ENZYME–MEMBRANE RELATIONSHIP IN PHENOBARBITAL INDUCTION OF SYNTHESIS OF DRUG–METABOLIZING ENZYME SYSTEM AND PROLIFERATION OF ENDOPLASMIC MEMBRANES

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ABSTRACT

The enzyme-membrane relationship in phenobarbital induction of synthesis of drug-metabolizing enzyme system and proliferation of endoplasmic membranes has been further studied. Ultrastructural observations suggest that newly formed endoplasmic membranes in rat liver parenchymal cells arise through continuous outgrowth and budding off from pre-existing cisternae and tubules of rough-surfaced endoplasmic reticulum. The membranes induced by phenobarbital treatment persist in the cytoplasm of the hepatocyte for up to 15 days after the last of a series of 5 phenobarbital injections; the phase of regression of the induced enzymes lasts for only 5 days. Disappearance of the membranes is gradual and does not seem to be associated with increased autophagic activity in the cell. A second series of injections of phenobarbital to previously induced rats—exhibiting normal drug-hydroxylating activity but an excess of liver endoplasmic membranes—is associated with a stimulation of the rate of P_i^{32} incorporation into microsomal phospholipid in vivo, similar to that found during the original induction process. Administration of Actinomycin D following a single phenobarbital injection delays the regression of the enhanced drug-hydroxylating activity. Finally, the effects of Actinomycin D and puromycin on the stimulated membrane formation are discussed.

INTRODUCTION

Administration in vivo of phenobarbital to rats is known to give rise to enhanced liver-microsomal drug-hydroxylating activities (1–3) and to a proliferation of the endoplasmic reticulum (ER) of the liver parenchymal cell (4). Previous findings (5, 6) have indicated that there exists an interrelationship between the induced enzyme synthesis and the stimulated formation of endoplasmic reticulum membranes. The aim of the present investigation was to study more closely the fine structural alterations, in an attempt to elucidate the origin and evolution of the newly formed membranes. Attention was also focused on the phase of enzyme and membrane regression following induction by phenobarbital. The effects of Actinomycin D and puromycin on the enhanced membrane formation will also be discussed.

MATERIALS AND METHODS

In all experiments, male Sprague-Dawley rats (200 to 300 g) were used. The animals were starved overnight and killed by decapitation.

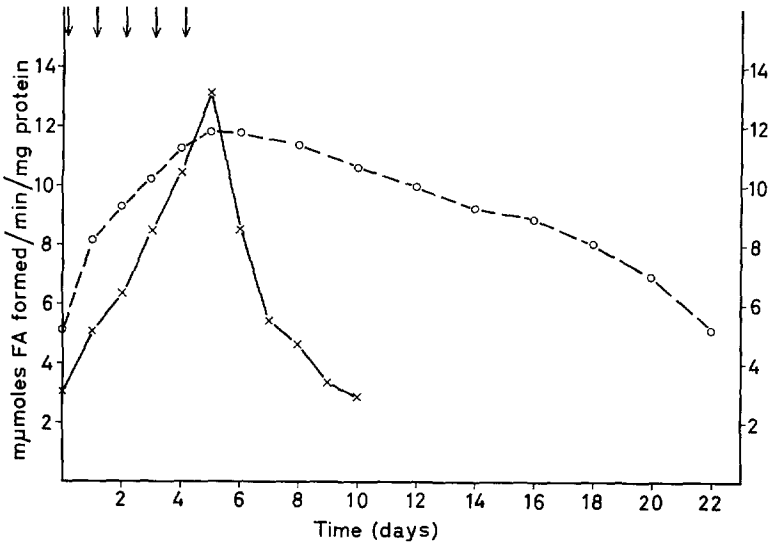


FIGURE 1 Effect of phenobarbital treatment on the oxidative demethylation activity and on the amount of liver-microsomal lipid phosphorus. Each value plotted represents an average of 6 animals. The arrows indicate the phenobarbital injections. *FA*, formaldehyde. Oxidative demethylation activity, x——x. Phospholipid mg/g liver, o---o.

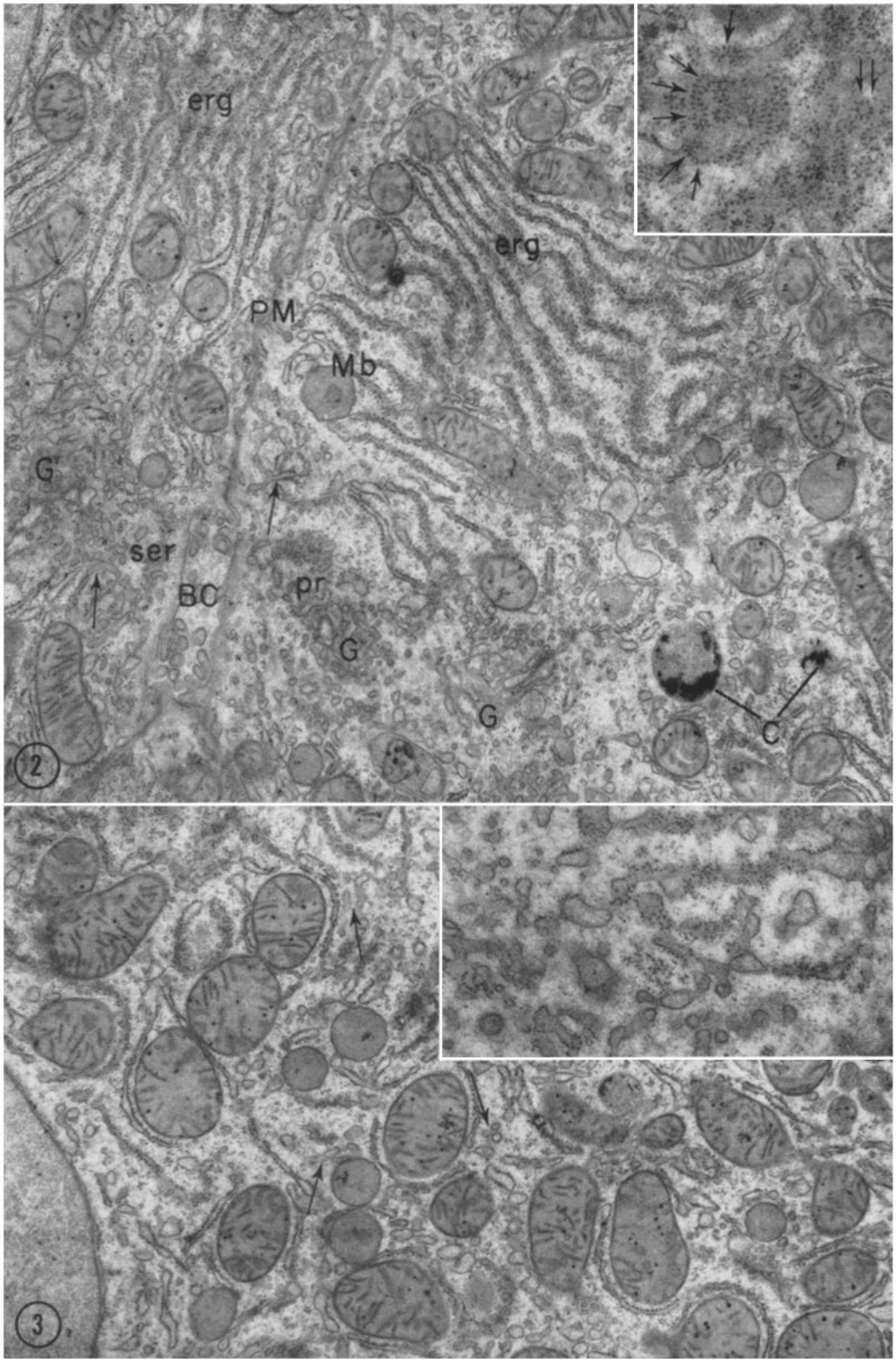
The experimental animals received daily one intraperitoneal injection of 100 mg phenobarbital per kg body weight. Actinomycin D or puromycin was dissolved in 0.9% NaCl and injected intraperitoneally in an amount of 8 μg per 100 g body weight, and 12.5 mg per 100 g of body weight, respectively. When the rate of P_i^{32} incorporation into microsomal phospholipid was investigated, 1 mc of "carrier-free" P_i^{32} was administered intraperitoneally 1 hr before the rats were killed.

