Induction of Drug Metabolism

I. Differences in the Mechanisms by Which Polycyclic Hydrocarbons and Phenobarbital Produce Their Inductive Effects on Microsomal *N*-Demethylating Systems

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(Received October 16, 1968)

SUMMARY

The administration of 3,4-benzpyrene, 3-methylcholanthrene, or phenobarbital to male rats stimulated the *N*-demethylation of 3-methyl-4-methylaminoazobenzene (3-MMAB) by hepatic microsomes, but only phenobarbital stimulated the *N*-demethylation of ethylmorphine. Simultaneous administration of maximum stimulatory doses of 3-methylcholanthrene and phenobarbital resulted in additive stimulation of *N*-demethylation of 3-MMAB, whereas similar treatment with 3,4-benzpyrene and 3-methylcholanthrene did not stimulate 3-MMAB *N*-demethylation beyond that observed when either was administered singly. Thioacetamide prevented increases in *N*-demethylation resulting from phenobarbital administration, but had little effect on increased drug metabolism resulting from 3-methylcholanthrene administration. It was concluded that 3,4-benzpyrene and 3-methylcholanthrene stimulate hepatic microsomal drug-metabolizing activity by the same mechanism and that phenobarbital stimulates drug metabolism through a different mechanism.

During phenobarbital administration parallel increases were observed in the *N*-demethylase activities and in the cytochrome P-450 content of microsomes; when phenobarbital was discontinued, microsomal *N*-demethylase activities and cytochrome P-450 content decreased in a parallel manner. Thioacetamide, administered alone, caused concomitant decreases in *N*-demethylase activities and cytochrome P-450 content. When given with phenobarbital, thioacetamide prevented the usual increases in *N*-demethylase activities and in cytochrome P-450 content seen when phenobarbital is administered. Polycyclic hydrocarbons caused an increase in microsomal P-450 content and in 3-MMAB *N*-demethylase activity, but the increases were not parallel. No increase in ethylmorphine *N*-demethylase activity occurred even though the content of microsomal hemoprotein was elevated. These studies suggested either that cytochrome P-450 is not rate-limiting in the over-all ethylmorphine *N*-demethylation reaction or that the administration of polycyclic hydrocarbons causes the synthesis of a microsomal hemoprotein different from that which was present initially in that it is capable of participating in the *N*-demethylation of 3-MMAB, but not of ethylmorphine.

This research was supported by United States Public Health Service Grant GM-12543. Part of this material has appeared in abstract form [*Pharmacologist* **6**, 186 (1964)] and in a thesis submitted by N. E. Sladek in partial fulfillment of the requirements for the degree of Doctor of Philosophy to the Department of Pharmacology, University of Minnesota, 1966.

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INTRODUCTION

It was recognized early that agents which stimulate increased activity of the microsomal enzymes involved in the metabolism of drugs can be classified into two groups: those which induce the increased metabolism of a wide variety of drugs, and those whose inductive effects are directed toward a much smaller number of substrates (1, 2). The first group is made up largely of drugs and insecticides. Phenobarbital is the agent in this group most frequently employed in induction studies. The second comprises largely polycyclic hydrocarbons. 3-Methylcholanthrene and 3,4-benzpyrene are the most frequently employed agents in the latter group. That phenobarbital and the polycyclic hydrocarbons differ in the specificity of their inductive effects immediately suggests that more than one mechanism of induction may exist.

The *N*-demethylation of 3-methyl-4-methylaminoazobenzene is stimulated by both phenobarbital and 3-methylcholanthrene. If these two agents produce their inductive effects on the *N*-demethylation of 3-methyl-4-methylaminoazobenzene by different mechanisms, and if they are given simultaneously in maximum stimulatory doses, the resulting increase in *N*-demethylating activity should equal the sum of the increases obtained when each is given alone. On the other hand, if a single mechanism is involved, the stimulatory effect seen when both agents are administered together should be no greater than when the more potent of the two is used singly, and it could be less.

Both types of inducing agents are thought to produce their effects by causing an increase in the synthesis of the microsomal enzymes responsible for drug metabolism. This conclusion is based largely on the observation that inhibitors of protein or nucleic acid synthesis, such as ethionine, actinomycin D, and puromycin, prevent induction (3–10). The work of Laird (11) and of Adams and Busch (12) suggested that thioacetamide might also be used to block induction. Thioacetamide caused a marked decrease in microsomal protein and RNA. At the same time RNA accumulated in the nucleus, and it was proposed that the decrease in protein and RNA in the microsomes was due to suppression of the release of RNA from the nucleus. Support for this proposal was provided by Stöcker and Yokoyama (13), who reported that thioacetamide inhibited the migration of RNA labeled with ^{3}H-cytidine from the nucleus to the cytoplasm. Thioacetamide was employed in the current studies with the expectation that it would block induction produced by both phenobarbital and 3-methylcholanthrene. Contrary to expectations, although thioacetamide prevented induction due to phenobarbital, it had little or no effect on induction caused by 3-methylcholanthrene.

For reasons already given, 3-methyl-4-methylaminoazobenzene was used as a substrate in these experiments. Ethylmorphine was also employed because its *N*-demethylation is stimulated by phenobarbital, but not by 3-methylcholanthrene.

Increases in the rates of drug metabolism that result after inducing agents are administered, and the declines in rates that follow when the inducing agents are withdrawn, have been shown to parallel closely the increases and decreases in levels of microsomal cytochrome P-450 (14, 15). Time course studies were therefore conducted to determine the effect of thioacetamide on cytochrome P-450 levels of microsomes from rats treated with 3-methylcholanthrene, 3,4-benzpyrene, and phenobarbital during periods when the inducing agents were administered and for several days after their withdrawal.

