 ± 0·001	0·009 ± 0·001
455 mμ Peak‖ / 430 mμ Peak¶	Control	0·69 ± 0·08	0·63 ± 0·06
	PB	0·89 ± 0·04	0·75 ± 0·05
	MC	1·72 ± 0·08	1·29 ± 0·20
pH intercepts	Control	7·7**	7·7
	PB	7·4	7·5
	MC	6·9	7·0
Protein††	Control	7·8 ± 0·5	2·8 ± 0·2
	PB	13·1 ± 0·4	2·3 ± 0·4
	MC	10·7 ± 0·6	3·0 ± 0·2

* Rats received PB, 40 mg/kg/day, or MC, 20 mg/kg/day, for 4 days and were sacrificed 24 hr after the last injection. Ethylmorphine *N*-demethylation and CO and ethyl isocyanide difference spectra were determined as described in Methods. To determine the pH intercepts, curves were constructed for the 430 and 455 mμ peaks obtained by determining the ethyl isocyanide spectra at different pH values. The "pH intercept" is the pH at which the 455 and 430 mμ curves intercept.

† Values represent mean ± S.E. of five experiments except where indicated otherwise; each experiment used a single rat.

‡ Micromoles of HCHO formed per milligram of protein per hour.

§ Δ O.D.$_{450-500}$ mμ/mg of protein.

‖ Δ O.D.$_{455-500}$ mμ/mg of protein.

¶ Δ O.D.$_{430-500}$ mμ/mg of protein.

** Values represent the mean of two experiments.

†† Milligrams per gram of wet liver.

SER from rats treated for 4 days with phenobarbital or with 3-methylcholanthrene are shown in Table 1. In control rats, the activity of ethylmorphine *N*-demethylase and content of cytochrome P-450 were about the same in RER and SER. Treatment of rats with phenobarbital caused increases in *N*-demethylase activity and cytochrome P-450 content in both subfractions, but increases were greater in RER. Treatment of rats with 3-methylcholanthrene caused a small but statistically significant increase in ethylmorphine *N*-demethylase activity in RER, but no induction was observed in the SER subfraction. This confirms previous observations[8,11,12] that phenobarbital induces increased ethylmorphine *N*-demethylase activity, but that 3-methylcholan-

threne administration produces little or no change in the velocity of this reaction Treatment with 3-methylcholanthrene caused a 2·5-fold increase in the P-450 hemoprotein concentration of RER, but no significant increase was seen in SER.

The peak heights of the maxima of the ethyl isocyanide difference spectra obtained with RER and SER from livers of untreated, phenobarbital- and 3-methylcholanthrene-treated rats are also given in Table 1. After treatment with phenobarbital, 2- to 4-fold increases in the 455 mμ and the 430 mμ peaks occurred in both subfractions. In contrast, the administration of 3-methylcholanthrene caused a preferential increase in the height of the 455 mμ maximum, particularly in RER, where a 4-fold increase was seen. These changes are reflected in the 455 mμ/430 mμ ratios, also given in the table. 3-Methylcholanthrene is seen to have caused large increases in the ratio in both RER and SER, but the increase was considerably greater in RER. The apparent increases in the ratios seen after phenobarbital administration did not prove to be statistically significant.

In developing their concept of a pH-dependent interconvertible cytochrome P-450, Imai and Sato[18] showed that the relative sizes of the 455 and 430 mμ peaks were pH dependent. At high pH, the height of the 455 mμ peak is maximal and that of the 430 mμ peak is minimal; at low pH, the reverse is seen. When curves are constructed for the 455 and 430 mμ peak heights at different pH values, and intercept (pH intercept) is obtained at the pH where the heights of the 455 and 430 mμ peaks are equal. The pH intercept for cytochrome P_1-450 is lower than that for cytochrome P-450.[8,12] RER and SER suspensions from control and phenobarbital-treated rats exhibited pH intercepts between pH 7·4 and 7·7 (Table 1). RER and SER from 3-methylcholanthrene-treated rats showed pH intercepts at pH 6·9 and 7·0, respectively, thus further establishing the presence of cytochrome P_1-450 in both microsomal fractions.

