

FURTHER STUDIES ON THE INDUCTION OF THE DRUG-HYDROXYLATING ENZYME SYSTEM OF LIVER MICROSOMES

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ABSTRACT

Further studies of the induction of the liver microsomal drug-hydroxylating enzyme system by pretreatment of rats with various drugs are presented. The phenobarbital-induced increase in the microsomal content of CO-binding pigment and in the activities of TPNH-cytochrome *c* reductase and the oxidative demethylation of aminopyrine is proportional, within certain limits, to the amount of phenobarbital injected. Removal of the inducer results in a parallel decrease in the levels of CO-binding pigment, TPNH-cytochrome *c* reductase, and aminopyrine demethylation. Other inducing drugs have been investigated and shown to act similarly to phenobarbital. The early increase in these enzymes is found in the microsomal subfraction consisting of rough-surfaced vesicles, whereas repeated administration of the inducing drug results in a concentration of the enzymes in the smooth-surfaced vesicles. The phenobarbital-stimulated formation of endoplasmic membranes is reflected in increased amounts of the various microsomal phospholipid fractions as revealed by thin layer chromatography. There is no significant difference between the stimulated rates of P_i^{32} incorporation into phospholipids of the two different microsomal subfractions in response to phenobarbital treatment. The drug-induced enzyme synthesis is unaffected by adrenalectomy.

INTRODUCTION

In previous papers (1, 2) it has been shown that the well recognized stimulation of the liver microsomal drug-metabolizing activity by treatment of rats with phenobarbital (3-5) is accompanied by an increase in the TPNH-cytochrome *c* reductase activity and the content of CO-binding pigment of the microsomes. These findings suggested that the TPNH-cytochrome *c* reductase and the CO-binding pigment are components of the oxidative drug-metabolizing enzyme system, a conclusion also supported by biochemical evidence (6, 7). Puro-

¹ Triphosphopyridine nucleotide, reduced form.

mycin and actinomycin D, which had been shown to inhibit the induction of stimulated drug metabolism (8, 9), abolished the increase in the levels of TPNH-cytochrome *c* reductase and CO-binding pigment (1, 2), indicating the involvement of a net synthesis of both enzymes which was preceded by the synthesis of messenger-RNA. Electron microscopy revealed (2) a marked proliferation of the smooth-surfaced endoplasmic membranes, in accordance with earlier findings of Remmer and Merker (10), and there was an increase in microsomal lipid which was reflected in an enhanced

incorporation of P_i^{32} into the microsomal phospholipids *in vivo* (2). The increase in drug-metabolizing activity appeared to be greater in the smooth than in the rough vesicle fraction of the microsomes (2, 10).

Results of further studies of the induction of the liver microsomal drug-hydroxylating enzyme system are reported in the present paper. The kinetics of the enzyme induction caused by phenobarbital administration has been studied. The effects of other inducing drugs upon the levels of TPNH-cytochrome *c* reductase and CO-binding pigment have also been investigated. Furthermore, the early effects of phenobarbital on the pattern of distribution of the drug-hydroxylating enzymes between the two microsomal subfractions and on the production of various microsomal phospholipids have been studied.

MATERIALS AND METHODS

In all experiments male Sprague-Dawley rats (200 to 300 gm) were used. The animals were starved overnight. Preparation of liver microsomes was performed as described by Ernster *et al.* (11). Rough and smooth-surfaced vesicles were separated by density gradient centrifugation in the presence of Cs^+ (12).

The experimental animals received one daily injection intraperitoneally of 100 mg phenobarbital per kg body weight, unless otherwise stated. The controls were given the same amount of 0.9 per cent NaCl. In one series of experiments the effects of *in vivo* treatment with other known inducing agents were tested. These were aminopyrine (4), 3-methylcholanthrene (13), and nikethamide (5); the doses were 150, 25, and 250 mg per kg body weight, respectively. These drugs also were injected intraperitoneally, once daily. Aminopyrine was administered in 0.9 per cent NaCl solution, and 3-methylcholanthrene in an appropriate amount of corn oil. Control rats were injected with either 0.9 per cent NaCl or corn oil.

Adrenalectomy was performed under ether anesthesia. Besides a standard diet and drinking water, the adrenalectomized rats were given a 0.9 per cent NaCl solution *ad libitum*. Treatment of these animals with phenobarbital (100 mg per kg body weight) was started 5 days after the operation.