MATERIALS AND METHODS

Male Holtzman rats (90–125 g) were employed. Phenobarbital sodium (in 0.9% NaCl) was injected in daily doses of 40 mg/kg for 4 days, or, if a maximum inductive effect was desired, 50 mg of phenobarbital sodium per kilogram were injected twice daily for 5 days. 3,4-Benzpyrene (in corn oil) and 3-methylcholanthrene (in corn oil) were administered in doses of 20 mg/kg/day for 4 days; this amount of either compound produced a

maximum inductive effect. Thioacetamide (in 0.9% NaCl), 50 mg/kg, was administered either alone or with the inducing agents. "Untreated" animals received either corn oil or 0.9% NaCl. All injections were made intraperitoneally.

Preliminary investigations showed that thioacetamide depressed food intake to about 50% of normal. Accordingly, in one series of investigations, where the effect of thioacetamide on induction was studied, pair-fed animals were used. In all other experiments the animals were fed ad libitum.

Livers were removed 20 hr after the last injection or, in the time course studies, at 24-hr intervals after injections were discontinued. The rats were stunned by a blow on the head, decapitated, exsanguinated, and quickly hepatectomized, after which the excised livers were placed in ice-cold 1.15% KCl solution. All further procedures for the preparation of the enzyme were carried out at 0–4°. A 25% homogenate was prepared in 1.15% KCl solution using a Dounce homogenizer fitted with a loose plunger (15 strokes). The homogenate was transferred to polypropylene centrifuge tubes and centrifuged at $9000 \times g_{max}$ for 20 min in a refrigerated Lourdes centrifuge, model LRA (rotor No. 9RA). The supernatant fraction, containing the microsomal plus soluble fraction, was diluted so that each milliliter contained the equivalent of 250 mg of liver tissue when untreated animals were used and the equivalent of 100 mg of liver tissue when animals treated with inducing agents were used. When microsomal cytochrome P-450 levels were determined, a $105,000 \times g$ fraction was prepared from livers perfused *in situ* with cold, 1.15% KCl solution to remove blood. This was prepared by centrifuging the $9000 \times g$ supernatant fraction in a Spinco model L ultracentrifuge (rotor No. 50) at $105,000 \times g_{avg}$ for 60 min at $2° \pm 2°$ and suspending the pellet in a 1.15% KCl solution so that each milliliter of the suspension contained the equivalent of 250 mg of wet liver. All fractions were used on the day they were prepared.

Microsomal ethylmorphine and 3-MMAB[2] *N*-demethylase activities were determined using the following reaction mixture adjusted to pH 7.4: TPN (Sigma), 2 μmoles; potassium phosphate buffer, 2 mmoles; semicarbazide hydrochloride, 37.5 μmoles; nicotinamide, 20 μmoles; magnesium chloride, 10 μmoles; disodium glucose 6-phosphate (Sigma), 20 μmoles; 3-MMAB,[3] 1 μmole, or ethylmorphine hydrochloride, 5 μmoles; 1 ml of tissue preparation; and sufficient 1.15% KCl solution to give a final volume of 5 ml. 3-MMAB was added to the reaction mixture in 10 μl of ethanol. Reactions were started by the addition of tissue preparation. The mixture was incubated with shaking (120 oscillations/min) at 37° in open, 25-ml Erlenmeyer flasks in a Dubnoff metabolic shaker. Incubation times were 10 min when 3-MMAB was the substrate and 15 min when ethylmorphine was used. Reaction rates were linear during these time intervals. The reactions were stopped in one of two ways. When 3-MMAB was used as the substrate, 2- or 4-ml aliquots of the reaction mixture were removed and placed in a 50-ml conical distilling flask containing 4 ml of a freshly prepared 30% trichloracetic acid solution. *N*-Demethylation of ethylmorphine was stopped by the addition of 2 ml of a 5.5% zinc sulfate solution.

Formaldehyde produced by the oxidative *N*-demethylation of ethylmorphine was measured by a modified method of Nash (16) as previously described (17), except that a 5.5% rather than a 5.0% $ZnSO_4 \cdot 7H_2O$ solution was employed. The method of MacFadyen (18) as described by Takemori and Mannering (19) was used for the determination of formaldehyde resulting from *N*-demethylation of 3-MMAB.

Microsomal cytochrome P-450 was determined essentially as described by Omura and Sato (20), using a Beckman

[2] The abbreviation used is: 3-MMAB, 3-methyl-4-methylaminoazobenzene.

[3] This compound was kindly supplied by Dr. J. A. Miller, McArdle Laboratory for Cancer Research, University of Wisconsin.

model DB dual-beam spectrophotometer and quartz cuvettes (1-cm light path). To two cuvettes containing the same fraction of microsomal preparation (equivalent to either 125 or 250 mg of wet liver) and 0.2 M potassium phosphate buffer, pH 7.4, in a volume of 3 ml, were added a few milligrams of solid sodium dithionite. The final pH was 7.0. Carbon monoxide was bubbled through one of the cuvettes for 30 sec. The difference in absorption at 450 mμ minus that at 500 mμ was used as the estimate of cytochrome P-450 concentration.

The glycogen content of the microsomes was determined by the method of Montgomery (21).

RESULTS

Effect of phenobarbital, 3-methylcholanthrene, and 3,4-benzpyrene on 3-MMAB N-demethylation. In agreement with the original observations of Conney and co-workers (3, 5), phenobarbital, 3-methylcholanthrene, and 3,4-benzpyrene treatment caused an increase in the 3-MMAB *N*-demethylase activity of hepatic microsomes (Fig. 1). Simultaneous administration of maximum stimulatory doses of 3-methylcholanthrene and phenobarbital resulted in additive stimulation of enzyme activity, indicating that these compounds produce their inductive effects by different mechanisms. On the other hand, the simultaneous administration of maximum stimulatory doses of 3,4-benzpyrene and 3-methylcholanthrene did not enhance the *N*-demethylation of 3-MMAB above that observed when either polycyclic hydrocarbon was administered singly, thus indicating that these compounds produce their inductive effects by the same mechanism.

