DRUGS AND ENZYME INDUCTION[1,2]

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In a short review, it is not possible to cover all aspects of "enzyme induction," but instead, this review will concentrate on certain facets of the induction of liver microsomal enzymes which have not been reviewed previously in the *Annual Review of Pharmacology*. The areas which will be covered include (*a*) certain aspects of the effect of drugs on the metabolism of foreign chemicals; and (*b*) the effect of drugs on the metabolism and concentration of normal body constituents such as steroids, sterols, fatty acids, microsomal hemoproteins, bilirubin, thyroxin, and melatonin. The possible therapeutic implications which arise as a result of this action will be discussed. Recently, comprehensive reviews have been written by Conney and by Mannering (1, 2) on various aspects of microsomal enzyme induction, and other material on enzyme induction can be found in these reviews.

Effect of Drugs on the Metabolism of Foreign Chemicals

Enzyme induction in chronic toxicity studies.—During chronic drug therapy, a gradual decline in the plasma level of the drug may be observed. This effect, which results from the ability of some compounds to stimulate their own metabolism by increasing liver microsomal enzymes was recently reviewed (1), and a discussion of the mechanisms responsible for the increased enzyme activity was presented. Recent studies have shown that benzypyrene hydroxylase activity is increased by phenothiazines and polycyclic hydrocarbons in organ and tissue culture, indicating the lack of hormonal control of the induction process (3, 4). This adaptation of the animal to the repeated administration of foreign compounds has special significance in chronic toxicity studies. For the first few days during the daily administration of phenylbutazone to dogs, a high plasma concentration of drug was found and certain side effects such as bloody stools and vomiting were observed. However, as the chronic administration continued, the plasma level of phenylbutazone decreased, and the side effects disappeared. When the daily dose was increased, the plasma level of phenylbutazone increased, and serious side effects were again observed (5, 6). Similarly, when rats were given 150 mg/kg of phenylbutazone, the plasma level of the drug the following day was 57 μg/ml and gastric ulcers were observed in 66 percent of

[1] The survey of literature pertaining to this review was concluded in May 1968.
[2] Abbreviations and symbols used: 3-MC *for* 3-methylcholanthrene.

the rats. After chronic administration of phenylbutazone for 2 weeks, gastric ulcers were not found and a plasma level of only 15 μg/ml of phenylbutazone was observed. Chronic treatment with phenobarbital also decreased the ulcerogenic action of a subsequent dose of phenylbutazone and decreased the plasma level of phenylbutazone. When the dose of phenylbutazone was increased in rats pretreated with phenobarbital, phenylbutazone again caused the formation of ulcers (5, 6). Zbinden (7) has found that the peritoneal adhesions and fibrinous peritonitis induced by an intraperitoneal injection of phenylbutazone in the rat were much less severe in rats that were chronically treated with phenylbutazone for 17 to 60 days and he has suggested that this effect may be due to an increased metabolism of phenylbutazone locally in the peritoneal cells rather than through increased metabolism by the liver. Many "foreign" compounds stimulate their own metabolism or the metabolism of other drugs. Among these are phenylbutazone, chlorcyclizine, probenecid, tolbutamide, hexobarbital, pentobarbital, phenobarbital, aminopyrine, meprobamate, glutethimide, chlorpromazine, chlordiazepoxide, DDT, methoxyflurane, 3,4-benzpyrene, and 9,10-dimethyl-1,2-benzanthracene (1). Chronic treatment with such compounds would be expected to decrease their effectiveness and their toxicity. Because of this, when toxicity studies with a new drug are planned one should consider including a high dose of the drug for only a few days, in addition to the usual chronic study.

Enzyme induction by environmental chemicals.—Several substances present in our environment have been shown to stimulate the metabolism of drugs and other foreign chemicals. These include insecticides and other agricultural chemicals, food additives, and polycyclic hydrocarbons found in polluted city air, cigarette smoke, and certain cooked foods (8, 9). During toxicity and metabolism studies care should be taken to exclude halogenated hydrocarbon insecticides from animals used for these studies, since these compounds have been shown to increase the metabolism of drugs (8–10). If insecticide is needed in animal quarters, the compound of choice would be pyrethrum, which has been shown not to stimulate drug metabolism (11). In some laboratories, animals are kept in cages that contain cedar shavings. These shavings increase liver microsomal enzymes and the use of cedar shavings should, therefore, be avoided (12, 13).