For the electron microscope investigations, cubes of liver tissue approximately 1 mm^3 were immersed into 2% osmium tetroxide buffered with purified *s*-collidine (7) or a phosphate buffer (pH 7.4) (8) for 1.5 to 2 hr at 0–4°. After dehydration in a graded series of ethanol solutions, starting with 70%, the tis-

Note: All the electron micrographs show the appearance of hepatic parenchymal cells from rats starved 12 to 16 hr prior to sacrifice. Sections were stained with lead hydroxide with the exception of that demonstrated in the inset in Fig. 11; for this a combined uranyl acetate-lead hydroxide procedure was utilized (12, 13).

FIGURE 2 Control. Survey picture of centrolobular cells with RER (*erg*) mainly stacked in parallel arrays. SER (*ser*) is sparse and is in some places (arrows) continuous with RER. The cytoplasmic matrix contains small numbers of free ribosomes. In areas in which RER is cut tangentially, the ribosomes are often grouped together to form polysome-like clusters (above area indicated by *pr*). Polysomes are shown at higher magnification in the inset (single arrows); some form ring-shaped elements (two parallel arrows). *BC*, bile capillary; *C*, cytosome; *G*, Golgi apparatus; *Mb*, microbody. *PM*, plasma membrane. × 11,500. *Inset*, × 30,000.

FIGURE 3 Control. From a cell adjacent to those shown in Fig. 1 (centrolobular area). RER in this cell was only present in the form of randomly distributed cisternae or tubules of highly variable length; parallel arrays of cisternae were not observed. SER is sparse and appears as short, sometimes branching tubules or as vesicular images (arrows). × 14,000. *Inset* shows an area with branching tubular elements which in places are coated with ribosomes and thus appear as RER. × 26,000.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sues were immersed into two changes of propylene oxide at room temperature and then embedded in Epon 812 (9). Sections 1 μ thick, cut with an LKB Ultrotome, were stained with alkaline toluidine blue solution for light microscopy (10). These sections were utilized for selection of proper areas for thin sectioning and for examination of tissue preservation. In agreement with preliminary observations by Klatskin (11), optimal preservation was obtained only in the outer 2 to 3 layers ($\sim$ 75 μ) of hepatic parenchymal cells fixed with OsO_4 in phosphate buffer. With *s*-collidine buffer, the zone of well fixed cells was at least 20 to 25 cell layers. In small blocks of tissue (largest diameter less than 1 mm), preservation was good throughout. Thin sections were stained with lead hydroxide (12) and/or uranyl acetate (13), and were examined in a Siemens Elmiskop I.

Although some observations were made on OsO_4 phosphate–fixed tissues, the more uniform preservation and higher contrast of membranes in OsO_4 collidine–fixed material made these specimens more suitable for the present study. The results to be described pertain exclusively to OsO_4 collidine–fixed hepatic parenchymal cells in peripheral areas of the tissue blocks.

Preparation of liver microsomes was performed as described by Ernster et al. (14). Protein was measured by the method of Lowry et al. (15). The total microsomal lipid was extracted with a chloroform-methanol mixture, and the extract was washed with 0.73% NaCl (16). The amount of phosphorus was determined in the perchloric acid–digested extract (17). Oxidative demethylation was assayed with aminopyrine as substrate, as described previously (5). Other enzyme assays were performed as described by Dallner (18).

Actinomycin D was a generous gift from Merck, Sharp & Dohme, International, Rahway, New Jersey. All chemicals employed were standard commercial products.

RESULTS

A. *Synthesis and Regression of Membranes and Enzymes*

Biochemical Observations

Fig. 1 shows that administration of phenobarbital to rats for 5 days caused a 4- to 5-fold increase in the specific activity of aminopyrine demethylation in the liver microsomes, and a 2- to 2.5-fold increase in the content of microsomal lipid phosphorus. After cessation of phenobarbital treatment, there was a symmetric and rather rapid decrease in the oxidative demethylation activity. The microsomal phospholipid, however, showed a much slower decrease in amount, and not until approximately 2 wk after the cessation of injections of phenobarbital had the amount of phospholipid declined to equal the original value.

Electron Microscopic Observations

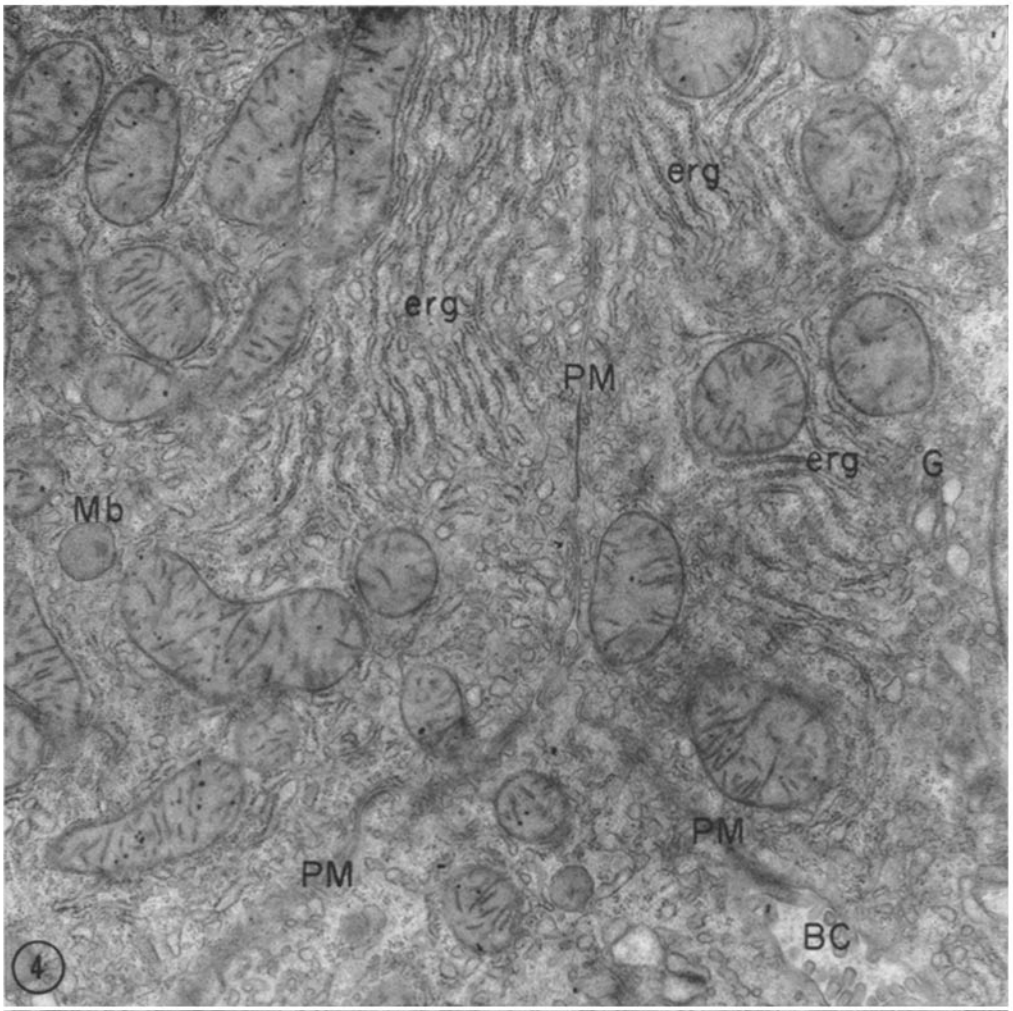
NORMAL STRUCTURE OF HEPATIC PARENCHYMAL CELLS: The general appearance of rat hepatic parenchymal cells has been described previously by a number of investigators (*see* 19) and does not need consideration here. Of particular interest in the present context were the appearance and distribution of the ER and the relationship between smooth and rough membranes. Since these features are in some respects incompletely described in earlier publications (19), and since they form the basis for the interpretation of the phenobarbital-induced alterations, a detailed description is given here.

Although rough-surfaced ER (RER, "ergastoplasm") (19, 20) stacked in parallel arrays (Fig. 2)

Note: Figs. 4 and 5 show the appearance after *one* injection of phenobarbital.

FIGURE 4 Centrolobular cells showing the same basic pattern of organization of cytoplasmic organelles as in control animals (*see* Fig. 2). The cytoplasmic matrix is, however, somewhat more prominent than in controls due to increased numbers of tubular and vesicular images with dimensions similar to those of the ER. A few cisternae of RER (*erg*) appear to be slightly dilated. *BC*, bile capillary; *G*, Golgi apparatus; *Mb*, microbody; *PM*, plasma membrane. × 13,000.