Protein was measured by the method of Lowry *et al.* (14). RNA was determined according to Ceriotti (15). The total microsomal lipids were extracted with a chloroform-methanol mixture, and the extract was washed with 0.73 per cent NaCl (16). The amount of phosphorus was determined in the perchloric acid-digested extract (17).

The various microsomal phospholipid fractions were separated by thin layer chromatography. Plates were prepared by standard technique (18) using Silica Gel G (Merck) as adsorbent. After extraction of the microsomes with chloroform-methanol and washing of the extract with 0.73 per cent NaCl, as described above, the chloroform-methanol mixture was evaporated. The samples were put on the plate in a minimal amount of chloroform-methanol (1:1). Chloroform-methanol-water (100:40:6) was used as solvent to develop the chromatograms, which were then exposed to iodine vapors to make the spots evident. Purified samples of phospholipids, obtained commercially, were run parallel. The phosphorus content of the various spots was determined (17). When the rate of P_i^{32} incorporation into microsomal phospholipid was investigated, 1 mc of P_i^{32} was administered intraperitoneally 1 hour before the rats were killed.

Oxidative demethylation activity was assayed with aminopyrine as substrate (7). Other enzyme assays were performed as described by Dallner (12).

All chemicals employed were standard commercial products.

RESULTS

Kinetics of Induction

Daily injections of phenobarbital in an amount of 100 mg per kg body weight for 5 days caused a *ca.* 5-fold enhancement of the activity of oxidative demethylation of aminopyrine in the liver microsomes (Fig. 1). This increase was reached after 5 injections, and further treatment did not elevate the activity above this value. When the dose was lowered, a less rapid increase in activity was observed. However, it was possible to reach a *ca.* 5-fold increase over controls by daily injections of 66 mg of phenobarbital per kg body weight, if the treatment was continued for another 5-day period. With the lowest dose tested, 33 mg of phenobarbital per kg body weight, a 3 to 4-fold enhancement in activity was reached after 15 daily injections. Doses of phenobarbital higher than 100 mg per kg body weight did not cause a more rapid increase than did 100 mg. For each dose tested, there was an increase in the amount of CO-binding pigment, as calculated on the protein basis, and in the specific activity of TPNH-cytochrome *c* reductase, parallel to the enhancement of the specific activity of aminopyrine demethylation. Cessation of the phenobarbital treatment after 5 days resulted in a symmetric and parallel decrease in the levels, and

after another 5 days the values were about the same as those for the control rats (Fig. 2).

Effects of Various Inducing Drugs

It has been shown (2) that the phenobarbital-induced increase in the levels of the TPNH-cyto-

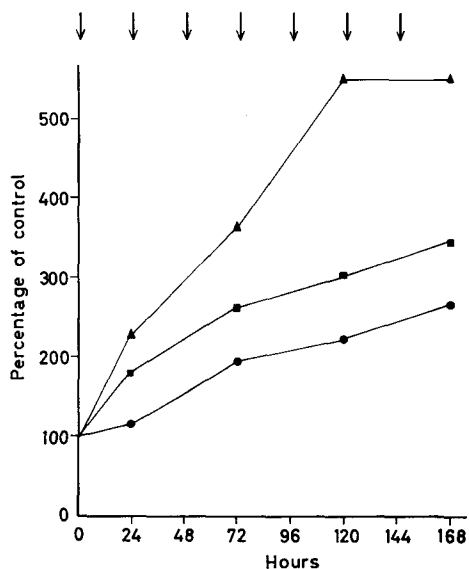


FIGURE 1 Enhanced rate of aminopyrine demethylation after treatment of rats with different daily doses of phenobarbital. Triangles, 100 mg/kg body weight; squares, 66 mg/kg body weight; circles, 33 mg/kg body weight. Each value plotted represents an average of the specific activities of 4 experimental animals as compared with those of 4 control rats. The arrows indicate phenobarbital injections.