Early effects of phenobarbital and 3-methylcholanthrene administration on the N-demethylase activities and P-450 hemoprotein concentrations in RER and SER. Phenobarbital and 3-methylcholanthrene differed markedly in the time course of their inductive effects and in the changes they caused in the distribution of *N*-demethylase activities and P-450 hemoprotein contents between RER and SER (Fig. 1). The major increase in *N*-demethylase activity of the RER occurred within 12 hr with both inducing agents, but although rising less abruptly, the increase was apparent earlier when 3-methylcholanthrene was employed. Increases in *N*-demethylase activities in SER lagged behind and never quite attained levels in RER. However, 3-methylcholanthrene caused a more rapid increase in activity in SER than seen when phenobarbital was the inducing agent; thus at 12 hr, activity had increased about 40 per cent of what it was to reach at the end of the experiment when 3-methylcholanthrene was employed, but was barely discernible at this time when phenobarbital was administered. Even more apparent differences in the two inducing agents are recognized when P-450 hemoprotein levels are compared. Excellent parallelism, both in time and quantity, is seen in the increases of *N*- demethylase activity and P-450 hemoprotein content of the RER when phenobarbital is employed, but no such parallelism is seen with 3-methylcholanthrene. Not only is the increase in P-450 hemoprotein proportionately much less than the increase in *N*-demethylase activity, especially in the SER, but during the first 12 hr, during which time the increase in *N*-demethylase is greatest, there is no apparent increase in P-450 hemoprotein. On the other hand, the qualitative change in P-450 hemoprotein from cytochrome P-450 to cytochrome P_1-450, as reflected by the increase

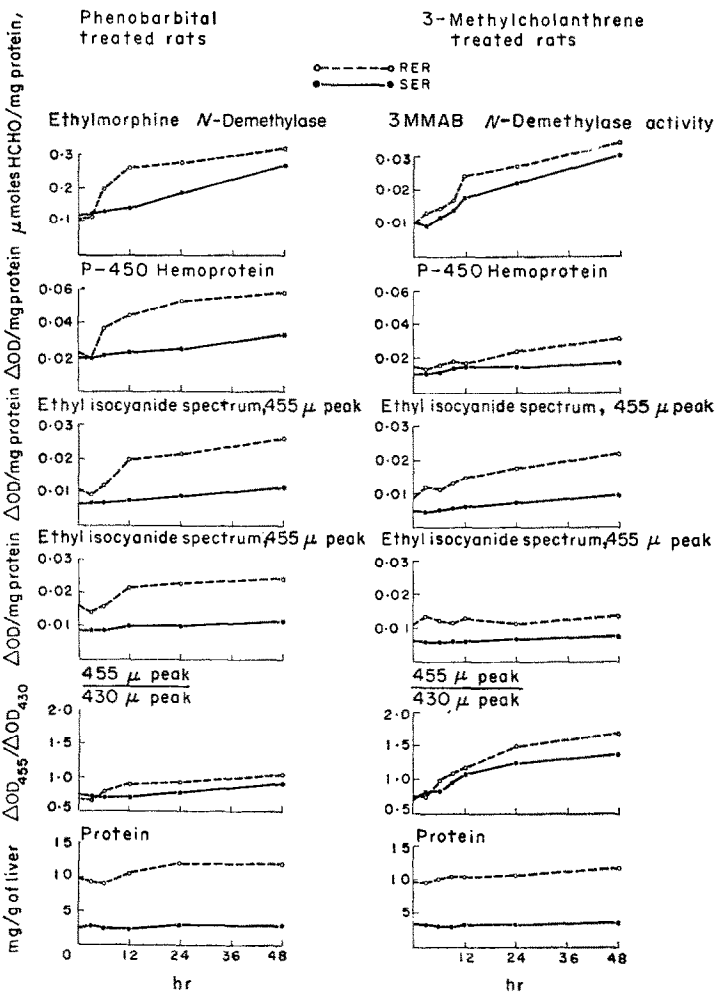

FIG. 1. Temporal effects of a single intraperitoneal injection of sodium phenobarbital or 3-methylcholanthrene (100 or 20 mg/kg of rat, respectively) on the *N*-demethylase activities and P-450 hemoprotein contents of hepatic smooth and rough microsomes (RER = rough microsomes; SER = smooth microsomes). The determinations of ethylmorphine- or 3-methyl-4-methylaminoazobenzene (3-MMAB)-*N*-demethylase activities, P-450 hemoprotein content (reduced-CO spectrum), reduced-ethyl isocyanide spectra (430 and 455 mμ peaks) and protein are given in Methods.

in the 455 mμ peak and the corresponding increase in the 455 mμ/430 mμ peak ratio, roughly parallels the increase in 3-MMAB *N*-demethylase activity.

DISCUSSION

The early recognition that inducing agents differed in the specificity of their inductive effects immediately suggested that more than one mechanism of induction of drug metabolism exists. Agents were classified roughly into two groups. Phenobarbital and many other drugs were placed in one group because of their ability to induce the

metabolism of a large variety of drugs; 3-methylcholanthrene and certain other polycyclic hydrocarbons were placed in a second group because of their less ubiquitous inductive properties.[25] More explicit evidence for different mechanisms of induction by the two classes of agents was obtained from studies which showed that inductive effects were additive when the two compounds were given together[11,16,25–29] and that qualitative differences in the substrate specificities of the enzyme systems induced by the two agents could be explained by a qualitative change in P-450 hemoprotein that occurs when 3-methylcholanthrene and other polycyclic hydrocarbons are given.[8–10,12,17] Several kinds of evidence obtained in our laboratory supported the view that the P-450 hemoprotein (cytochrome P_1-450) synthesized in response to the administration of 3-methylcholanthrene is a specific molecular entity differing from native cytochrome P-450, that it is not simply a complex of polycyclic hydrocarbon with native cytochrome P-450, and that it is not found in appreciable amounts in hepatic microsomes from animals not exposed to polycyclic hydrocarbons or to other agents with similar inductive capabilities.[7]