Since 3,4-benzpyrene and several other polycyclic aromatic hydrocarbons are potent inducers of benzpyrene hydroxylase in rat liver and are environmental carcinogens that are present in tobacco smoke, experiments were carried out to determine the effect of cigarette smoke on the metabolism of 3,4-benzpyrene by enzymes in human placenta. The enzymatic hydroxylation of 3,4-benzpyrene was not detected in human placentas obtained after childbirth from women who did not smoke cigarettes, whereas this enzyme activity was present in all placentas obtained from individuals who smoked cigarettes (9). The induction of carcinogen-metabolizing enzymes, such as benzpyrene hydroxylase, has been suggested as a mechanism through which

people protect themselves and their fetuses from polycyclic hydrocarbons found in their environment (9).

Enzyme induction in multiple drug therapy.—Since patients are often given several drugs at the same time, it is possible that one of the drugs may stimulate the metabolism of a second drug and decrease its action. In addition, removal of an enzyme inducer from patients treated with drug combinations may be hazardous. A classic example of this is in patients treated with barbiturates and anticoagulants. When a patient receiving 75 mg/day of bishydroxycoumarin was given in addition, 60 mg/day of phenobarbital, a substantial decrease in the plasma level of bishydroxycoumarin and a decrease in anticoagulant activity was observed. When phenobarbital was discontinued, the plasma level of the anticoagulant and the prothrombin time returned to their pre-phenobarbital values (14). Goss & Dickhaus have reported that phenobarbital treatment increased the dosage of bishydroxycoumarin required for anticoagulant maintenance in eight patients with ischemic heart disease (15), and Robinson & MacDonald have shown that the administration of phenobarbital to patients antagonized the anticoagulant response to warfarin (16). The possible hazardous consequences following the removal of phenobarbital from a drug combination has recently been reported by Welch, Harrison & Burns (17). Bishydroxycoumarin was administered to dogs until a constant plasma level and prothrombin time was obtained. Then phenobarbital treatment was started and the plasma level of bishydroxycoumarin and its anticoagulant activity decreased. The dose of anticoagulant was then increased five times without any ill effects. However, when phenobarbital administration was stopped and the high dose of bishydroxycoumarin continued, severe hemorrhage resulted. More recently, Hunningshake & Azarnoff have shown that the administration of glutethimide, amobarbital, secobarbital, and meprobamate to dogs markedly reduced the plasma half-life of warfarin and increased the warfarin required for maintenance of adequate anticoagulation (18). That this type of situation also occurs in man has recently been implied by MacDonald & Robinson (19). They have suggested that in 14 of 67 bleeding reactions observed in humans on anticoagulant therapy, the causative factor was barbiturate interference with anticoagulant activity by enzyme induction. Examples of drug combinations in which one drug alters the disposition of the second drug in man are: phenobarbital-diphenylhydantoin, phenobarbital-griseofulvin, phenylbutazone-aminopyrine, barbiturates-Dipyrone, and phenobarbital-digitoxin (1). In each case, evidence was presented that the first drug stimulates the metabolism of the second drug.

Effect of Drugs on the Metabolism and Concentration of Normal Body Constituents

Steroids.—Studies in several laboratories have shown that liver microsomes contain NADPH-dependent enzyme systems which hydroxylate steroids such as estradiol, testosterone, androsterone, progesterone, and the

corticoids (see 1). Additional studies have demonstrated many similarities between the enzyme systems which hydroxylate steroids and those which oxidize drugs, and these observations suggested that steroid hormones are naturally occurring substrates for drug-metabolizing enzymes in liver microsomes (20). Further support for this concept came fom finding that the Michaelis constants for the hydroxylation of testosterone, estradiol, and progesterone were lower than those for several representative drug substrates (21). More recent studies by Tephly & Mannering have shown that estradiol, testosterone, androsterone, progesterone, and hydrocortisone competitively inhibit the oxidation of ethylmorphine and hexobarbital by rat liver microsomes and that the K_i for this inhibition is similar to the K_m previously reported for the hydroxylation of these steroids (22). These results have led Tephly & Mannering to conclude that certain drugs and steroids are alternate substrates for a common microsomal mixed-function oxidase system. Studies on the microsomal enzyme systems which hydroxylate drugs have shown that these enzymes are inhibited by carbon monoxide and that this inhibition can be reversed by monochromatic light at 450 mμ (23) (discussed further under *Microsomal hemoprotein*). Further evidence supporting the concept that drugs and steroids are hydroxylated by the same enzyme system in liver microsomes was obtained from studies which showed that the hydroxylation of testosterone in the 6β-, 7α- and 16α-positions of the steroid nucleus is inhibited by carbon monoxide and that this inhibition is also reversed by monochromatic light at 450 mμ (24, 25).