FIGURE 5 Portion of cytoplasm containing ergastoplasm (*erg*). The cisternae of RER are shorter and more irregularly grouped than in corresponding areas of controls (*see* Fig. 2). The cisternae are surrounded by smooth, vesicular images (*v*), probably representing slightly dilated SER. Some of these vesicles may consist of focal distensions of RER (arrow). *PM*, plasma membrane; *ser*, conglomerate of SER. × 24,000.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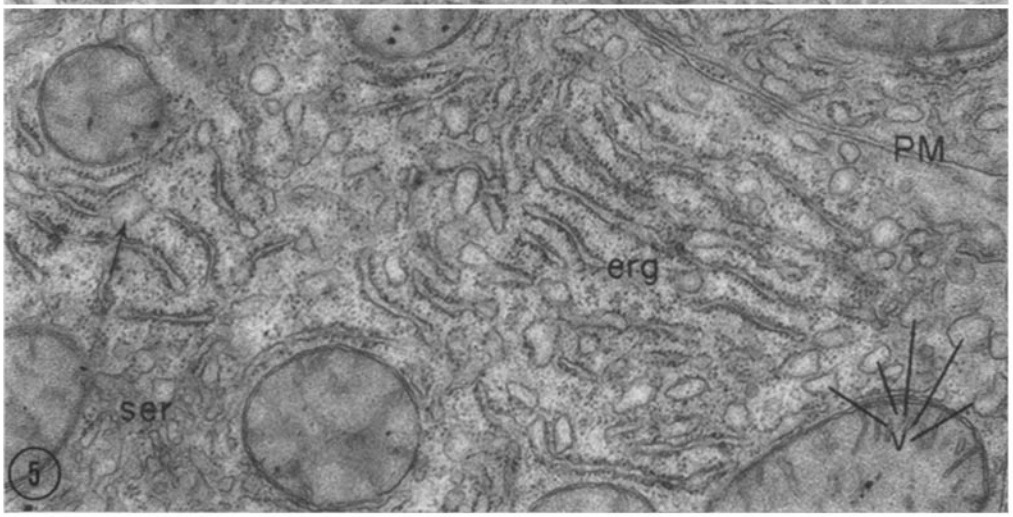
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was most abundant in centrolobular cells (21), it was also present in many of the cells in the midportions and peripheral areas of the lobules. In addition, RER occurred as isolated, haphazardly distributed cisternae (Fig. 3), often curving around other cytoplasmic organelles such as mitochondria, or, more rarely, it appeared as vesicular profiles. Such a scattered distribution of RER was observed in cells throughout the lobule but was usually much less prominent in cells with well developed aggregates of parallel cisternae (*see* Figs. 2 and 3). The contents of the cisternae were pale or sometimes finely flocculent or granular. In some areas, ribosome-coated, branching tubular and vesicular elements of RER were intimately related to smooth-surfaced ER (SER) (Fig. 3, inset) and appeared to show the same spatial arrangement as the latter.

SER was much less abundant than RER and formed loose lattices of tubular (partly interconnected) elements and vesicular structures. It was found mainly in three locations in the cells: (1) along the margins of the cells, notably near the sinusoidal surface; (2) in the vicinity of the Golgi apparatus; and (3) in central portions of the cells, corresponding to "glycogen areas" (22). As a rule, portions of cytoplasmic matrix containing SER were closely related to RER. Direct continuity between smooth- and rough-surfaced membranes of ER was frequently encountered. Occasionally, rosettes of dense particles, 200 to 400 A in diameter, probably representing particulate glycogen, were found in areas with SER.

In the normal parenchymal cells, the ribosomes were usually attached to the membranes of ER. A small proportion of ribosomes appeared to be free in the cytoplasm (Fig. 2). When seen on cross-sectioned cisternae of ER, the ribosomes appeared as rows of granules with 100 to 200 A interspaces; occasional uncoated areas of membrane were sometimes encountered. When the cisternae were cut tangentially, the ribosomes usually appeared in orderly rows forming semicircular or helical patterns (Fig. 2). These elements had the appearance generally attributed to "polysomes" (23). Some polysomes were also free in the cytoplasm.

ALTERATIONS FOLLOWING ONE INJECTION: Although many cells appeared unaltered, some appeared to show increased numbers of short tubular or vesicular elements (Fig. 4). Occasionally, some disorganization of the RER was noted, with irregular dilatations of the cisternae which also were shorter and less regularly arranged than in normal cells (Fig. 5).

ALTERATIONS FOLLOWING TWO INJECTIONS: The cytoplasmic matrix in many cells was filled with vesicular and tubular elements (Figs. 6 to 8); some of the latter were branching and formed a loose network. In their dimensions, these tubular and vesicular elements were similar to the SER.[1] Many were coated with ribosomes. Free ribosomes were somewhat more abundant than in controls. In cells with well developed aggregates of parallel cisternae of RER, dense conglomerates of tubular and vesicular elements of SER appeared to be growing out from the ends of the cisternae (Fig. 7). Continuity between RER and SER was frequently observed in such areas. Similar conglomerates were also present along the

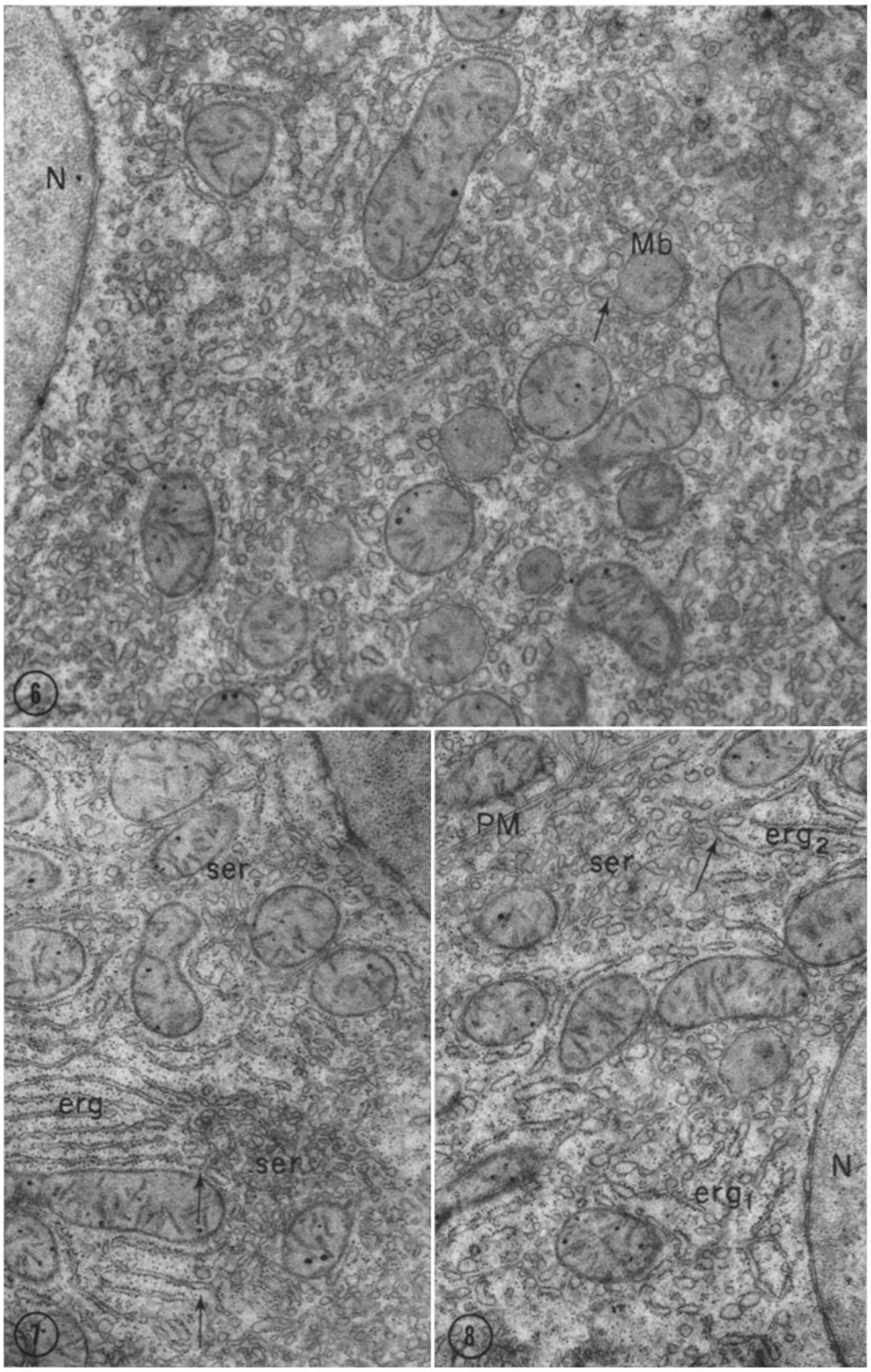
[1] Although only peripheral portions of the blocks were used, it is probable that the presence of vesicular elements of the SER is due to inadequate fixation.