Effect of phenobarbital, 3-methylcholanthrene, and 3,4-benzpyrene on ethylmorphine N-demethylation. Administration of 3-methylcholanthrene or 3,4-benzpyrene to male rats did not increase the microsomal *N*-demethylation of ethylmorphine (Fig. 2). As reported previously (22), however, hepatic microsomes from phenobarbital-treated rats showed enhanced ethylmorphine *N*-demethylase activity. In accordance with expectation, simultaneous administration of 3,4-benzpyrene and 3-methylcholanthrene had no stimulatory effect on ethylmorphine *N*-demethylation, nor did the simultaneous administration of 3-methylcholanthrene and phenobarbital stimulate activity above that observed when phenobarbital alone was given.

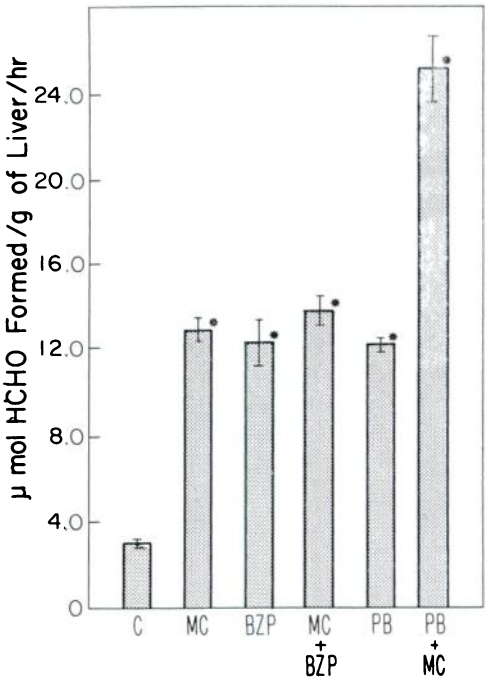

FIG. 1. *Effect of phenobarbital, 3-methylcholanthrene, and 3,4-benzpyrene administration on the N-demethylation of 3-MMAB by hepatic microsomes*

Rats were treated for 4 days with 0.9% NaCl or corn oil (C); 3-methylcholanthrene, 20 mg/kg daily (MC); 3,4-benzpyrene, 20 mg/kg daily (BZP); or phenobarbital sodium, 50 mg/kg twice daily (PB). Each bar represents the mean and standard error of at least five animals.

* $p < 0.01$ when compared to controls (C).

Effects of thioacetamide on stimulation of N-demethylation by phenobarbital and 3-methylcholanthrene. The effects of thioacetamide on the ethylmorphine and 3-MMAB *N*-demethylase activities of hepatic microsomes from untreated, 3-methylcholanthrene-treated, and phenobarbital-treated rats are shown in Figs. 3 and 4.

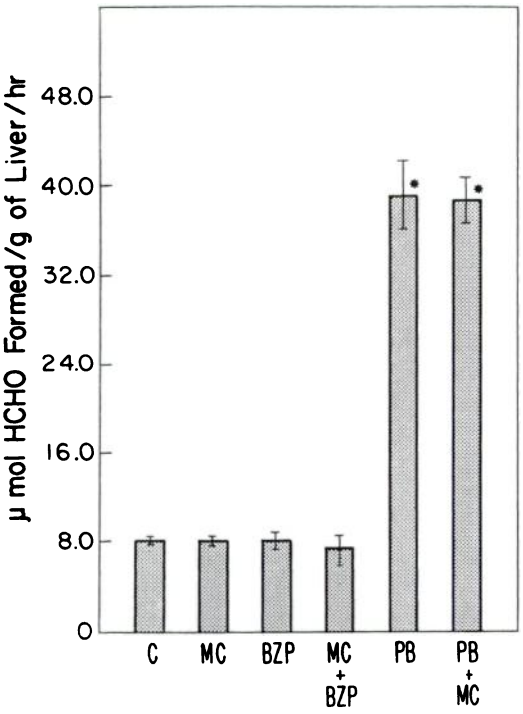

FIG. 2. *Effect of phenobarbital, 3-methylcholanthrene, and 3,4-benzpyrene administration on the N-demethylation of ethylmorphine by hepatic microsomes*

Rats were treated as described in Fig. 1. Each bar represents the mean and standard error of at least five animals. For abbreviations, see the legend to Fig. 1.

* $p < 0.01$ when compared to controls (C).

Thioacetamide alone decreased both *N*-demethylase activities. When thioacetamide was given concurrently with 3-methylcholanthrene, ethylmorphine *N*-demethylase activity was decreased (Fig. 3). This was expected because 3-methylcholanthrene does not stimulate the activity of this enzyme system. When given simultaneously with thioacetamide, phenobarbital no longer produced a stimulatory effect on the *N*-demethylation of ethylmorphine or 3-MMAB (Figs. 3 and 4). In contrast thioacetamide did not prevent the stimulation of 3-MMAB *N*-demethylation by 3-methylcholanthrene (Fig. 4). However, the total *N*-demethylating activity was not as great as when 3-methylcholanthrene was administered without thioacetamide. This need not be interpreted to mean that the stimulatory effect had been partially inhibited by thioacetamide. Evidence will be presented to show that the enzyme system resulting *de novo* from 3-methylcholanthrene administration differs from that which existed initially (23). The 3-MMAB-*N*-demethylating activity seen after 3-methylcholanthrene treatment without thioacetamide administration represents the sum of the activities of the normally occurring enzyme, the level of which is maintained during the course of 3-methylcholanthrene administration, and the enzyme that results *de novo* after 3-methyl-