Studies during the last 10 years have shown that the chronic administration of many drugs causes a nonspecific increase in the metabolism of drugs by microsomal enzymes in the liver, and thereby decreases the duration and intensity of drug action *in vivo* (see 1). Therefore, enhanced steroid hydroxylation in animals treated chronically with drugs would be an important consequence of the hypothesis that drugs and steroids are metabolized by the same enzymes. Treatment of animals with phenobarbital increased the hydroxylation *in vitro* of testosterone (26), Δ^4-androstene-3,17-dione (26), estradiol-17β (20), estrone (27), progesterone (28, 29), deoxycorticosterone (29), cortisol, corticosterone, and cortisone (30, 31) by enzymes in liver microsomes. The administration of many drugs and insecticides which stimulate the oxidative metabolism of drugs also increased the hydroxylation of steroids. These included diphenylhydantoin, chlorcyclizine, norchlorcyclizine, orphenadrine, phenylbutazone, chlordane, DDT, and *o,p'*-DDT (27, 29, 31, 32). The administration of DDT (10 ppm) or dieldrin (2 ppm) for 1 week to male and female pigeons stimulated the metabolism of testosterone and progesterone by pigeon liver microsomes (33). The stimulatory effect of insecticides on steroid metabolism in birds may help to explain the decrease in the population of certain species of birds which has been observed recently.

The increase in steroid hydroxylation *in vitro* caused by chronic drug

treatment is paralleled by increased metabolism and decreased physiological action of steroids *in vivo*. The increased hydroxylation of progesterone caused by phenobarbital treatment is associated with a decreased anesthetic action of progesterone and decreased amounts of this steroid and its metabolites in the brain and total body of the rat (29, 34). Phenobarbital treatment for 4 days also decreased the central nervous system activity of other steroids such as deoxycorticosterone, androsterone, Δ^4-androsterone, Δ^4-androstene-3,17-dione, and testosterone. Similarly, chronic treatment of rats with chlorcyclizine, phenylbutazone, chlordane, and DDT shortened the anesthetic action of progesterone and increased its metabolism by liver microsomal enzymes (29, 34).

The administration of physiological amounts of estradiol to immature female rats increased uterine weight, protein synthesis, and the activity of some enzymes in the uterus such as phosphofructokinase (35). Chronic treatment with phenobarbital decreased the effect of estradiol in eliciting these responses (27, 36–38), but a single high dose of phenobarbital administered 1 hr before the steroid did not inhibit estradiol action (27). Chronic phenobarbital treatment also inhibited the uterotropic action of estrone and several synthetic estrogens and progestational steroids, used as oral contraceptives (27, 39). The decreased activity of estrogens in phenobarbital-pretreated rats was associated with decreased amounts of estrogen in the uterus and with increased metabolism of the steroid *in vivo* by the whole animal (27, 40). When estrone was administered to control rats, 18 per cent of the dose remained unmetabolized after 40 min; in contrast, following the administration of the steroid to rats previously treated with 75 mg/kg of phenobarbital daily for 4 days, only 4 percent of the steroid was unmetabolized after 40 min. The smallest amount of phenobarbital which inhibited estrogen action, decreased the amount of estradiol or estrone in the uterus, and increased the hydroxylation of these steroids *in vitro* was 2 to 5 mg/kg per day for 4 days. The administration of norchlorcyclizine, chlorcyclizine, phenylbutazone, orphenadrine, and chlordane for 4 days also decreased the effect of estradiol on the weight of the uterus and decreased the amount of estradiol found in the uterus following the administration of physiological amounts of tritiated estradiol. Drug treatment with all of the above compounds increased the liver microsomal metabolism of estradiol and estrone (27). Chronic treatment with pentobarbital inhibited the precocious ovulation elicited in immature female rats following estradiol treatment (41), and it is possible that this effect was caused by enhanced metabolism of estrogens.

Some studies have suggested that barbiturate administration may alter the action of endogenous estrogens. Fahim, King & Hall (42) have shown that the administration of phenobarbital (50 mg/kg per day) for 4 days to adult female mice markedly decreased the weight of the uterus when no exogenous estradiol was administered, and they suggested that this effect of phenobarbital was caused by a stimulation of the metabolism of endogenous

estradiol. Interestingly, the decrease in uterine weight was not observed in ovariectomized mice. Welch, Levin & Conney have also noted an inhibitory effect of phenobarbital treatment on uterine weight in rats not treated with exogenous estrogen. During the postnatal development of female rats, the weight of the uterus increases with age; however, when phenobarbital was included in the food of these developing rats, the rate of increase in uterine size was decreased for several days when the rats reached 10 days (68 gm) of age (43).