Note: Figs. 6 to 8 show the appearance after *two* injections of phenobarbital.

FIGURE 6 Numerous vesicular images and branching, partially distended, tubular images are present in the cytoplasmic matrix. Some of these are coated with ribosomes. Free ribosomes are moderately abundant. Note apparent continuity between a microbody (*Mb*) and a tubular structure (arrow). *N*, nucleus. × 18,000.

FIGURE 7 Ergastoplasmic cisternae (*erg*) stacked in parallel arrays are peripherally closely associated with abundant SER (*ser*). Apparent continuity between RER and SER is marked by arrows. × 18,000.

FIGURE 8 Peripheral portions of cells showing moderately abundant SER (*ser*) near the plasma membrane (*PM*). RER either is forming irregular branching tubular images (erg_1) or shows a tendency towards parallel stacking (erg_2). Note continuity between SER and RER (arrow). *N*, nucleus. × 18,000.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cell borders (Fig. 8), notably near the sinusoidal surface. In some cells, especially those containing large aggregates of rough-surfaced cisternae (mostly centrolobular cells), proliferation of smooth membranes was not so prominent, but the lumina of the cisternae were considerably dilated and contained abundant, finely granular, or flocculent material. Although glycogen particles were noted in some areas of cytoplasm containing smooth-surfaced vesicular and tubular elements, particulate glycogen was lacking completely in most cells. In comparison with the controls, these cells revealed no definite difference in the amount or distribution of particulate glycogen.

ALTERATIONS FOLLOWING THREE INJECTIONS: The alterations were essentially similar to those after two injections, although generally more pronounced. Greatly distended peripheral portions of RER filled with granular or flocculent material were observed in some cells (Fig. 9). In other areas, the cisternae of the RER appeared to break up into smaller units (Fig. 10), and vesicles studded with ribosomes were frequently associated with such areas. These may possibly represent fixation artifacts. The proliferating tubular and vesicular elements in the cytoplasmic matrix outside parallel cisternae of RER tended to be less frequently coated by ribosomes than at earlier intervals. In some areas most of these elements appeared as isolated vesicles. Particulate glycogen was present in some cells (Fig. 9).

ALTERATIONS FOLLOWING FOUR AND FIVE INJECTIONS: The cytoplasmic matrix was now tightly packed with tubular and vesicular profiles (Fig. 11), most of which were smooth-surfaced. Although RER was abundant in some cells, and was often present in the form of cisternae packed in parallel arrays, it was almost completely lacking in other cell sections. Polysomes were frequently identified on the membranes of RER.

Alterations in polysomes, cytosomes, microbodies, Golgi apparatus, or mitochondria were not observed.

ALTERATIONS 1 TO 5 DAYS AFTER CESSATION OF INJECTIONS: These were essentially similar to those after 5 injections.

ALTERATIONS 8 TO 9 DAYS AFTER CESSATION OF INJECTIONS: Although some cells showed an appearance similar to that after five injections (Fig. 12), a moderate general reduction in the numbers of smooth tubular and vesicular elements in the cytoplasmic matrix was observed. Evidence of increased autophagic activity was lacking.

APPEARANCE 10 DAYS AFTER CESSATION OF INJECTIONS: A further reduction in the amounts of membranes in the cytoplasmic matrix was observed.

APPEARANCE 15 DAYS AFTER CESSATION OF INJECTIONS: Some cells showed slightly increased numbers of tubular and vesicular elements in the cytoplasmic matrix, but the majority appeared similar to those of untreated animals (Figs. 13 and 14).

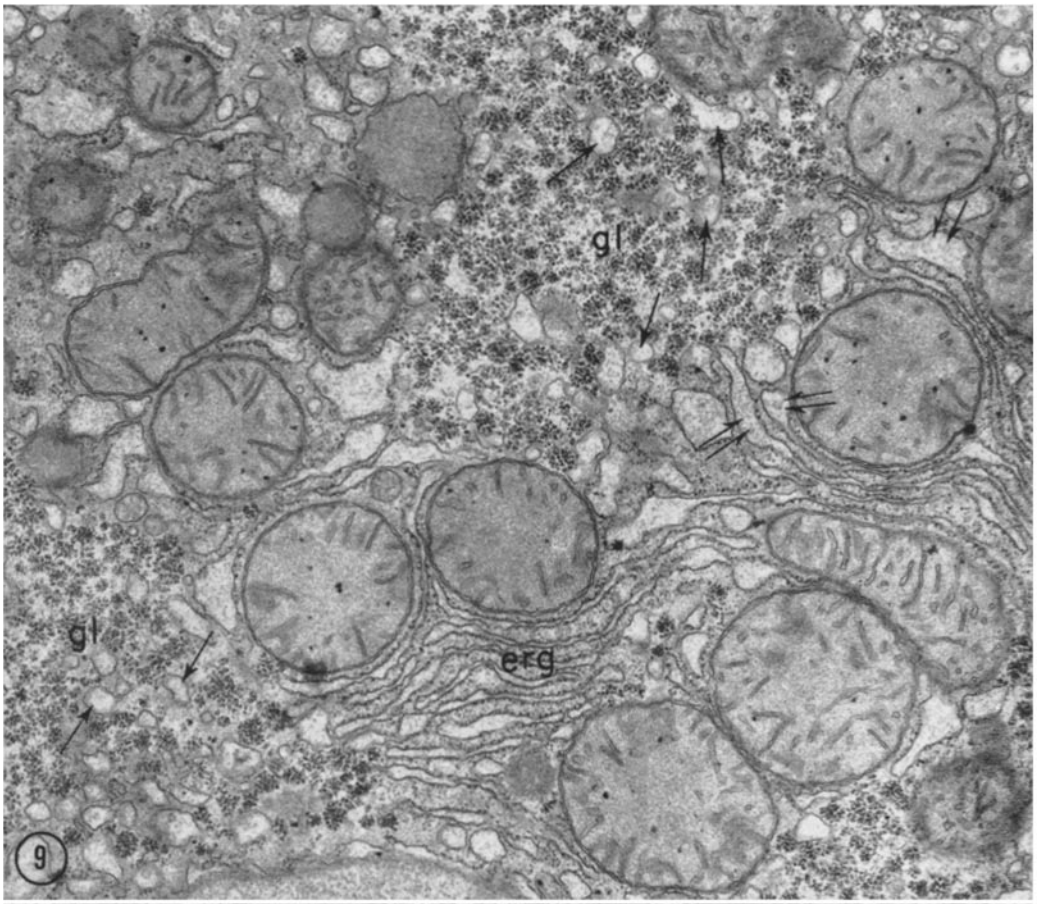
B. *"Reinduction Experiments"*

The findings described above indicated an apparent discrepancy between the phases of regression of the induced enzymes and membranes. In order to ascertain whether the existence of a previously induced proliferation of endoplasmic reticulum membranes would influence the "reinduction" of the hydroxylating enzyme system, experiments were performed in which rats were reinjected with phenobarbital. The second period of treatment was started on the 10th day after the

Note: Figs. 9 and 10 show the appearance after *three* injections of phenobarbital.

FIGURE 9 Cisternae of RER (*erg*) tend to be arranged in a parallel fashion. Most of them show focal, irregular expansions, notably at their ends (two parallel arrows) which usually are devoid of ribosomes. The cisternae contain abundant, moderately electron-opaque, flocculent, or finely granular "proteinaceous" material. Smooth-surfaced tubular or vesicular images marked by single arrows appear to represent SER (or transversely sectioned expansions of RER devoid of ribosomes). Particulate glycogen (*gl*) is abundant. × 20,000.

