ON THE RELATIONSHIP OF LIVER GLUCOSE-6-PHOSPHATASE TO THE PROLIFERATION OF ENDOPLASMIC RETICULUM IN PHENOBARBITAL INDUCTION

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ABSTRACT

The differentiated effects of phenobarbital treatment on liver microsomal enzymes have been further studied. The relationship between the resulting decrease in the specific glucose-6-phosphatase activity and the enhancement of formation of endoplasmic reticulum membranes with high drug-hydroxylating activity has been investigated with biochemical and histochemical methods. Biochemically and histochemically demonstrable glucose-6-phosphatase activity was found to be present in all endoplasmic reticulum membranes, including the phenobarbital-induced smooth-surfaced proliferates, even though there was an over-all decrease in activity. Actinomycin D did not inhibit the decrease in glucose-6-phosphatase activity. The findings are discussed with reference to the enzyme-membrane relationship in phenobarbital induction.

INTRODUCTION

Treatment of animals with phenobarbital in vivo is known to give rise to an increased metabolism of drugs and other lipid-soluble foreign compounds (1–3). It also results in an enhanced formation of the endoplasmic reticulum of the liver parenchymal cell (4–6). Various microsomal enzymes show different responses to phenobarbital induction (5, 7). Whereas the levels of the TPNH[1]-oxidizing flavoenzyme and the CO-binding pigment cytochrome P-450 are increased parallel to the enhanced over-all drug-hydroxylating activity, the amount of cytochrome b_5 and the nucleoside diphosphatase activity display only a minor increase, roughly equivalent to the increase in microsomal protein. Another group of enzymes, i.e. glucose-6-phosphatase, ATPase, and DPNH-cytochrome *c* reductase, respond to phenobarbital treatment by significantly decreased specific activities. This decrease in enzyme activities has been investigated in the present communication in order to obtain further information on the enzyme-membrane relationship in liver microsomes during phenobarbital induction. Most of the results refer to studies of changes in the glucose-6-phosphatase level during phenobarbital-enhanced synthesis of the drug-hydroxylating enzyme system and formation of endoplasmic membranes. The glucose-6-phos-

[1] Abbreviations used are: DPNH and TPNH, di- and triphosphopyridine nucleotides, reduced forms; ATP, adenosine-5′-triphosphate; G-6-P, glucose-6-phosphate; FA, formaldehyde.

phatase activity has been studied both biochemically and histochemically, and the results will be discussed with special reference to the phenobarbital-induced formation of new endoplasmic membranes.

MATERIALS AND METHODS

In all experiments male Sprague-Dawley rats (200 to 300 g) were used. Unless otherwise stated, the animals were fasted overnight and sacrificed by decapitation.

The experimental animals received daily one intraperitoneal injection of 90 mg of sodium phenobarbital per kg body weight. Actinomycin D was dissolved in 0.9% NaCl and injected intraperitoneally in an amount of 8 μg per 100 g body weight.

For the morphological and cytochemical studies, the liver tissue was fixed by either (*a*) perfusion fixation or (*b*) immersion fixation. The fixative solution consisted of 3% purified glutaraldehyde in 0.1 M cacodylate or phosphate buffer, pH 7.4. Experimental and control animals were sacrificed after a fasting period of 14 to 16 hr. In addition, 2 nonfasted controls were investigated.

(*a*) *Perfusion Fixation*

Rats were anesthetized with Nembutal or ether, and the livers were perfused via the portal vein (8). For the cytochemical studies of glucose-6-phosphatase, perfusion with glutaraldehyde was discontinued after 1 min and was immediately followed by perfusion with pure buffer for another 2 to 3 min; pieces of the liver (~1 mm thick) showing gross evidence of good fixation were then transferred to 0.1 M cacodylate or tris-maleate buffer, and were washed for 1 to 2 hr at +4°C. When livers were fixed for purely morphological studies, perfusion times were 10 to 15 min. Wedges, ~1 mm thick were subsequently immersion-fixed in glutaraldehyde for another 3 hr. Some of these tissues were immediately postfixed in osmium tetroxide (OsO_4) (see below); others were first washed at +4°C in 0.1 M tris-maleate or cacodylate buffer, pH 7.4, for 24 hr.

(*b*) *Immersion Fixation*

Rats were sacrificed by decapitation, and ~1 mm thick slices of the livers were immediately immersed in 3% buffered glutaraldehyde at +4°C as described above. Some of these slices were transferred to 0.1 M tris-maleate or cacodylate buffer, pH 7.4 for the cytochemical studies of glucose-6-phosphatase; other slices were fixed for 3 hr (for purely morphological studies) and were then treated as perfusion-fixed tissues (see above).