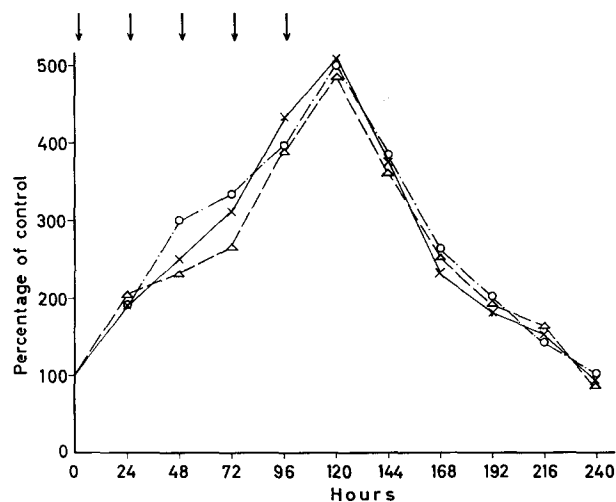


FIGURE 2 Decrease of enzyme activities after cessation of phenobarbital treatment. Each value plotted is an average of 6 experiments. The injections (each 100 mg/kg body weight) are indicated by the arrows. Crosses, oxidative demethylation activity; circles, CO-binding pigment; triangles, TPNH-cytochrome c reductase activity.

chrome *c* reductase and the CO-binding pigment is paralleled by a similar enhancement of the *in vitro* rates of N-demethylation of aminopyrine and dimethylnitrosamine, O-demethylation of codeine, and hydroxylation of phenobarbital. It is also accompanied by increased activities of side chain oxidation of hexobarbital and sulfoxide formation from chlorpromazine (19). If the phenobarbital-induced enhancement of the metabolism of these different drugs is directly dependent on the stimulation of the synthesis of the hydroxylating system, *i.e.* the TPNH-cytochrome *c* reductase and the CO-binding pigment, it seems probable that other drugs, which are able to act as inducers of enhanced drug metabolism, also should act by causing increased levels of these enzymes. Fig. 3 shows that this was the case for the drugs tested here. Aminopyrine, 3-methylcholanthrene, and nikethamide all caused a parallel increase in the content of CO-binding pigment and in the activities of TPNH-cytochrome *c* reductase and oxidative demethylation. As with phenobarbital, the stimulating effects were limited to the parameters mentioned, whereas the levels of other enzymes, as exemplified in Fig. 3 by cytochrome *b*₅, revealed no appreciable changes.

Phospholipids

It was found previously (2) that treatment of rats with phenobarbital caused a greatly enhanced incorporation *in vivo* of P₁³² into the microsomal phospholipid, whereas the incorporation into the phospholipid of mitochondria was only slightly increased. Since phospholipid composition is

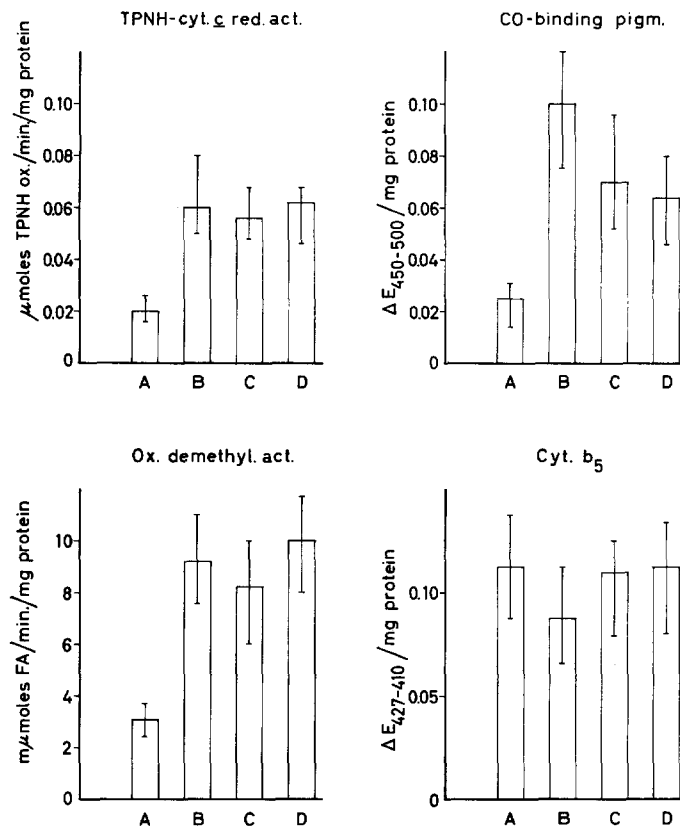


FIGURE 3 Induction of increased rates of aminopyrine demethylation and TPNH-cytochrome *c* reductase and increased content of CO-binding pigment in liver microsomes by pretreatment of the rats *in vivo* with aminopyrine (B), 3-methylcholanthrene (C), and nikethamide (D), in comparison with control rats (A).