FIGURE 10 Cisternae of RER (*erg*) appear to be breaking up into small vesicular or short tubular units (arrows). SER (*ser*) is abundant and is partially closely related to RER. *Mb*, microbodies. × 21,000.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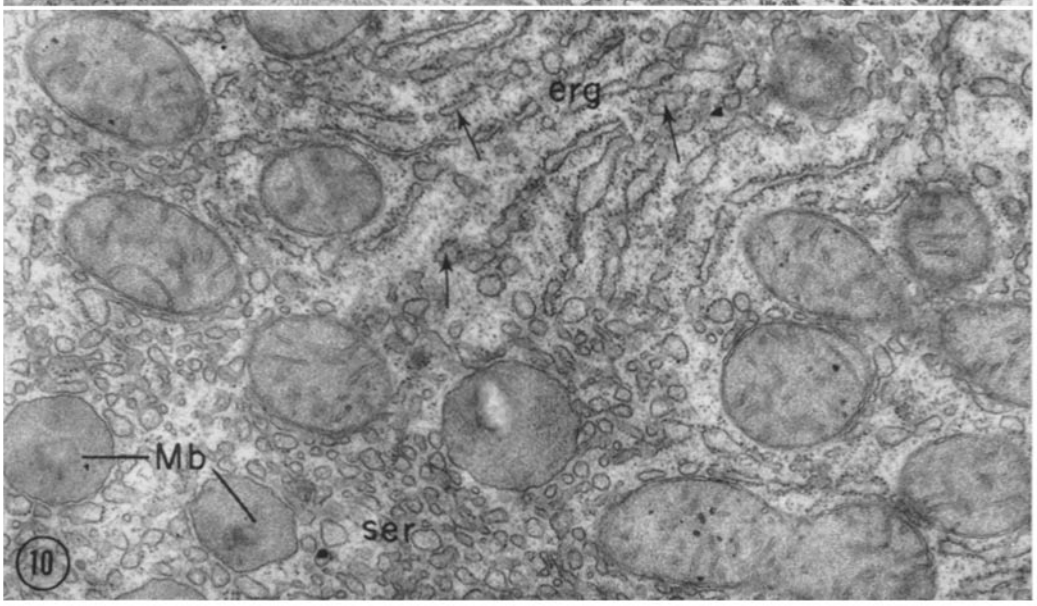
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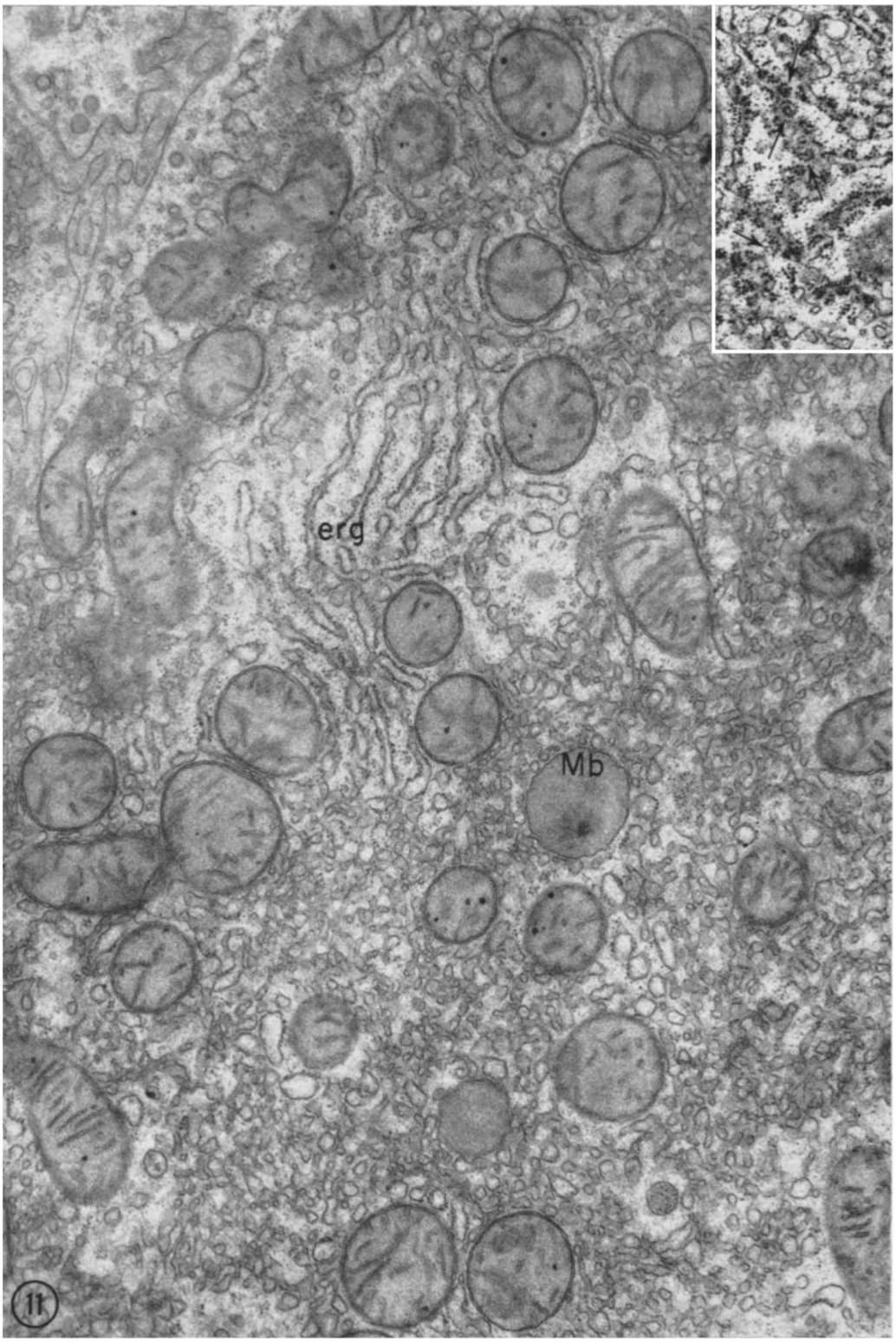


FIGURE 11 *Five* injections of phenobarbital. A small area of RER (*erg*) is surrounded by closely packed, diffusely distributed vesicular or tubular profiles of SER. *Mb*, microbody. × 20,000. *Inset* shows numerous polysomes (arrows) on RER. × 22,000.