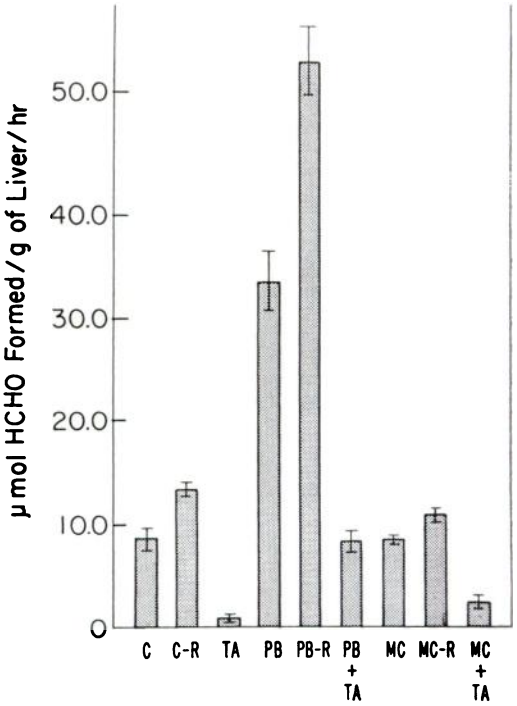

FIG. 3. *Effects of thioacetamide administration on the N-demethylation of ethylmorphine by hepatic microsomes from phenobarbital- and 3-methylcholanthrene-treated rats*

Rats were treated for 4 days with 0.9% NaCl or corn oil (C); thioacetamide, 50 mg/kg daily (TA); phenobarbital sodium, 40 mg/kg daily (PB); or 3-methylcholanthrene, 20 mg/kg daily (MC). All animals were fed ad libitum except where the designation R appears, in which cases the food intake was restricted to the amounts consumed by thioacetamide-treated rats. Each bar represents the mean and standard error of at least four animals.

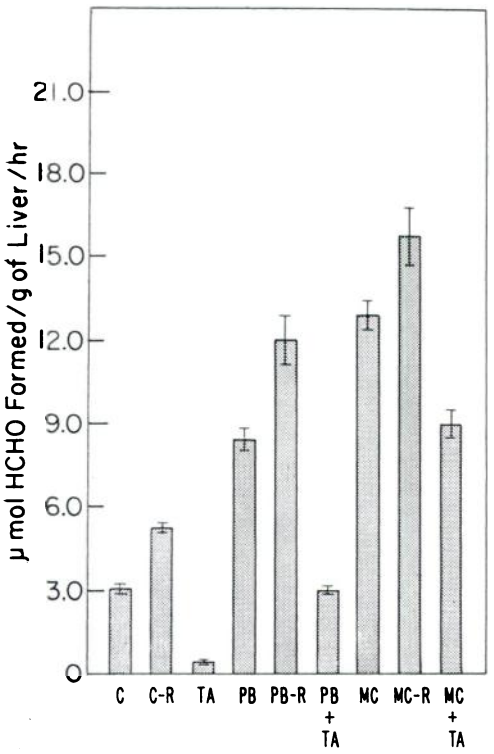

FIG. 4. *Effects of thioacetamide administration on the N-demethylation of 3-MMAB by hepatic microsomes from phenobarbital- and 3-methylcholanthrene-treated rats*

Rats were treated as described in Fig. 3. Each bar represents the mean and standard error of at least four animals. For abbreviations, see the legend to Fig. 3.

cholanthrene treatment. The reduction of 3-MMAB *N*-demethylase activity resulting from the administration of thioacetamide alone can be interpreted to mean that the synthesis of the normally occurring enzyme is prevented by thioacetamide. Accordingly, when thioacetamide is given with 3-methylcholanthrene, the total activity is reduced by about the same amount that had previously been contributed by the normally occurring enzyme. Thus, while the synthesis of the normally occurring enzyme is inhibited by thioacetamide, synthesis of the enzyme system appearing in response to 3-methylcholanthrene does not appear to be inhibited by thioacetamide.

During the course of these experiments, it was noted that thioacetamide decreased food intake by approximately 50%. It was thus considered necessary to repeat the experiments using pair-fed rats. As may be seen in Figs. 3 and 4, restricted feeding increased the levels of the drug-metabolizing enzymes, but net differences obtained with the various treatments were essentially no different from those obtained with animals fed ad libitum. The values in Figs. 3 and 4 are expressed in terms of activity per gram of liver; had they been expressed in terms of activity per milligram of protein, it is conceivable that no differences would have been observed.

Thioacetamide did not exert its effect on the *N*-demethylase activities by causing a deficiency in glucose 6-phosphate dehydrogenase levels, as shown by the failure of exogenous glucose 6-phosphate dehydrogenase (Sigma Chemical Company) to restore activity. When added directly to the incubation medium in a concentration of 1×10^{-4} M, thioacetamide did not inhibit the *N*-demethylation of ethylmorphine or 3-MMAB.

Rats did not lose weight during the 4 days of thioacetamide treatment, but, unlike control animals, they did not gain weight (Table 1). Nevertheless, liver weights were the same in treated and un-

TABLE 1

Effect of thioacetamide treatment on body weight, liver weight, and liver glycogen levels in male rats

Thioacetamide, 50 mg/kg, was administered daily for 4 days. All determinations were made 20 hr after the last injection. Each value represents the mean of four animals.

Treatment	Body wt (A)	Liver wt (B)	B:A × 100	Liver glycogen
	g	*g*	%	*g/100 g*
None, day 0	125	5.33	4.26	
None, day 4	147	6.20	4.22	6.75
Thioacetamide, day 4	119	6.18	5.17	3.29

treated animals. Glycogen levels were reduced about 50%, but returned to normal within 3 days after the cessation of thioacetamide treatment. The rats had a normal appearance during thioacetamide treatment; no overt toxic symptoms were observed.