Pretreatment of the rats was performed as described in Materials and Methods. Three daily injections of each drug were given. The bars represent mean values from 5 experiments. FA, formaldehyde.

known to differ between different biological membranes (20), it seemed to be of interest to study the influence of phenobarbital treatment on the liver microsomal phospholipids in greater detail. This was done by determining individual components of the phospholipid fraction at different intervals after phenobarbital treatment, and also by measuring their specific radioactivity following injection of P_i^{32} into the rats. Most of the phospholipid was found to be in the form of phosphatidylcholine, whereas sphingomyelin, phosphatidylethanolamine, and phosphatidylinositol were minor components² (Fig. 4). These data are in agreement with those reported elsewhere (21). As shown in Table I, 12 hours after a single injection of pheno-

² This method did not provide a separation of phosphatidylserine, which was probably recovered in the phosphatidylcholine fraction (18).

barbital there was a more than 1.5-fold increase in the total microsomal lipid phosphorus and a *ca.* 2.5-fold enhancement of the specific activity. Most of this increase was due to the phosphatidylcholine fraction. Repeated phenobarbital injections gave rise to a further increase in the various phospholipid fractions (Fig. 4).

Adrenalectomy

Thyroidectomy has previously been shown (2) not to influence the phenobarbital-induced activation of the drug-hydroxylating enzyme system. In Table II it is shown that the same holds for adrenalectomized animals. These results are in line with the observation of Kato *et al.* (22) that adrenalectomy did not affect the drug-induced activation of microsomal strychnine metabolism, and with the results of Conney *et al.* (23) from stud-

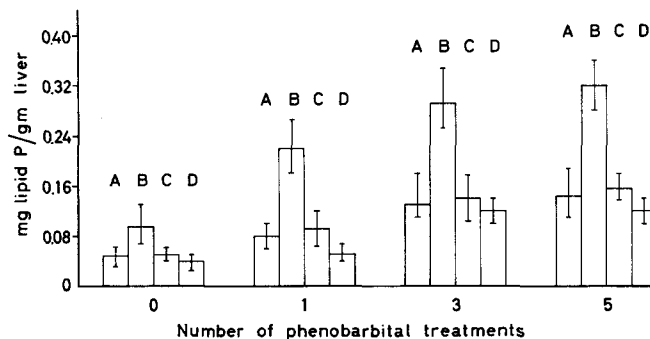


FIGURE 4 Effect of phenobarbital treatment on the content of various microsomal phospholipids: sphingomyelin (A); phosphatidylcholine (B); phosphatidylethanolamine (C); phosphatidylinositol (D). The bars represent mean values from 5 experiments.

TABLE I

Stimulation of the Rate of P_i^{32} Incorporation into Liver Microsomal Phospholipids by Treatment of Rats with Phenobarbital

The treated rats received one injection of 100 mg of phenobarbital per kg body weight 12 hours before they were killed. The controls received the same volume of 0.9 per cent NaCl. The table shows the mean values and standard deviations from 6 experiments.

Fraction	Control		Treated	
	$\mu\text{g lipid P}$ per gm liver	CPM per $\mu\text{g lipid P}$	$\mu\text{g lipid P}$ per gm liver	CPM per $\mu\text{g lipid P}$
Total phospholipids	340 \pm 40	260 \pm 48	560 \pm 65	640 \pm 120
Sphingomyelin	50 \pm 12	185 \pm 27	80 \pm 19	350 \pm 56
Phosphatidylcholine	130 \pm 22	250 \pm 60	290 \pm 38	775 \pm 149
Phosphatidylethanolamine	60 \pm 10	300 \pm 56	90 \pm 16	500 \pm 96
Phosphatidylinositol	50 \pm 11	200 \pm 33	60 \pm 16	300 \pm 49

TABLE II

Phenobarbital-Induced Enzyme Synthesis in Adrenalectomized Rats

Adrenalectomy and treatment of the rats with phenobarbital was performed as described in Materials and Methods. FA, formaldehyde.