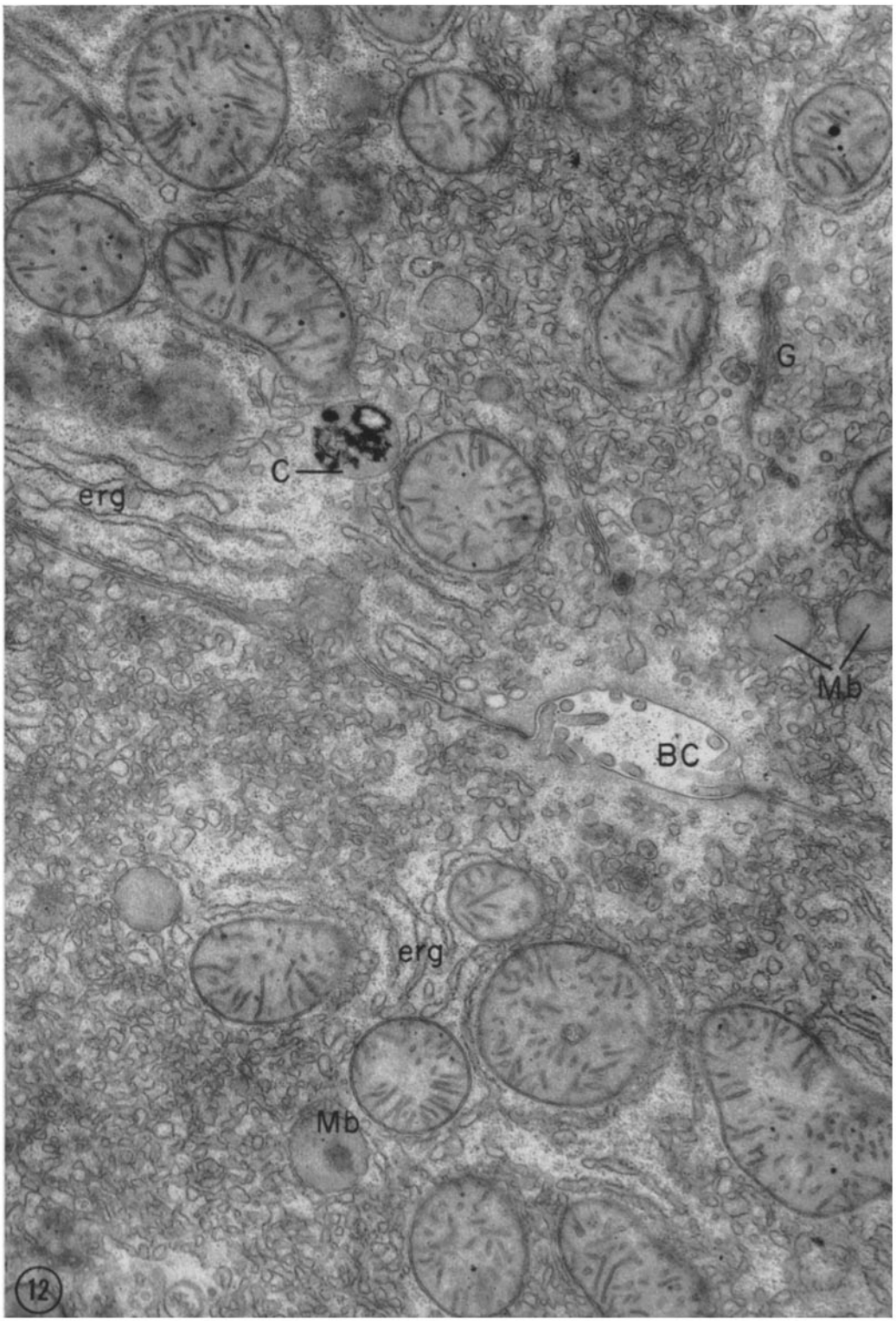


FIGURE 12 *Five* injections of phenobarbital. Animal sacrificed *9 days after the last injection.* RER (*erg*) is sparse. SER, on the other hand, forms compact masses of tightly packed tubular and vesicular images. *BC*, bile capillary; *C*, cytosome; *G*, Golgi apparatus; *Mb*, microbodies. × 17,000.

beginning of the original five injections of phenobarbital. At that time, the oxidative demethylation activity of the liver microsomes equalled the normal control value (see Fig. 1), whereas the content of microsomal phospholipid was still more than twice as much as the original amount. Fig. 15 shows that the second period of treatment with phenobarbital caused an increase in the oxidative demethylation activity which was less rapid than that caused by administration of phenobarbital to previously untreated animals. This phenomenon was most pronounced during the early phase of the reinduction, i.e. after the first and second injections. On the other hand, it may be seen in Fig. 15 that the reinduction also involved a marked increase in the rate of P_i^{32} incorporation into microsomal phospholipid. These findings suggest that the reinduction of the hydroxylating enzyme system requires the synthesis of new endoplasmic reticulum membranes, and that the membranes remaining in excess, after the original induction, cannot be used again for this purpose. Garren et al. (24) have reported that the repression of hydrocortisone-induced enzyme synthesis is due to the formation of a rapidly turning-over repressor, the synthesis of which is sensitive to Actinomycin D. To test whether the same holds for the present system, rats were injected with Actinomycin D 24 hr after a single injection of phenobarbital. Fig. 16 shows that the decrease in oxidative demethylation activity, which started 48 hr after injection with phenobarbital, was turned into a continued increase by administration of Actinomycin D. A 3-fold increase in oxidative demethylation activity—and also in the TPNH–cytochrome *c* reductase activity, and in the concentration of CO-binding pigment—was reached on the 4th day after the injection with phenobarbital, after which there was no further increase in the levels of these enzymes.

C. *Effects of Inhibitors*

It has been reported (5, 25, 26) that simultaneous administration of phenobarbital and Actinomycin D or puromycin inhibits the phenobarbital-induced increase in the level of the drug-hydroxylating enzyme system. Table I shows that puromycin almost completely blocked the stimulation by phenobarbital of the rate of P_i^{32} incorporation into microsomal phospholipid, whereas Actinomycin D was only partially effective. The same result was obtained when the microsomal content of lipid phosphorus was determined after three phenobarbital injections (Fig. 17); i.e. puromycin inhibited the phenobarbital-induced increase in lipid phosphorus almost totally, while the effect of Actinomycin D was much weaker.

The electron microscope investigation revealed a moderate proliferation of endoplasmic reticulum membranes after 3 injections of Actinomycin D and phenobarbital (Fig. 19). At 5 days, this alteration was rather pronounced but not so severe as in animals given phenobarbital alone. Repeated injections of puromycin and phenobarbital caused no appreciable morphological alterations in the liver cell (Fig. 18).

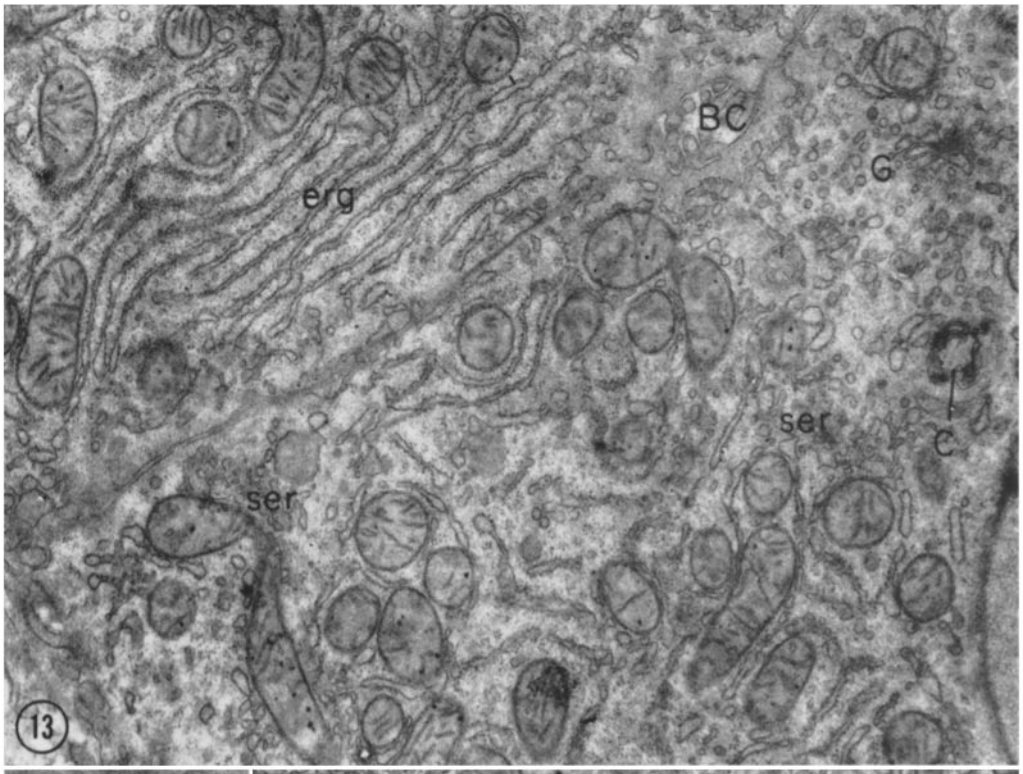
DISCUSSION

From the present data, as well as from those previously published (2, 4, 5, 27), it seems highly probable that the induction of enhanced drug metabolism by the in vivo administration of phenobarbital involves an enhanced synthesis of

Note: Figs. 13 and 14 show the appearance *15 days after the end of a 5-day period of injections.*

FIGURE 13 Portions of parenchymal cells showing an appearance similar to that in controls. Note RER stacked in parallel arrays (*erg*). SER (*ser*) is sparse. *BC*, bile capillary; *C*, cytosome; *G*, Golgi apparatus. × 15,000.