Effects of thioacetamide on changes in cytochrome P-450 levels produced by polycyclic hydrocarbons and by phenobarbital. Time course studies relating microsomal cytochrome P-450 levels to *N*-demethylase activities are shown in Figs. 5–11. During the 4 days of treatment with thioacetamide, cytochrome P-450 levels and the activities of both *N*-demethylases decreased in a parallel manner until values were only about 10–20% of control values (Fig. 5). When thioacetamide was discontinued, *N*-demethylase activities and cytochrome P-450 levels increased in a parallel manner and reached at least 80% of normal values within 5 days. Assuming from these observations that thioacetamide almost completely blocks the synthesis of cytochrome P-450, it would appear that the half-life of this cytochrome is less than 2 days.

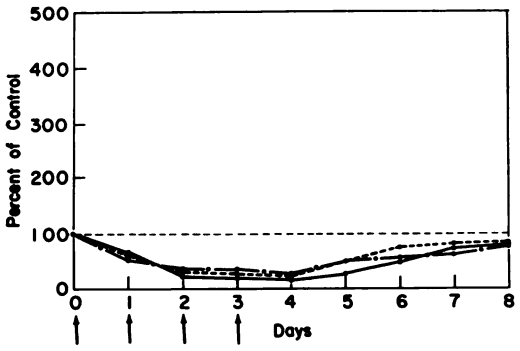

FIG. 5. *Effect of thioacetamide administration on microsomal N-demethylation and cytochrome P-450 levels*

Rats received thioacetamide, 50 mg/kg, daily for 4 days (arrows). O–●–O, Cytochrome P-450 levels; O——O, 3-MMAB *N*-demethylation; O– – –O, ethylmorphine *N*-demethylation. Mean control values were: ethylmorphine *N*-demethylase, 8.1 μmoles of HCHO formed per gram of liver per hour; 3-MMAB *N*-demethylase, 2.9 μmoles of HCHO formed per gram of liver per hour; cytochrome P-450, 0.085 ($\Delta A_{455} - A_{500}$). Each point represents the mean of three animals.

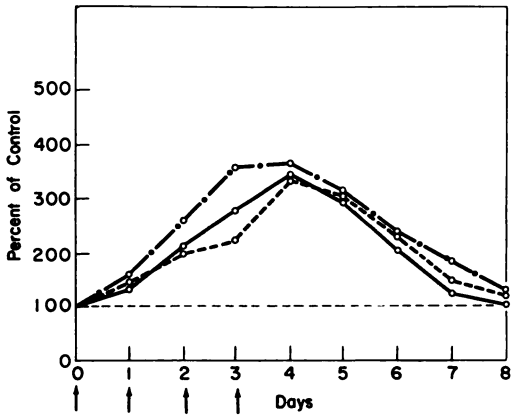

FIG. 6. *Effect of phenobarbital administration on microsomal N-demethylation and cytochrome P-450 levels*

Rats received phenobarbital sodium, 40 mg/kg, daily for 4 days (arrows). O–●–O, Cytochrome P-450 levels; O——O, 3-MMAB *N*-demethylation; O– – –O, ethylmorphine *N*-demethylation. Mean control values are given in Fig. 5. Each point represents the mean of three animals.

The administration of phenobarbital to rats caused parallel increases in ethylmorphine and 3-MMAB *N*-demethylase activities and in the cytochrome P-450 content of microsomes; cessation of phenobarbital treatment resulted in parallel

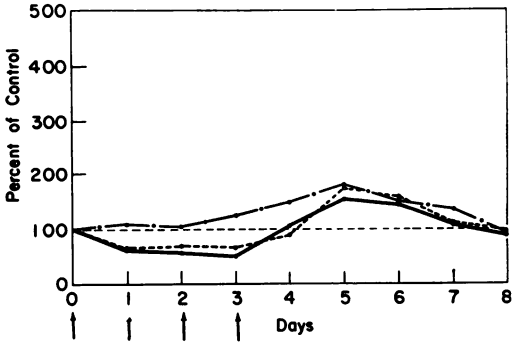

FIG. 7. *Effect of concurrent administration of phenobarbital and thioacetamide on microsomal N-demethylation and cytochrome P-450 levels*

Rats received phenobarbital sodium, 40 mg/kg, plus thioacetamide, 50 mg/kg, daily for 4 days (arrows). O–●–O, Cytochrome P-450 levels; O——O, 3-MMAB *N*-demethylation; O– – –O, ethylmorphine *N*-demethylation. Mean control values are given in Fig. 5. Each point represents the mean of three animals.

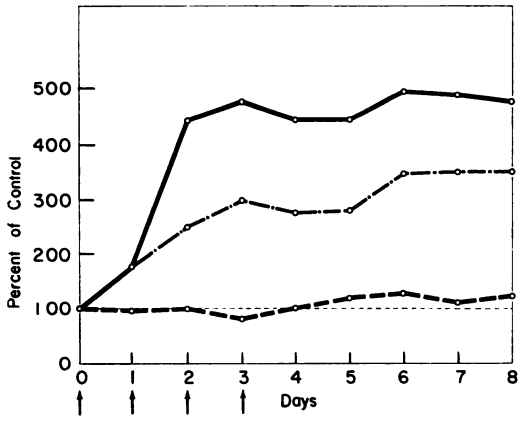

FIG. 8. *Effect of 3-methylcholanthrene administration on microsomal N-demethylation and cytochrome P-450 levels*

Rats received 3-methylcholanthrene, 20 mg/kg, daily for 4 days (arrows). O-·-O, Cytochrome P-450 levels; O——O, 3-MMAB *N*-demethylation; O- - -O, ethylmorphine *N*-demethylation. Mean control values are given in Fig. 5. Each point represents the mean of three animals.

returns to normal values (Fig. 6). Thioacetamide administered concurrently with phenobarbital prevented the increases in cytochrome P-450 content and in both *N*-demethylase activities (Fig. 7).