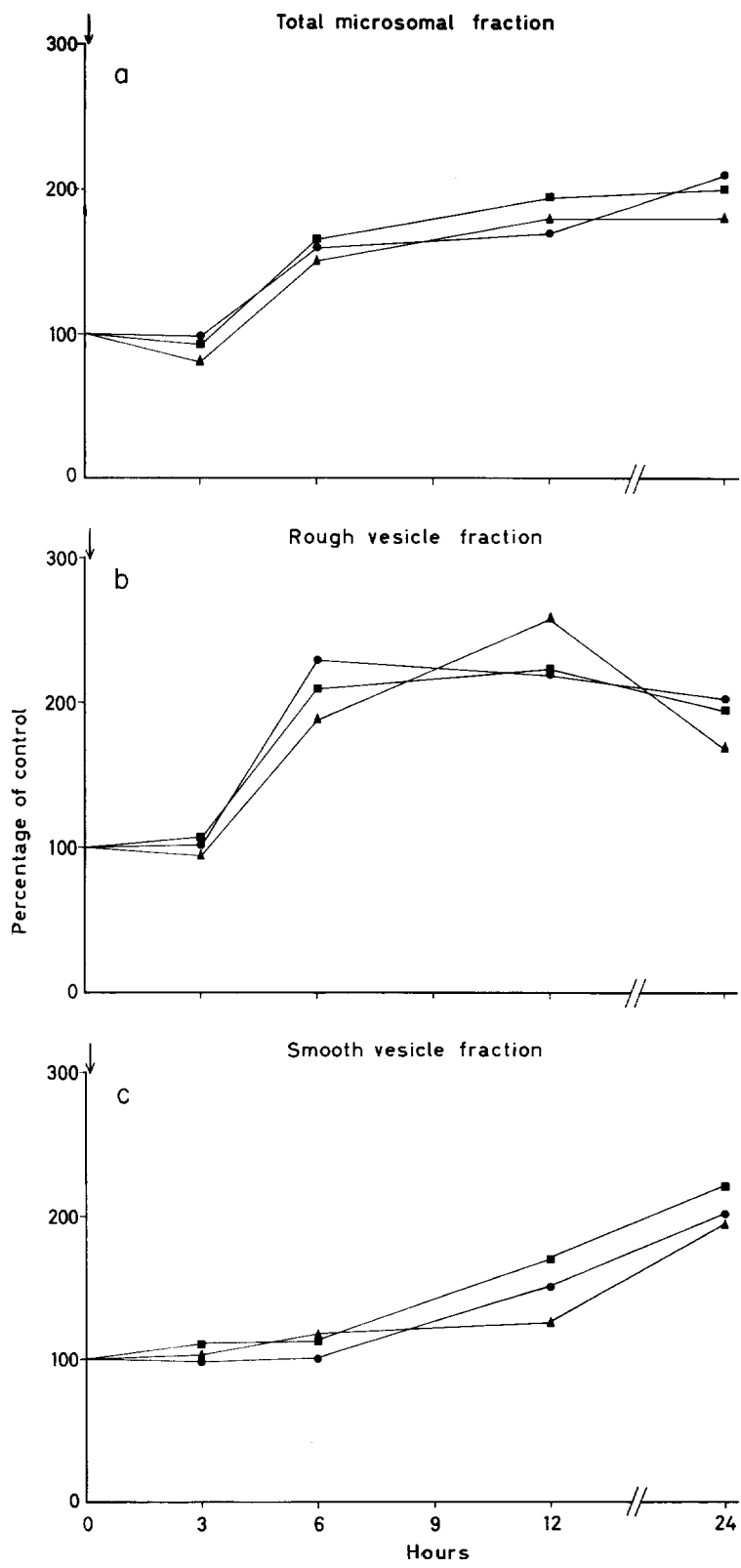
No. of phenobarbital treatments	Protein mg/gm liver	TPNH-cytochrome <i>c</i> reductase activity	CO-binding pigment	Oxidative demethylation activity
		$\mu\text{moles TPNH}$ ox./min./mg protein	$\Delta E_{400-600}$ /mg protein	$\mu\text{moles FA/min./mg protein}$
0	19.0	0.022	0.020	3.35
1	20.1	0.038	0.035	5.20
2	22.0	0.061	0.056	7.70
3	24.2	0.078	0.082	13.80

ies of the induction of enhanced N-demethylase activity by 3-methylcholanthrene.