FIGURE 14 Cells with RER mainly forming randomly distributed cisternae; in one area there is formation of parallel arrays (*erg*). Note the cytosegresome (*CS*) containing what appears to be a partially digested mitochondrion. *BC*, bile capillary; *ser*, SER. × 16,000. *Inset* shows cytosegresome containing vesicular and tubular profiles, probably representing SER. × 22,000.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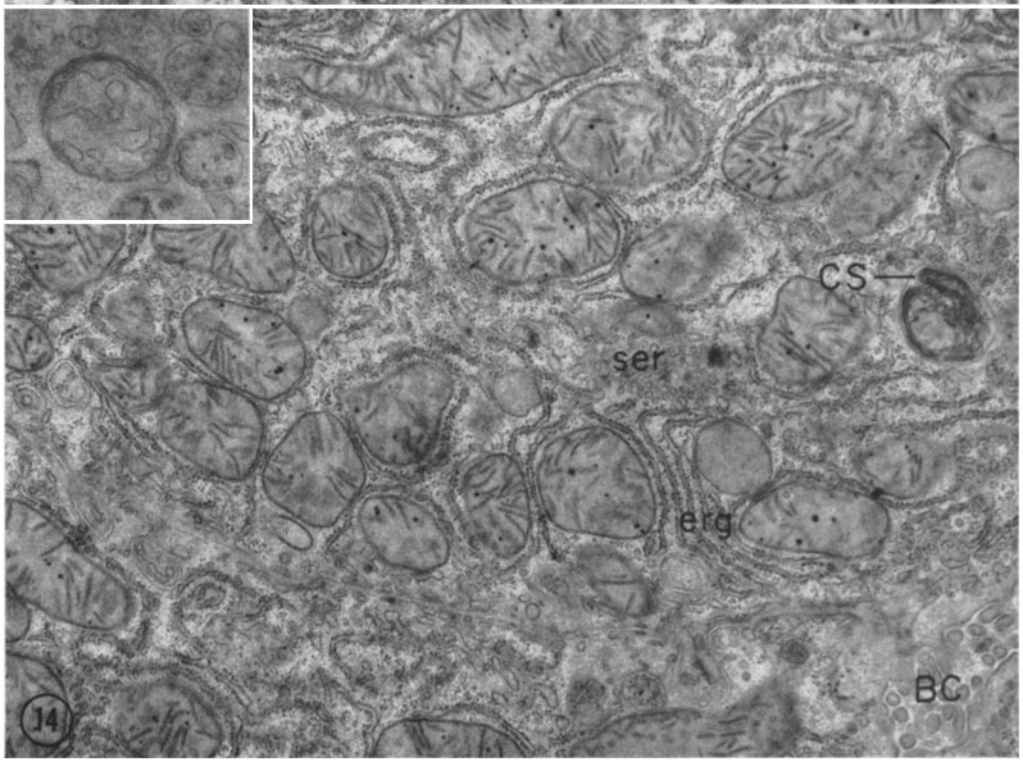
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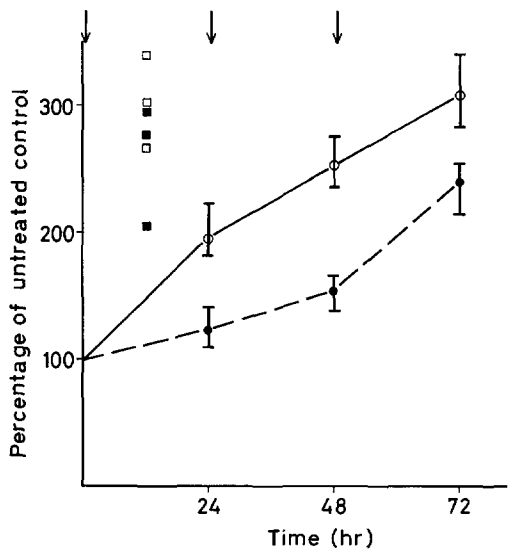


FIGURE 15 Enhancement of the rate of P_i^{32} incorporation into microsomal phospholipid, and of the oxidative demethylation activity, after administration of phenobarbital to untreated rats and to rats previously treated with phenobarbital. The previously treated rats had received five injections of phenobarbital on the 10th to 6th days before the second period of treatment was started. Each value represents an average of the specific activities of three animals plotted as percentage of the corresponding value from three untreated controls. ○——○, oxidative demethylation activity, previously untreated rats; ●——●, oxidative demethylation activity, previously treated rats; □, P_i^{32} incorporation, previously untreated rats; ■, P_i^{32} incorporation, previously treated rats. Arrows indicate phenobarbital injections.

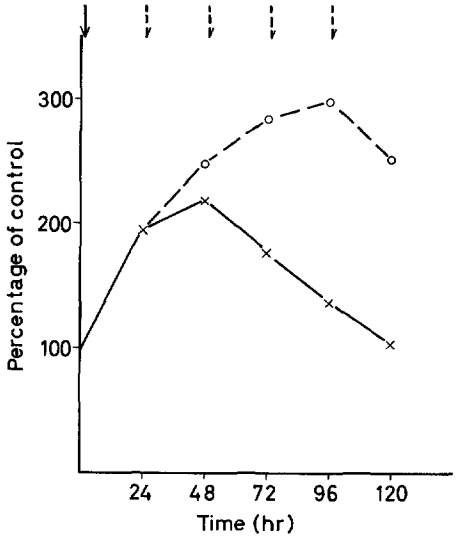


FIGURE 16 Effect of treatment with repeated injections of Actinomycin D on the regression of the enhanced oxidative demethylation activity following one injection of phenobarbital. ×——×, one injection of phenobarbital; ○——○, one injection of phenobarbital followed by repeated injections of Actinomycin D. Solid arrow, phenobarbital injection. Dashed arrow, Actinomycin D injections.

the drug-hydroxylating enzyme system which is attended by a rapid proliferation of endoplasmic reticulum membranes. A stimulated rate of P_i^{32} incorporation into microsomal phospholipid has proved to be an early step in the induction process (5) which is evident before the first measurable changes in enzyme levels appear in the rough-

TABLE I

Effects of Puromycin and Actinomycin D on the Stimulation by Phenobarbital of the Rate of P_i^{32} Incorporation in vivo into Liver-Microsomal Phospholipid

Injections with phenobarbital and with phenobarbital plus inhibitor were performed 12 hr before the rats were killed.

Exp. No.	Treatment	Lipid P
		CPM per μg
1	None (2)*	263 (223, 303)‡
	Phenobarbital (3)	751 (716-804)
	Phenobarbital + Puromycin (7)	340 (291-401)
2	None (2)	289 (267, 311)
	Phenobarbital (2)	850 (802, 898)
	Phenobarbital + Actinomycin D (6)	521 (430-567)

* Number of animals.

‡ Average value and range.

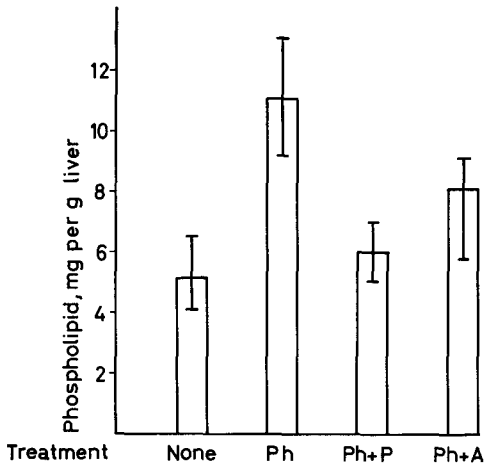


FIGURE 17 Effects of puromycin and Actinomycin D on the phenobarbital-induced increase in microsomal phospholipid. 3 injections with phenobarbital or with phenobarbital plus inhibitor were given. Each bar represents average and range from 7 rats. *Ph*, phenobarbital; *P*, puromycin; *A*, Actinomycin D.

surfaced part of the ER (6). These findings suggested that there may exist an intimate interplay between the synthesis of membranes and the synthesis of enzymes in the induction of a membrane-bound enzyme system which has now been further studied.

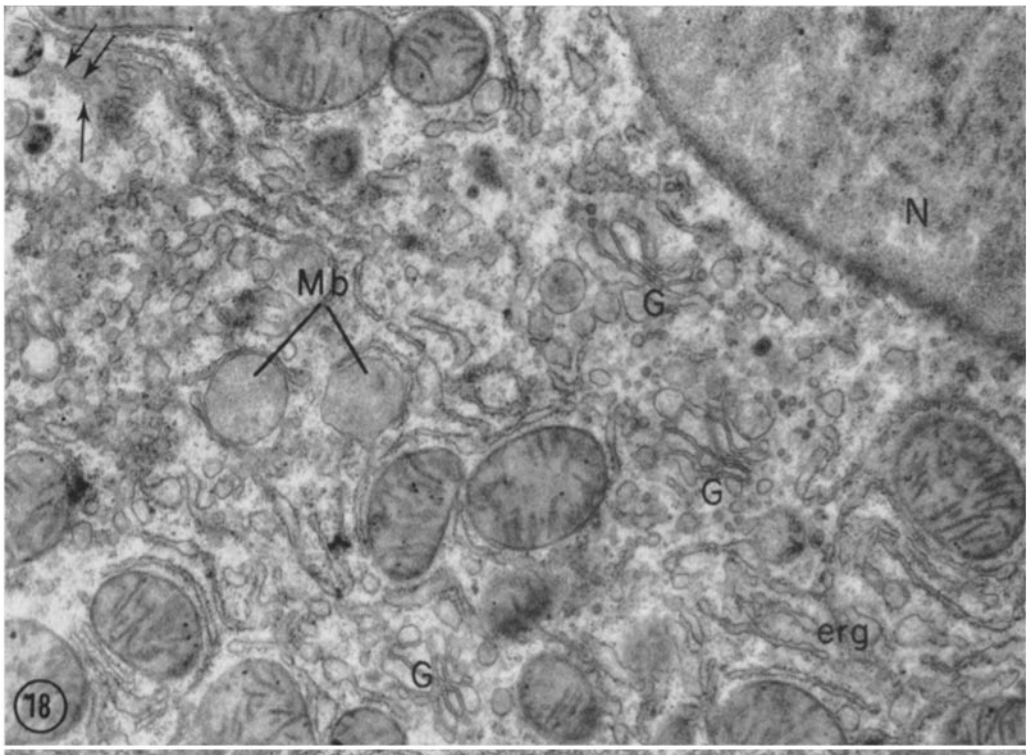
There is now good experimental evidence that the synthesis of microsomal enzymes takes place exclusively in the rough endoplasmic reticulum membranes (6, 28). Repeated administration of phenobarbital gives rise to an accumulation of smooth-surfaced vesicles exhibiting high drug-hydroxylating activities (5, 27). The ultrastructural observations suggest that the newly formed membranes arise through a process of continuous outgrowth and budding off from preexisting rough-surfaced membranes. Most of the branching, tubular elements are studded with ribosomess especially at early intervals. Later, the membranes are usually devoid of ribosomes. These observations indicate that ribosomes are associated with the membranes during the formation of the latter but are later detached. It is conceivable that, in the normal cell, SER is formed in a similar way, although at a much lower rate. This is suggested by the presence in normal cells of similar branching tubular elements studded with ribosomes (see Fig. 3). The results are compatible with the contention that the ribosomes are detached when the presumed synthetic functions on the membranes have been performed.