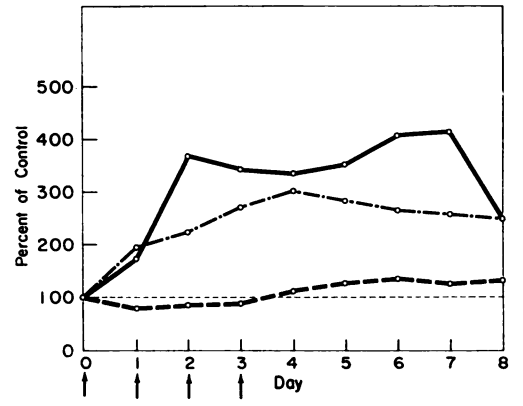

FIG. 9. *Effect of 3,4-benzpyrene administration on microsomal N-demethylation and cytochrome P-450 levels*

Rats received 3,4-benzpyrene, 20 mg/kg, daily for 4 days (arrows). O-·-O, Cytochrome P-450 levels; O——O, 3-MMAB *N*-demethylation; O- - -O, ethylmorphine *N*-demethylation. Mean control values are given in Fig. 5. Each point represents the mean of three animals.

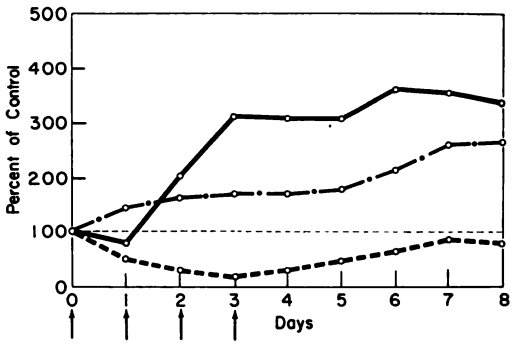

FIG. 10. *Effect of concurrent administration of 3-methylcholanthrene and thioacetamide on microsomal N-demethylation and cytochrome P-450 levels*

Rats received 3-methylcholanthrene, 20 mg/kg, plus thioacetamide, 50 mg/kg, daily for 4 days (arrows). O-·-O, Cytochrome P-450 levels; O——O, 3-MMAB *N*-demethylation; O- - -O, ethylmorphine *N*-demethylation. Mean control values are given in Fig. 5. Each point represents the mean of three animals.

Parallel increases in the two *N*-demethylase activities with increases in cytochrome P-450 content were not seen when 3-methylcholanthrene or 3,4-benzpyrene was employed as the inducing agent (Figs. 8 and 9). In agreement with previous results, no increases in ethylmorphine *N*-

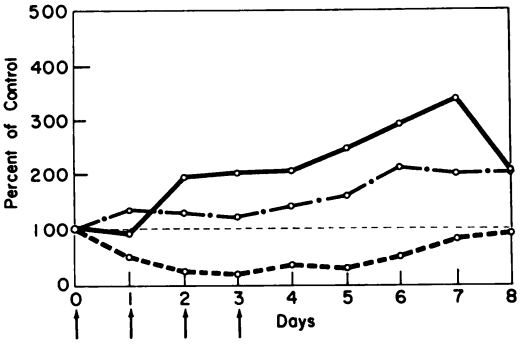

FIG. 11. *Effect of concurrent administration of 3,4-benzpyrene and thioacetamide on microsomal N-demethylation and cytochrome P-450 levels*

Rats received 3,4-benzpyrene, 20 mg/kg, plus thioacetamide, 50 mg/kg, daily for 4 days (arrows). O-·-O, Cytochrome P-450 levels; O——O, 3-MMAB *N*-demethylation; O- - -O, ethylmorphine *N*-demethylation. Mean control values are given in Fig. 5. Each point represents the mean of three animals.

demethylase activities were observed. The increases in 3-MMAB *N*-demethylase activities were considerably greater than the increases in cytochrome P-450 content. Results were quite similar when thioacetamide was given simultaneously with the polycyclic hydrocarbons, but all values were lower (Figs. 10 and 11). These values were lower by nearly the amount observed when thioacetamide alone was administered. It is to be noted that 3-MMAB *N*-demethylase activity and cytochrome P-450 levels did not return to normal values within the 5 days following the cessation of treatment with the polycyclic hydrocarbons, as was the case when phenobarbital treatment was discontinued. This more persistent effect of the polycyclic hydrocarbons has usually been explained on the basis that these compounds are stored in fat depots and released over a prolonged period. An alternative explanation might be that the hemoprotein formed as a result of polycyclic hydrocarbon administration is less readily degraded than that formed as a result of phenobarbital administration.

DISCUSSION

Gillette (24, 25) determined the activities of several drug-metabolizing enzymes in hepatic microsomes from immature female rats receiving maximum stimulatory doses of 3,4-benzpyrene and phenobarbital, administered singly or in combination. Microsomes from rats given both 3,4-benzpyrene and phenobarbital metabolized acetanilide, monomethyl-4-aminoantipyrene, zoxazolamine, and phenacetin more rapidly than did microsomes from rats given only phenobarbital or 3,4-benzpyrene, although these effects were not additive in all cases. On the other hand, microsomes from rats that received maximum stimulatory doses of both 3,4-benzpyrene and 3-methylcholanthrene oxidized zoxazolamine, acetanilide, and phenacetin at about the same rates as did those from animals given either 3,4-benzpyrene or 3-methylcholanthrene alone. Hart and Fouts (26) reported that simultaneous administration of chlordane and phenobarbital to male rats did not stimulate the metabolism of hexobarbital or aminopyrine beyond the rate observed when either was given alone. Mullen *et al.* (27) observed additive stimulatory effects on zoxazolamine hydroxylase activity when chlordane was administered with either 3,4-benzpyrene or 3-methylcholanthrene. These studies and the present investigation strengthen the view that inducing agents such as phenobarbital and chlordane stimulate the metabolism of drugs by one mechanism and that the polycyclic hydrocarbons stimulate drug metabolism by a different mechanism.