Pretreatment of immature rats with phenobarbital decreased the action of exogenous androgens. Treatment with 75 mg/kg of phenobarbital per day inhibited the growth-promoting effect of testosterone propionate, testosterone, 17α-methyl-testosterone, and fluoxymesterone on the seminal vesicles. It was also found that the increase in the weight of the seminal vesicle which occurs as rats mature was inhibited in animals treated with phenobarbital (44).

Not only can treatment of animals with drugs alter the metabolism of steroids, but several reports indicate that treatment with steroids can alter the metabolism of drugs. The administration of progesterone and norethynodrel to rats 1 to 2 hr prior to sacrifice inhibited the metabolism of hexobarbital and zoxazolamine by liver microsomes, while enhanced metabolism of hexobarbital and zoxazolamine was observed when rats were treated with norethynodrel at 18 to 48 hr before sacrifice (45). Studies in women on the urinary excretion of pethidine, promazine and their metabolites have shown that the metabolism of these drugs was deficient during pregnancy and that their metabolism could be influenced by some oral contraceptive agents (46). In pregnancy, more unchanged and less demethylated metabolites of promazine and pethidine were found in the urine. A circadian pattern of drug metabolism with a maximum and minimum separated by about 12 hr was recently observed in normal male and female rats and this rhythm was abolished by adrenalectomy (47). The authors suggested that this was caused by the diurnal variation in corticosteroid level in the rat.

The induction of liver microsomal oxidases following chronic drug treatment suggests possible therapeutic uses for "enzyme induction" in cases of steroid hormone overproduction. Werk, MacGee & Sholiton (48) have shown that treatment of humans with diphenylhydantoin increased the urinary excretion of 6β-hydroxycortisol and decreased the excretion of the less polar cortisol metabolites. Other drugs shown recently to produce this same effect are phenobarbital (49), phenylbutazone (50), *o,p'*-DDD (51, 52), and N-phenylbarbital (53). The finding that chronic treatment of guinea pigs with several of these drugs stimulated the formation of 6β-hydroxycortisol from cortisol by liver microsomal enzymes (31, 53) suggested a possible explanation for the increased excretion of 6β-hydroxycortisol in man. Treatment of fourteen-month-old female guinea pigs with phenobarbital (75 mg/kg per day) for 18 days increased 3.8 or 3.6 times respectively the initial rates of 6β-hydroxylation and 2α-hydroxylation of

cortisol (54). When male guinea pigs were given phenobarbital (64 mg/kg per day) for only 5 days, an increase in 2α-hydroxylation of cortisol was observed, but the increase in 6β-hydroxylation was not significant (55).

The observation that compounds which stimulate drug metabolism also increase the excretion of 6β-hydroxycortisol in man has led to the suggestion that the level of urinary 6β-hydroxycortisol in man may be useful as a measure of "enzyme induction." In humans, less than 400 μg of 6β-hydroxycortisol are excreted in the urine per day, but after treatment of eight subjects with either N-phenylbarbital or phenylbutazone, the excretion of 6β-hydroxycortisol always exceeded this amount (50, 53).

Studies by Werk, Sholiton & Olinger (56) and Southren et al. (52) have suggested that the increased metabolism of cortisol observed in man following diphenylhydantoin or *o,p'*-DDD treatment may have therapeutic value in the treatment of Cushing's syndrome. A decrease in the biochemical and clinical signs of this disease was observed following treatment of two subjects with 300 to 400 mg per day of diphenylhydantoin for 3 months (56). The administration of *o,p'*-DDD also reduced the symptoms of Cushing's syndrome before the drug produced any effect on the rate of cortisol secretion from the adrenal gland (52). Southren has suggested that the increased amounts of hydroxylated cortisol metabolites which are formed following treatment with these compounds may inhibit the action of cortisol and thereby ameliorate the symptoms of the Cushing's syndrome (52).

Bilirubin.—Compounds which stimulate drug and steroid metabolism also stimulate the metabolism of bilirubin in animals (1). Catz & Yaffe have found that following chronic administration of barbital to mice, a 2.5-fold increase in hepatic bilirubin-conjugating activity occurred (57). Roberts & Plaa have shown that treatment of mice with phenobarbital enhanced the disappearance of exogenously administered bilirubin from the plasma. Indirect evidence obtained in their study suggests that increased bilirubin conjugation plays a role in the enhanced hepatic uptake and excretion of bilirubin after phenobarbital treatment (58). The hyperbilirubinemia found after the administration of the antibiotic, novobiocin, could be reduced by prior treatment with phenobarbital; however, this effect may be due to either the increased metabolism of bilirubin or novobiocin (59).