A considerable variation in the amount and distribution of newly formed membranes was noted between individual cells. For instance, some cells showed large accumulations of smooth-surfaced tubules and vesicles, while others showed very slight proliferation (Figs. 7 and 9). It is conceivable that these variations in appearance among the cells reflect differences in their functional state. In some cells—usually those lacking RER arranged in parallel cisternae—the proliferation appeared to be diffuse throughout the cytoplasm; in cells with well developed ergastoplasm forming piled cisternae, on the other hand, proliferation was more focal. These differences may depend upon the original distribution of RER in the cells as shown in Figs. 2 and 3.

The marked sensitivity of the enzyme induction to the simultaneous administration of Actinomycin D strongly suggests that the formation of new messenger-RNA is involved in this process. Furthermore, it has recently been shown by Loeb and Gelboin (29) that administration of methylcholanthrene to rats—which is known to give rise to enhanced drug-metabolizing activities in the liver microsomes (30)—causes an increase in the uptake of orotic acid into nuclear RNA in liver cell nuclei. Thus, the induction of the drug-hydroxylating enzyme system apparently is dependent upon gene activation which leads to the formation of rather short-lived messenger-RNA.

There are, however, marked differences in the response to phenobarbital between the hydroxylating enzyme system and the ER. The discrepancies between the two phenomena were most pronounced during the regression phase. 5 days after the cessation of phenobarbital treatment, the enzyme levels had declined to original values but there was still a 2-fold increase in the amount of microsomal phospholipid. It appeared that the newly formed vesicular and tubular elements gradually disappeared during a 15-day period. Although occasional "cytosegresomes" (31) ("autophagic vacuoles" (32)) containing remnants of ER were encountered, these images were not clearly more frequent than in normal cells.

Puromycin, besides blocking the induction of increased enzyme synthesis, was also found to exert a strong inhibitory action on the stimulation by phenobarbital of the rate of P_i^{32} incorporation into microsomal phospholipid. This inhibition may



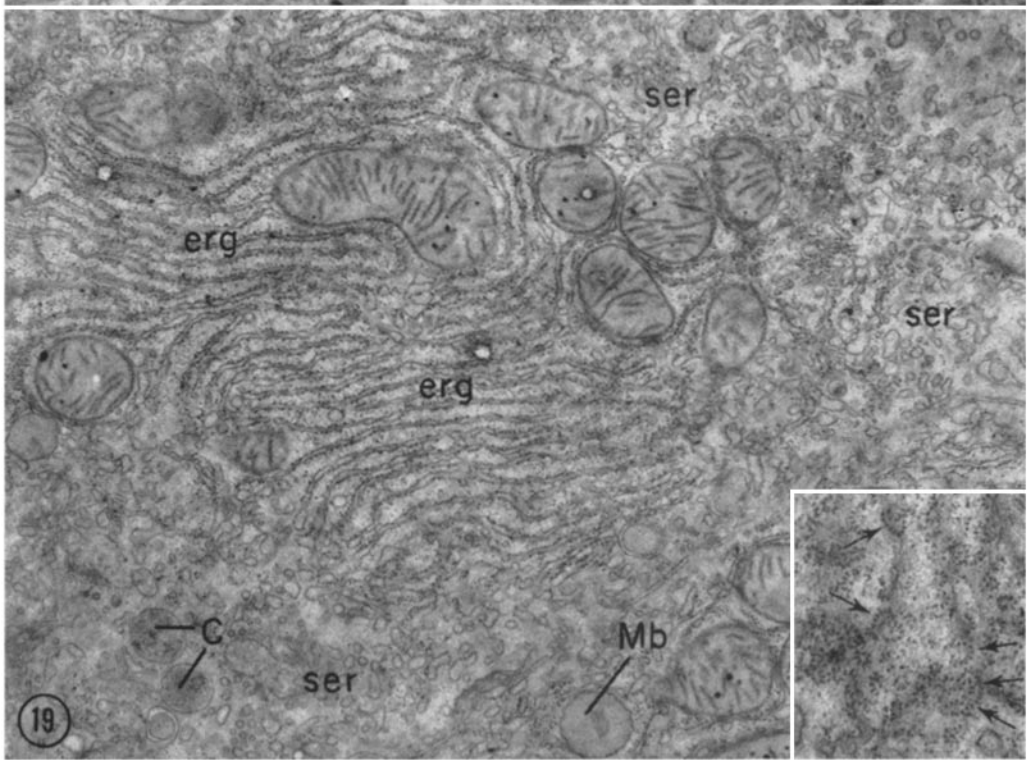


FIGURE 18 *Three* injections of phenobarbital and puromycin. Appearance similar to that in controls. Arrows point to polysomes. *erg*, RER; *G*, Golgi apparatus; *Mb*, microbodies; *N*, nucleus. × 16,000.

FIGURE 19 *Three* injections of phenobarbital and Actinomycin D. A large area of RER stacked in parallel arrays (*erg*) is surrounded by moderately abundant SER (*ser*). × 18,000. *Inset* shows polysomes (arrows) on tangentially sectioned membranes of RER. *C*, cytosomes; *Mb*, microbody. × 26,000.

be due to either an inhibition of the synthesis of enzymes involved in phospholipid synthesis, or a blocking of the formation of the protein part of the membrane which, in that case, should be the rate-limiting step in new membrane formation. Actinomycin D, which strongly inhibits the enzyme induction, has a much weaker effect on the enhanced phospholipid synthesis caused by phenobarbital. These findings suggest that the two processes are regulated by different genes and involve different messengers—that the messenger involved in membrane synthesis is more long-lived, or, alternatively, that the stimulation of formation of endoplasmic reticulum membranes may not require the formation of new messenger-RNA. A similar dissociation of enzyme and membrane syntheses has recently been suggested to occur in rat skeletal muscle mitochondria during different thyroid states (33).

Even if there are different mechanisms for the regulation of enzyme synthesis and membrane synthesis, the fact remains that the two processes are intimately correlated. The reinduction experiments recorded above indicate that the preexisting membranes may not be used for the synthesis of new enzymes, since the reinduction was associated with a marked stimulation of phospholipid synthesis. Furthermore, reinduction was found to be slower than the original induction, suggesting the presence within the cell of a repressor, which may be synthesized during—and be responsible for—the regress in enzyme levels. In line with the observations of Garren et al. (24), we noticed that treatment with Actinomycin D, after a single injection of phenobarbital, delayed the regress in the activity of oxidative demethylation. It appears that one of the interesting problems remaining to be solved is how phenobarbital triggers the induction of enhanced formation of endoplasmic reticulum membranes.

We wish to acknowledge the valuable advice and criticism of Dr. Lars Ernster throughout the investigation.

We are also indebted to Miss Hjördis Berg and Miss Gesa Thies for valuable technical assistance.

This study has been supported by grants from the Swedish Cancer Society and the "Stiftelsen Therese och Johan Anderssons Minne," Stockholm, Sweden.

Received for publication 13 August 1965.

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