Early Effects of Phenobarbital Stimulation

Previous studies have revealed (1, 2) that the phenobarbital-induced enhancement of the hy-

droxylation of various drugs is paralleled by an increased amount of endoplasmic membranes. Morphologically there was mainly an increase in the content of smooth-surfaced endoplasmic reticulum; also the isolated smooth-surfaced vesicle fraction displayed a greater enhancement of oxi-



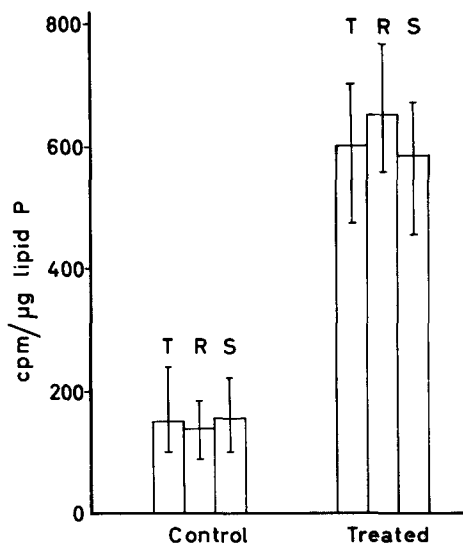


FIGURE 6 Increased rate of incorporation of P_i^{32} into phospholipid of the total liver microsomal fraction and of isolated rough and smooth vesicle fractions 6 hours after one injection of 100 mg of phenobarbital per kg body weight. Mean values of 4 experiments. T, total liver microsomes; R, rough-surfaced vesicles; S, smooth-surfaced vesicles.

ductive demethylation activity after repeated injections of phenobarbital than did the rough-surfaced vesicle fraction. In order to study the earliest measurable changes in the levels of the hydroxylating enzymes of the two microsomal sub-fractions, experiments were performed in which the rats were killed 3, 6, 12, and 24 hours after a single phenobarbital injection. The liver microsomes were divided into smooth- and rough-surfaced vesicle fractions by density gradient centrifugation in the presence of Cs^+ (12). As shown in Fig. 5 a, the total microsomal fraction exhibited a ca. 1.5-fold increase over controls in the amount of CO-binding pigment and the specific activities of

TPNH-cytochrome *c* reductase and aminopyrine demethylation as measured 6 hours after the injection of phenobarbital. This increase continued and was about 2-fold 24 hours after the treatment. Comparison of the rough and smooth vesicle fractions (Fig. 5 b and c) reveals that the rough vesicle fraction almost exclusively accounts for the initial rise of the enzymes, observed between 3 and 6 hours, after which the activation tends to level off in this fraction. Conversely, the increase in enzyme levels in the smooth vesicle fraction is negligible after 6 hours, but proceeds then at a progressive rate. After 24 hours, the enzyme levels are equal in the two fractions, in accordance with previous findings (1, 2).

The striking difference in enzyme levels between smooth- and rough-surfaced vesicles at the early stages of enzyme induction, described above, prompted interest in comparing the rates of incorporation of P_i^{32} into phospholipids *in vivo* into the two types of endoplasmic membranes. As shown in Fig. 6, both the rough- and smooth-surfaced vesicle fractions revealed markedly enhanced rates of P_i^{32} incorporation into phospholipid 6 hours after phenobarbital treatment, and there was no significant difference between the two fractions.³

DISCUSSION

From the data presented in this paper it is evident that the activity of the TPNH-cytochrome *c* reductase and the amount of CO-binding pigment

³ Under the conditions employed here, *i.e.* injection of P_i^{32} 1 hour before the animals were killed, the specific radioactivity of the total liver microsomal phospholipid P in the phenobarbital-treated animals was about 20 per cent of that of the liver or plasma P_i . Isotopic equilibrium between liver microsomal phospholipid P and the liver or plasma P_i was reached about 8 hours after the intraperitoneal injection of P_i^{32} .