The finding that barbiturates increased bilirubin conjugation in animals suggested that they might have a therapeutic effect in diseases of hyperbilirubinemia in man. Yaffe et al. (60) and Crigler & Gold (61) recently found that, in two infants with congenital nonhemolytic jaundice, treatment with 15 mg of phenobarbital two to three times a day caused a decrease in the plasma bilirubin levels and a disappearance of the jaundice (60, 61). When the treatment with phenobarbital was stopped, the jaundice returned and the plasma bilirubin levels were elevated. Further treatment with phenobarbital again decreased the hyperbilirubinemia (60). Thompson & Williams recently treated four subjects with chronic intrahepatic cholestasis with 180 mg of phenobarbital per day for up to 50 days. In all of the sub-

jects, plasma bilirubin decreased up to 50 per cent and all four subjects reported a striking decrease in itching and an improved feeling of well being (62). In some humans with hyperbilirubinemia, phenobarbital treatment did not lower serum bilirubin, which is similar to results obtained in the Gunn rat (63). In this animal, bilirubin conjugation is deficient and, although the metabolism of drugs can be induced by phenobarbital administration, the conjugation of bilirubin is not enhanced.

Phenobarbital treatment increased the incorporation of C^{14}-glycine into the rapidly labeled bilirubin in rats (64). The rapidity of this incorporation excluded the possibility that the early labeled pigment arose from hemoglobin breakdown, and it has been suggested that it may reflect the turnover of liver microsomal hemoproteins, especially cytochrome P_{450}. The incorporation of δ-aminolevulinic acid into bilirubin was increased in a human subject given phenobarbital, but no increased incorporation of C^{14}-glycine was found (65).

In the first week of life, human infants develop a transient hyperbilirubinemia composed mainly of unconjugated bilirubin, and a positive relation between the degree of neonatal hyperbilirubinemia and the incidence of low motor or mental scores, or both, attained at 8 months of age has been reported (66). In a retrospective study, Trolle found that
binemia which occurred in the newborn was decreased if the mothers received phenobarbital and primidone throughout pregnancy for the treatment of epilepsy (67). Recently, Maurer et al. (68) have found that when phenobarbital (30 to 120 mg per day) was administered orally to pregnant women for 2 weeks or longer prior to delivery, a marked decrease in neonatal serum bilirubin levels was found in their offspring. Three days after birth, serum bilirubin averaged 5.7 mg per cent in 16 control babies, whereas in ten babies delivered by mothers treated with phenobarbital, the average serum bilirubin level at 3 days after birth was only 2.5 mg per cent (68). This effect of phenobarbital may also be important in the treatment of erythroblastosis fetalis, where high levels of bilirubin, capable of causing kernicterus, occur. However, studies such as these should be done with caution, for inducers of liver microsomal enzymes may produce undesirable effects such as an alteration in normal body steroid metabolism, displacement of bilirubin from binding sites on plasma protein, or stimulation of the formation of polar metabolites in the fetus, which cannot be eliminated from the body until after birth.

Lipids.—Christensen & Fawcett have suggested that some of the enzymes necessary for cholesterol synthesis are associated with the smooth endoplasmic reticulum (69). Treatment of hamsters with phenobarbital (80 mg/kg per day) increased the incorporation by hamster liver slices of C^{14}-acetate into cholesterol (70). More recently, Wada, Hirata & Sakamoto showed that the incorporation of C^{14}-mevalonate into cholesterol was increased in rat liver by phenobarbital treatment to a greater extent than was the incorporation of acetate (71). These investigations also showed that

carbon monoxide inhibited the incorporation of mevalonate into cholesterol, and the authors have suggested that cytochrome P_{450} is a necessary component for cholesterol synthesis in the liver (71). Phenobarbital treatment may also lead to increased cholesterol metabolism to bile acids *in vivo,* since this requires hydroxylation of cholesterol in the 7-position in a manner similar to the 7-hydroxylation of testosterone which has already been shown to be increased by drug treatment. Phenobarbital administration has been shown to stimulate bile flow markedly in the rat (58). In the rabbit, phenobarbital treatment did not change the endogenous serum cholesterol levels, but did decrease the hypercholesterolemia and the incidence of aortic plaque formation in rabbits that were fed exogenous cholesterol in the diet (72). These results would be consistent with the view that phenobarbital increased both the synthesis and metabolism of cholesterol.