There are several ways by which inducing agents can increase enzyme activity: (a) by increasing enzyme synthesis, (b) by causing the synthesis of a more active enzyme, (c) by directly activating existing enzyme, (d) by indirectly activating existing enzyme, or (e) by stabilizing the enzyme. Agents that induce drug metabolism are not effective *in vitro*, and experiments designed to show that they produce their effects by altering the level of some enzyme activator or inhibitor were negative (28). The stimulatory effect of these compounds is not mediated through hormones of the testis, adrenal, pituitary, or thyroid gland, since removal of these glands does not prevent induction of drug metabolism (3, 29, 30). All these findings argue against direct or indirect activation of the enzymes immediately involved in drug metabolism. Because inhibitors of protein or nucleic acid synthesis, such as ethionine, actinomycin D, and puromycin, also inhibit the stimulation of drug-metabolizing enzymes by agents such as phenobarbital and the polycyclic hydrocarbons, it has been argued that induction is due to increased synthesis of enzyme protein (3–10). However, the concept of increased enzyme synthesis is not required to explain induction; increased activity would also result if the stimulatory compounds stabilized the drug-metabolizing enzymes to resist catabolism or if they prevented the synthesis of the catabolizing enzymes. Blockers of protein synthesis would still prevent induction because they would prevent the accumulation of the new enzyme being formed at its usual rate.

Another possibility to be considered is that synthesis may take place in such a way that there is no net increase in the amount of enzyme but that the newly formed enzyme possesses a higher specific activity. Induction of alkaline phosphatase activity in growing HeLa cells by prednisolone appears to occur in this manner (31). This mechanism might better explain the action of 3-methylcholanthrene, which, unlike phenobarbital, produces its inductive effects without causing great increases in microsomal protein (3, 8, 32, 33).

Actinomycin D, an inhibitor of DNA-directed RNA synthesis, inhibits induction by both phenobarbital and 3-methylcholanthrene. Thus, it has been postulated that these agents initiate their inductive effects at the transcriptional level of protein synthesis (7, 8). If this hypothesis is correct, and if thioacetamide depresses microsomal protein synthesis by suppressing the release of RNA from the nucleus (12, 13), thioacetamide should also inhibit induction of drug metabolism by phenobarbital or 3-methylcholanthrene since it would prevent the release of mRNA from the nucleus into the cytoplasm. In view of these considerations, some explanation is needed to account for the finding that thioacetamide prevented induction by phenobarbital, but not by 3-methylcholanthrene. The following speculations are offered.

1. Phenobarbital and 3-methylcholanthrene each might cause the synthesis of a unique mRNA; the release from the nucleus of the mRNA caused by phenobarbital treatment might be prevented by thioacetamide, but that caused by 3-methylcholanthrene treatment might not. This hypothesis requires that the two inducing agents cause the synthesis of different messages which code for the synthesis of two different enzymes *N*-demethylating 3-MMAB. Evidence for the presence of two such enzymes in rat liver will be presented (23).

2. Phenobarbital might stimulate induction at the transcriptional level of protein synthesis, but 3-methylcholanthrene might cause its effect at the translational level. This hypothesis requires that phenobarbital increase the synthesis of a relatively unstable mRNA. Thioacetamide would block phenobarbital induction by preventing the release of this unstable message from the nucleus. 3-Methylcholanthrene would not induce enzyme activity by this mechanism, but would act by allowing the expression of a relatively stable cytoplasmic mRNA. Although thioacetamide would be expected to block the release of this mRNA from the nucleus, an immediate supply from the nucleus would not be necessary for induction to occur. If 3-methylcholanthrene acts through a relatively stable cytoplasmic mRNA, the thioacetamide block at the level of mRNA release from the nucleus would be circumvented temporarily. The existence of unstable and stable mRNA is well documented (34). It is understandable that actinomycin D should inhibit phenobarbital induction, for it is known to act at the transcriptional level. If, as this hypothesis demands, however, 3-methylcholanthrene causes its effect at the translational level, some explanation is needed for the action of actinomycin D in preventing induction by 3-methylcholanthrene. The well-established action of actinomycin D on mRNA synthesis does not exclude the possibility that it may also affect other cellular mechanisms.

3. A third possibility is that the mRNA involved in phenobarbital stimulation is templated in the nucleus, but that involved in 3-methylcholanthrene stimulation is templated in the cytoplasm. Thus actinomycin D, which inhibits transcription, and puromycin and ethionine, which inhibit translation, would inhibit induction by both agents, but thioacetamide, which prevents the release of mRNA from the nucleus, would prevent induction by phenobarbital but not by 3-methylcholanthrene. This hypothesis is consistent with recent reports that RNA is templated off cytoplasmic DNA and the suggestion that this RNA codes for the synthesis of microsomal and mitochondrial membrane protein (35).

4. A fourth possibility is that the enzyme activity resulting from phenobarbital treatment is sensitive to some other cellular effect of thioacetamide, e.g., alteration of

the endoplasmic reticulum (36), whereas that resulting from 3-methylcholanthrene treatment is not.