FIGURE 5 Effect of one phenobarbital injection on the content of CO-binding pigment and on the activities of TPNH-cytochrome *c* reductase and aminopyrine demethylation of the total microsomal fraction and of isolated rough and smooth vesicle fractions. The averages of the amount of CO-binding pigment per mg protein and of the specific activities of TPNH-cytochrome *c* reductase and aminopyrine demethylation of the phenobarbital-treated group (5 rats) are plotted in relation to the corresponding values for the control group (5 rats). Triangles, TPNH-cytochrome *c* reductase activity; circles, CO-binding pigment; squares, oxidative demethylation activity. The arrow indicates the phenobarbital injection.

change in a fashion parallel to each other, and to the over-all drug-hydroxylating activity, during both the induction phase following drug administration, and the regression phase after its cessation. The changes of the three parameters were also parallel regardless of the absolute intensity of the induction, when the latter was varied by using different doses of drug or different drugs. Besides once more emphasizing the involvement of the TPNH-cytochrome *c* reductase and the CO-binding pigment in the drug-hydroxylating enzyme system—a conclusion already supported by several lines of evidence (2, 7)—these results suggest that the turnover of the two enzymes probably is regulated by a common mechanism. Such a conclusion would be consistent with the concept of Jacob and Monod (24) that the genes controlling the single enzymes in a metabolic sequence may be present in a cluster, called “operon,” regulated by a common operator gene. That the drug-induced changes in enzyme levels observed here reflect net enzyme synthesis is very probable in view of the fact, reported earlier (2), that the effects are abolished by actinomycin D and puromycin.

Repeated administration of an inducing drug has previously been shown to cause an accumulation of smooth-surfaced endoplasmic membranes of the liver, highly active in drug metabolism (1, 2). The present studies have revealed that the rough vesicle fraction first exhibits increased levels of the induced enzymes. Similar observations have recently been reported by Dallner *et al.* (25) from studies of the postnatal development of liver microsomal enzymes. It is well established that the rough-surfaced and smooth-surfaced canals of the endoplasmic reticulum represent a continuous system (26, 27), and there are indications that the two structures can readily change from one form to the other under different conditions (28, 29). It appears conceivable, therefore, that the newly formed enzyme protein is synthesized by and built into the rough-surfaced membranes of the endoplasmic reticulum, and that these membranes, once “saturated” with enzymes, release the ribosomes and accumulate as smooth-surfaced profiles. Electron micrographs suggestive of such a separation of ribosomes from endoplasmic membranes have recently been obtained by Heuson-Stiennon (30) in studies of myofilament synthesis in embryonic rat muscle.

The existence of a large number of hydroxylases

(*i.e.* enzymes transferring the active hydroxylating radical to the drug), each one specific for one drug or a group of drugs, has repeatedly been postulated on the basis of observed species differences and stereospecificity of various hydroxylations (*cf.* 31). However, the formation of an active hydroxylating radical may in itself be sufficient to bring about hydroxylations, without the intervention of any additional enzyme, as pointed out by Staudinger *et al.* (32). The concept of specific hydroxylating enzymes is not supported by the widely recognized fact, also revealed by the present work, that a wide variety of drugs can induce an enhanced metabolism of another wide variety of drugs.⁴ Admittedly, it may be assumed that the specific hydroxylases are not rate-limiting for the over-all process, and that an elevation of the TPNH-cytochrome *c* reductase and CO-binding pigment levels, as induced by any given drug, may enhance the metabolism of a number of other drugs as well. It appears equally possible, however, that the apparent preference of the hydroxylating system for one or another drug may be explained in terms of different accessibilities of the different drugs to a common enzymic site in the microsomal membrane structure (*cf.* 32, 33). Studies of the binding of drugs to microsomes may be helpful in deciding this question. Such investigations are now in progress in this laboratory.

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⁴ How generally this statement holds cannot be decided on the basis of the information available. It has been reported, for example, that the metabolism of only certain foreign compounds, such as benzpyrene, acetanilid, and 3-methyl-4-monomethylaminoazobenzene, was markedly enhanced by treatment with polycyclic hydrocarbons, whereas the metabolism of other drugs, such as aminopyrine and hexobarbital, was not influenced (4, 34), and furthermore that TPNH-cytochrome *c* reductase is not appreciably enhanced after one injection of 3-methylcholanthrene (35).

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