An enzyme system catalyzing ω-hydroxylation of fatty acids in the presence of NADPH and molecular oxygen has been obtained in a soluble form from rabbit liver microsomes. The solubilized preparation contained a hemoprotein which has been characterized as cytochrome P_{450}; it is increased in concentration by phenobarbital treatment and the enzymatic ω-hydroxylation is inhibited by carbon monoxide (73). These investigators are the first to report on a solubilized preparation of cytochrome P_{450}, which still retains enzymatic activity.

Microsomal hemoprotein—The microsomal hemoprotein just mentioned has been implicated as the terminal oxidase of the enzyme system which occurs in the liver and which hydroxylates a number of drugs and steroids. Evidence for the existence of this cytochrome was first obtained by Klingenberg and by Garfinkel (74, 75) in 1958, and it was more recently studied extensively by Omura & Sato (76, 77). This hemoprotein can be characterized by its reactivity with carbon monoxide and ethylisocyanide, and has been termed cytochrome P_{450}, since in its reduced form, the complex of hemoprotein with carbon monoxide has a maximum absorption at 450 mμ. Estabrook, Cooper & Rosenthal recognized that the oxygen-activating enzyme which was present in the adrenal cortex and which was responsible for the hydroxylation of steroids was a cytochrome similar to the hemoprotein found by Klingenberg in liver microsomes (78). Soon thereafter, Cooper et al. demonstrated that cytochrome P_{450} in liver microsomes was responsible for drug hydroxylation (23). They showed that the liver microsomal oxidation of codeine, 4-monomethylamino-antipyrine, or acetanilide was inhibited by carbon monoxide and that the inhibition was reversed by oxygen or by monochromatic light at 450 mμ, which liberates carbon monoxide from binding sites on cytochrome P_{450}. Similarly, the finding that the hydroxylation of testosterone by liver microsomes was inhibited by carbon monoxide and that the inhibition was reversed by monochromatic light at 450 mμ has also implicated cytochrome P_{450} as the terminal oxidase responsible for steroid hydroxylation in liver microsomes (24, 25).

Treatment of rats with phenobarbital or the polycyclic hydrocarbon,

3-methylcholanthrene (3-MC), increases the apparent concentration of the cytochrome P_{450} present in liver microsomes (79, 82). Marver has recently shown that hemin administration prevented the effects of phenobarbital on liver microsomal enzymes, cytochrome P_{450}, cytochrome b_5, NADPH cytochrome-*c* reductase, microsomal protein, and phospholipid. Part of this action may be a via a feedback inhibition of heme synthesis but since not all of the effects of hemin were on heme proteins, it has been suggested that hemin acts by interfering with the uptake of phenobarbital at extranuclear binding sites (83). Recent studies have indicated that the hemoprotein increased by phenobarbital is not the same as that found in liver microsomes after 3-MC treatment (84–88). Carbon monoxide interacts with reduced microsomal hemoprotein obtained from 3-MC-treated rats, as expected, but the maximum absorption of this complex was found at 448 mμ, instead of at 450 mμ which is the maximum for the hemoprotein from microsomes of normal or phenobarbital-treated rats (85, 86, 88). From a recent report from Estabrook's laboratory, one may conclude that 3,4-benzpyrene administration to female rats also changes the absorption maximum from 450 to 448 mμ; however, the change that had occurred was not noted [Fig. 4 (81)]. Similar results have been obtained in rabbits treated with 3-MC, where an absorption maximum at 446 mμ was observed. The reduced hemoprotein CO complex at 446 mμ had an extinction coefficient of about 200 $mM^{-1}cm^{-1}$, while that from phenobarbital-treated rabbits at 450 mμ was about 50 $mM^{-1}cm^{-1}$ (87). The previously reported extinction coefficient of about 91 $mM^{-1}cm^{-1}$ apparently results from the presence of both hemoproteins in liver microsomes. These differences in extinction coefficients will require a re-evaluation of the extent of cytochrome induction by phenobarbital and 3-MC. The difference spectrum of the oxidized hemoprotein obtained when the spectrum of the oxidized hemoprotein in control microsomes was subtracted from the spectrum of the hemoprotein in microsomes from phenobarbital-treated rabbits, had a peak at 418 mμ. In contrast, the difference spectrum obtained by subtracting the normal hemoprotein spectrum from the 3-MC hemoprotein spectrum had a peak at 394 mμ (87). Differences in the oxidized spectrum of microsomal hemoprotein, obtained from phenobarbital and 3-MC-treated rabbits, were also found between 500 and 650 mμ (87).