The thioacetamide-treated rats appeared quite normal grossly and did not lose weight, although they did not gain weight at the normal rate. This suggests that thioacetamide does not have an all-or-none effect on protein synthesis, but that its action is selective. Conceivably, the inductive effects of both phenobarbital and the polycyclic hydrocarbons might have been achieved if higher doses of thioacetamide or a different injection schedule had been employed. In any event, with the doses and dosage schedule used in the current study, it can be said that thioacetamide was selective in its inhibition of the inductive effects caused by phenobarbital and the polycyclic hydrocarbons, and this serves to distinguish the two inductive processes.

The parallel increases in rates of drug metabolism and cytochrome P-450 levels during the administration of phenobarbital and their subsequent parallel decreases to normal values after the withdrawal of the barbiturate (14, 15) have led to the conclusion not only that cytochrome P-450 is a necessary component of the drug-metabolizing system, but that it may be the rate-limiting component. The observation that the polycyclic hydrocarbons cause an increase in cytochrome P-450 content without increasing ethylmorphine *N*-demethylase activity (Figs. 8 and 9) suggests either that cytochrome P-450 is not rate-limiting in the over-all reaction involving the *N*-demethylation of ethylmorphine in microsomes from untreated rats or that the polycyclic hydrocarbons cause the production of a new cytochrome P-450 different from that which existed previously in that it functions in the *N*-demethylation of 3-MMAB, but not of ethylmorphine. Evidence that the latter possibility is the case is presented in the following publication (23).

ACKNOWLEDGMENT

The authors gratefully acknowledge the able technical assistance of Mrs. Sheila Ham.

REFERENCES

1. A. H. Conney, *Pharmacol. Rev.* **19**, 317 (1967).
2. G. J. Mannering, *in* "Pharmacological Testing Methods" (A. Burger, ed.), p. 51. Dekker, New York, 1968.
3. A. H. Conney, E. C. Miller and J. A. Miller, *Cancer Res.* **16**, 450 (1956).
4. A. H. Conney, E. C. Miller and J. A. Miller, *J. Biol. Chem.* **228**, 753 (1957).
5. A. H. Conney, C. Davison, R. Gastel and J. J. Burns, *J. Pharmacol. Exp. Ther.* **130**, 1 (1960).
6. S. Orrenius and L. Ernster, *Biochem. Biophys. Res. Commun.* **16**, 60 (1964).
7. H. V. Gelboin and N. R. Blackburn, *Cancer Res.* **24**, 356 (1964).
8. A. H. Conney and A. G. Gilman, *J. Biol. Chem.* **238**, 3682 (1963).
9. A. H. Conney, *Proc. 2nd Int. Pharmacol. Meeting, Prague* **4**, 277 (1965).
10. S. Orrenius, J. L. E. Ericsson and L. Ernster, *J. Cell Biol.*, **25**, 627 (1965).
11. A. K. Laird, *Arch. Biochem. Biophys.* **46**, 119 (1953).
12. H. R. Adams and H. Busch, *Cancer Res.* **23**, 576 (1963).
13. E. Stöcker and T. Yokoyama, *Beitr. Pathol. Anat. Allg. Pathol.* **134**, 1 (1966).
14. S. Orrenius, *J. Cell Biol.* **26**, 725 (1965).
15. H. Remmer and H. J. Merker, *Ann. N. Y. Acad. Sci.* **123**, 79 (1965).
16. T. Nash, *Biochem. J.* **55**, 416 (1953).
17. M. W. Anders and G. J. Mannering, *Mol. Pharmacol.* **2**, 319 (1966).
18. D. A. MacFadyen, *J. Biol. Chem.* **158**, 107 (1945).
19. A. E. Takemori and G. J. Mannering, *J. Pharmacol. Exp. Ther.* **123**, 171 (1958).
20. T. Omura and R. Sato, *J. Biol. Chem.* **239**, 2379 (1964).
21. R. Montgomery, *Arch. Biochem. Biophys.* **67**, 378 (1957).
22. A. Rubin, T. R. Tephly and G. J. Mannering, *Biochem. Pharmacol.* **13**, 1007 (1964).
23. N. E. Sladek and G. J. Mannering, *Mol. Pharmacol.* **5**, ??? (1969).
24. J. R. Gillette, *Advan. Enzyme Regul.* **1**, 215 (1963).
25. J. R. Gillette, *Progr. Drug Res.* **6**, 13 (1963).
26. L. G. Hart and J. R. Fouts, *Biochem. Pharmacol.* **14**, 263 (1965).
27. J. O. Mullen, M. R. Juchau and J. R. Fouts, *Biochem. Pharmacol.* **15**, 137 (1966).
28. A. H. Conney and J. J. Burns, *Advan. Enzyme Regul.* **1**, 189 (1963).

29. A. H. Conney, I. A. Michaelson and J. J. Burns, *J. Pharmacol. Exptl. Ther.* **132**, 202 (1961).
30. A. H. Conney and L. Garren, *Biochem. Pharmacol.* **6**, 257 (1961).
31. M. J. Griffin and R. P. Cox, *Proc. Nat. Acad. Sci. U. S. A.* **56**, 946 (1966).
32. J. C. Arcos, A. H. Conney and N. P. Buu-Hoi, *J. Biol. Chem.* **236**, 1291 (1961).
33. J. R. Fouts and L. A. Rogers, *J. Pharmacol. Exp. Ther.* **147**, 112 (1965).
34. H. C. Pitot, C. Peraino, C. Lamar, Jr., and A. L. Kennan, *Proc. Nat. Acad. Sci. U. S. A.* **54**, 845 (1965).
35. B. Attardi and G. Attardi, *Proc. Nat. Acad. Sci. U. S. A.* **58**, 1051 (1967).
36. W. Thoenes and P. Bannasch, *Virchows Arch. Pathol. Anat. Physiol. Klin. Med.* **335**, 556 (1962).