The current time-course experiment employing rough and smooth microsomal fractions provides additional evidence for different mechanisms of induction by phenobarbital and 3-methylcholanthrene: (a) during the first 12-hr period after phenobarbital administration, *N*-demethylase activity rose rapidly only in RER, but during the same time interval, 3-methylcholanthrene caused a rapid rise in *N*-demethylase activity in both SER and RER, although to a somewhat lesser degree in the former; (b) during the first 12-hr period after phenobarbital administration, the increase in *N*-demethylase activity in SER was accompanied by a parallel increase in P-450 hemoprotein (reduced-CO spectrum); no such parallelism was seen between *N*-demethylase activity and P-450 hemoprotein content (reduced-CO spectrum) in either SER or RER during the 12-hr period after administration of 3-methylcholanthrene; indeed, there was no apparent increase in the P-450 hemoprotein content of either microsomal fraction during this period when *N*-demethylase activity was increasing rapidly; (c) no qualitative change in P-450 hemoprotein, as determined by changes in the reduced-ethyl isocyanide spectra, was seen throughout the 48-hr experimental period after phenobarbital administration; qualitative changes in P-450 hemoprotein (reduced-ethyl isocyanide spectrum) were seen in both SER and RER soon after 3-methylcholanthrene administration and these changes persisted throughout the 48-hr experimental period; qualitative changes in P-450 hemoprotein paralleled increases in *N*-demethylase activity, but as stated previously, quantitative changes did not.

While these studies add to existing evidence that phenobarbital and 3-methylcholanthrene produce their effects by different mechanisms, they contribute little to the elucidation of the mechanisms. On the other hand, they raise some interesting questions regarding the location of newly synthesized P-450 hemoprotein in microsomal membranes. There is much evidence to support the concept that membrane synthesis occurs in RER and that SER derives from RER.[30–32] In fact, the time lag seen after phenobarbital administration between the early appearance of *de novo* cytochrome P-450 in the RER and the more gradual appearance of cytochrome P-450 in the SER has been used as evidence for the derivation of SER from RER.[5,33] This also implies that P-450 hemoproteins are synthesized in RER, but not in SER. How then do we explain the rapid increase in P-450 hemoprotein content of both RER and SER after 3-methylcholanthrene administration? If we assume that the P-450 hemoprotein

is synthesized in RER, we might conclude that either 3-methylcholanthrene accelerates the conversion of RER to SER, or that normally not all membrane is converted at the same rate. In the latter case, the *de novo* P-450 hemoprotein that forms after 3-methylcholanthrene would have to be located in a different part of the membrane than that occupied by the *de novo* P-450 hemoprotein formed after phenobarbital administration, and membrane from the two locations would be transferred at different rates. One might also consider the possibility that phenobarbital induces synthesis of P-450 hemoprotein in SER, but that 3-methylcholanthrene induces the synthesis of P-450 hemoprotein by some organelle other than the endoplasmic reticulum, for example, by unattached ribosomes, in which case cytoplasmic P-450 hemoprotein formed in this manner might be sequestered freely by both SER and RER. Finally, the more straightforward possibility should be recognized that P-450 hemoprotein synthesis, or partial P-450 hemoprotein synthesis, may occur in both RER and SER. This possibility is raised by studies of Levin *et al.*[34] who showed that when ^{3}H-leucine was used to label microsomal proteins, the initial rate of incorporation was greater in RER than in SER, but that when ^{3}H-δ-aminolevulinic acid, a precursor of heme, was used, the initial rates of incorporation of labeled heme into hemoprotein were similar in RER and SER.

It should be recognized that the administration of 3-methylcholanthrene may affect sedimentation rates and thereby prevent a separation of RER and SER comparable to that achieved with microsomes from control and phenobarbital-treated rats. Gram *et al.*[6] showed that 3-methylcholanthrene caused morphological changes in the endoplasmic reticulum and that sedimentation was different from that seen with microsomes from untreated and phenobarbital-treated rabbits; there was greater contamination of RER with SER when 3-methylcholanthrene was administered. It is reassuring with respect to interpretation of current results that RNA, which can serve as an indicator of the presence of RER, was distributed between RER and SER in about the same proportions regardless of whether microsomes were obtained from control rats or from rats which had been given 3-methylcholanthrene.[35]

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