The addition of ethylisocyanide to microsomes, reduced chemically with dithionite, caused an unusual difference spectrum which was characterized by two absorption maxima in the Soret region, one at 455 mμ and one at 430 mμ (89). Imai & Sato have suggested that this two-banded Soret spectrum arose from the possible presence in microsomes of two forms of cytochrome P_{450}, which appear to be in a pH-dependent equilibrium (89). Using ethylisocyanide as the ligand for reduced hemoprotein, Sladek & Mannering were the first to observe differences in the induction of hemoprotein by phenobarbital and 3-MC, and have suggested the name $P_{1\ 450}$ for the hemoprotein induced by 3-MC (84). They showed that pheno-

barbital treatment of rats induced the hemoprotein associated with the absorption maximum at 455 mμ to about the same extent as it induced the hemoprotein associated with the 430 mμ absorption maximum. In contrast, treatment with 3-MC preferentially increased the absorption obtained at 455 mμ. Thus, the ratio of the absorption at 455 to that at 430 mμ was increased by 3-MC treatment, and the pH at which this ratio became 1.0 shifted from pH 7.4 in normal and phenobarbital-treated rats to 6.9 in rats treated with 3-MC (84).

Recently treatment with 3-MC has been shown to change the Michaelis constant for the hydroxylation of 3,4-benzpyrene, and it is possible that this effect is due to a greater affinity of 3,4-benzpyrene for the hemoprotein preferentially formed in 3-MC-treated rats (90, 91). Although the V_{max} was increased, the K_m for the hydroxylation of 3,4-benzpyrene and other drugs did not change when rats were treated with phenobarbital (see 90). It has long been known that hexobarbital is metabolized more rapidly in the male rat than in the female. Recent studies have shown that the affinity of hexobarbital for cytochrome P_{450} is greater in the male than in the female rat (92) and that the K_m for the demethylation of ethylmorphine is lower in the male than in the female rat (93).

The spectral changes in the microsomal hemoprotein that are caused by 3-MC administration to animals could be due to (*a*) synthesis of a new hemoprotein or a different form of the hemoprotein, (*b*) the conversion of one form of hemoprotein to another form, or (*c*) the binding of 3-MC to hemoprotein, thereby changing its spectral properties. The third possibility was excluded since the *in vitro* addition of 3-MC to rat liver microsomes did not change the spectral properties of the cytochrome P_{450} and since the spectral change did not occur immediately after the administration of 3-MC *in vivo* (86). Studies which show that prior treatment of rats with either ethionine or actinomycin D prevented the spectral changes in microsomal hemoprotein caused by 3-MC treatment, suggest that this hydrocarbon caused the synthesis of a new hemoprotein or a different form of the original hemoprotein (85, 86, 88, 94). Studies by Hildebrandt & Estabrook suggest the presence in rabbit liver microsomes of two spectrally distinct hemoproteins that are interconvertible forms of a single hemoprotein. The addition of aniline to microsomes obtained from 3-MC-treated rabbits changed the spectrum of microsomal hemoprotein to the one seen in microsomes from phenobarbital-treated animals, while the addition of hexobarbital to microsomes from phenobarbital-treated rabbits caused a partial conversion to the spectral type observed in 3-MC-treated rabbits (87).

A direct answer to the question of whether more than one hemoprotein or more than one form of a hemoprotein having the characteristics of P_{450} exists in liver microsomes will have to await the solubilization and characterization of the hemoprotein(s) found in normal, phenobarbital- and 3-MC-treated animals. However, these studies, which demonstrate more than one spectrally distinct microsomal hemoprotein, are consistent with the view that

more than one cytochrome is responsible for the oxidation of drugs and steroids in liver microsomes (86, 95, 96). The results of several studies on testosterone hydroxylation also indicate that more than one rate-limiting component participates in microsomal hydroxylation. Chronic treatment of rats with phenobarbital, phenylbutazone, or DDT stimulated the hydroxylation of testosterone in the 16α-position to a greater extent than in the 6β- and 7α-positions (30, 97). In contrast, chronic treatment with 3-methylcholanthrene, a hydrocarbon known to induce only a very few drug oxidations when compared to phenobarbital, caused a marked increase in the hydroxylation of testosterone in the 7α-position and inhibited the hydroxylation at the 16α-position (86, 95). In dogs, the administration of phenylbutazone caused marked increases in the hydroxylation of testosterone in the 6β- and 16α-positions, but no increase was observed in the hydroxylation at the 7α-position (30). Studies on the inhibition of testosterone hydroxylation by carbon monoxide showed that the hydroxylation in the 16α-position was inhibited to a greater extent than hydroxylation in the 6β-position which was inhibited more than 7α-hydroxylation (25). Studies of testosterone hydroxylation as a function of age indicated that although the 6β- and 16α-hydroxylation increased as the animals matured, the 7α-hydroxylation of testosterone decreased (98). The rate of 7α-hydroxylation of testosterone was the same when either the activity of freshly prepared microsomal suspensions or those stored frozen for 20 days were assayed. However, the ability of microsomes to hydroxylate testosterone in the 16α- and 6β-positions decreased upon storage (86). The administration *in vivo* or the addition *in vitro* of chlorthion inhibited the conversion of testosterone to 16α-hydroxytestosterone, but had little or no effect on the 7α-hydroxylation reaction (28, 97). These studies and others which have used several drugs as substrates (1, 2, 82, 96, 99) indicate that more than one rate-limiting component participates in the various hydroxylation reactions and suggests that more than one hemoprotein or form of a hemoprotein are involved in microsomal drug and steroid oxidation (25, 86, 95, 96, 99).

The addition of drugs to liver microsomes changes the absorption spectrum of oxidized cytochrome P_{450} and gives rise to two different types of difference spectra which have been called type I and type II (100, 102). These spectral changes have been used to measure the interaction of cytochrome P_{450} with drugs. The type I spectral change, which is caused by the addition of hexobarbital, aminopyrine, phenobarbital, chlorpromazine, and imipramine to liver microsomes, is characterized by a difference spectrum with a trough at 420 mμ and a peak at 385 to 390 mμ. The type II spectral change, which is caused by the addition of aniline, nicotine, nicotinamide, pyridine, and *p*-aminophenol to liver microsomes, is characterized by a difference spectrum having a peak at 430 mμ and a trough at about 390 mμ. The magnitude of the spectral change is dependent on the concentration of drug added to microsomal suspensions, as well as to the concentration of microsomal protein. The concentration of drug which will produce half

maximum spectral change has been calculated and, with several drugs, this concentration is the same as the K_m for the enzymatic oxidation of the substrate by liver microsomes. These results suggested that the spectral changes observed after adding hexobarbital or aminopyrine may be an expression of an enzyme (cytochrome P_{450})-substrate complex (101, 102). The binding of C^{14}-aniline to microsomes has been studied by Orrenius & Ernster (103). They observed increased binding with microsomes obtained from phenobarbitol-treated rats, release of bound C^{14}-aniline upon incubation aerobically with NADPH, and a prevention of binding in the presence of carbon monoxide (103). These results suggest that the C^{14}-aniline was bound to cytochrome P_{450} in microsomes.

The amount of cytochrome P_{450} in microsomes of various species does not differ markedly and these small differences cannot explain the large species, strain, and individual variations in rates of drug metabolism by liver microsomes. Recently, Davies, Gigow & Gillette have found a good correlation between the amount of NADPH cytochrome-P_{450} reductase and the rate of metabolism of ethylmorphine in the rat, rabbit, mouse, and guinea pig and they suggest that this reductase may be the rate-limiting enzyme in the oxidation of drugs and steroids by liver microsomes. Alternatively, the microsomes of each species may contain a different form of cytochrome P_{450} which can have different affinities for drugs (104, 105).

Other endogenous compounds.—Phenobarbital and chlordane administered in dosage schedules known to induce liver microsomal enzymes increased the concentration ratio of I^{125}-thyroxin between liver and plasma in rats but did not change the ratio between kidney and plasma (106). Further studies by Oppenheimer, Bernstein & Surks (107) indicated that increased deiodination and biliary clearance of thyroxine accompanied the enhanced hepatocellular binding of thyroxine induced by phenobarbital. The enhanced clearance of thyroxine appeared to cause increased thyroidal function and the maintenance of a normal serum-protein bound iodine. In contrast, thyroidectomized animals maintained on a constant replacement dose of *L*-thyroxine and treated with phenobarbital exhibited a marked fall in serum-protein bound iodine (107). Klaassen & Plaa have recently shown that phenobarbital treatment caused a similar increase in the biliary excretion of several dyes which are not metabolized (108).

Melatonin is secreted from the mammalian pineal gland and appears to produce changes in the functional activity of the gonads, pituitary, and the thyroid. Chronic treatment with phenobarbital caused a decreased H^3-melatonin concentration in the brain and blood 30 min. after the injection of H^3-melatonin (109).

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