For the light and electron microscope studies of glucose-6-phosphatase, frozen sections approximately 6 and 40 μ thick were prepared on a regular freezing microtome. The sections were incubated in the Wachstein-Meisel medium (9) for 10 to 20 min and were briefly washed in 2 changes of 0.1 M acetate buffer, pH 5. Sections cut at 6 μ were briefly immersed in dilute ammonium sulfide for light microscopy. If blackening of perfusion-fixed tissues was uniform following treatment with ammonium sulfide, the 40-μ thick sections were directly transferred to OsO_4 after the rinse in acetate buffer. Immersion-fixed tissues showed a very irregular blackening with a 200 to 300 μ wide unreactive peripheral zone. The 40-μ thick sections of such tissues as well as perfusion-fixed tissues with uneven blackening were briefly immersed in ammonium sulfide for localization of reactive areas. The sections were washed in 0.1 M tris-maleate buffer and were then placed on a plate of dental wax under a dissecting microscope and portions of tissue showing moderate blackening were rapidly trimmed out and were subsequently postfixed in OsO_4 (see below). In immersion-fixed tissues the area immediately subjacent to the peripheral nonreactive zone was chosen. Control sections were incubated in a medium lacking glucose-6-phosphate.

All tissues primarily fixed in glutaraldehyde were postfixed for 1 to 1½ hr at +4°C in 2% OsO_4 buffered with *s*-collidine, pH 7.4 (10). Tissues that had not been utilized for the cytochemical studies were divided into cubes with a side of approximately 1 mm prior to fixation in OsO_4. Following fixation, tissues were dehydrated in a graded series of ethanol solutions at +4°C, starting with 70%, and propylene oxide (at room temperature). Embedding was performed in Epon 812 (11). Thin sections were cut on a LKB ultrotome, and the sections were studied in a Siemens Elmiskop I either unstained or stained with lead hydroxide (12).

Preparation of liver microsomes was performed as described by Ernster et al. (13). Microsomes were subfractionated into smooth- and rough-surfaced vesicle fractions by density gradient centrifugation in the presence of CsCl (14).

Protein was determined according to Lowry et al. (15). The enzyme assays were performed as described earlier (5, 14).

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

RESULTS

Biochemistry

Fig. 1 shows the effects of administration to rats of phenobarbital in vivo on the levels of oxidative demethylation of aminopyrine and glucose-6-phosphatase. Whereas there was a rapid increase

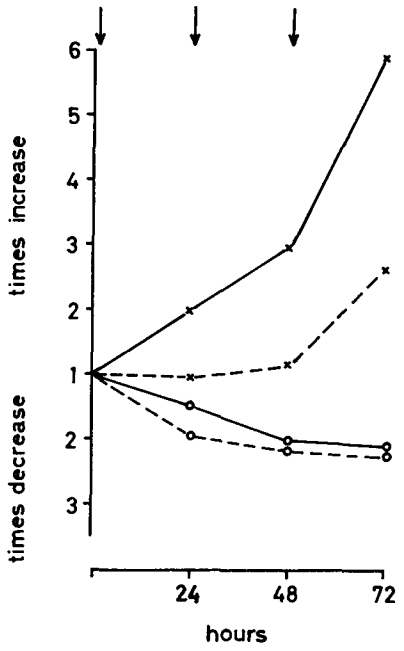


FIGURE 1 Effects of administration in vivo of actinomycin D on the phenobarbital-induced increase in the rate of oxidative demethylation of aminopyrine and decrease in glucose-6-phosphatase activity in rat liver microsomes. The values plotted represent averages of the specific activities for four experimental animals in relation to the same values for four control rats. The arrows represent the injections; ×——×, oxidative demethylation activity, phenobarbital treatment; ×----×, oxidative demethylation activity, phenobarbital + Actinomycin D treatment; ○——○, glucose-6-phosphatase activity, phenobarbital treatment; ○-----○, glucose-6-phosphatase activity, phenobarbital + actinomycin D treatment. (When administered, actinomycin D was injected intraperitoneally simultaneously with each one of the three phenobarbital injections.)

in oxidative demethylation activity, glucose-6-phosphatase revealed a less pronounced but significant decrease in specific activity. It can also be seen that simultaneous administration of actinomycin D inhibited the increase in oxidative demethylation activity while it had little or no inhibitory effect on the decrease in glucose-6-phosphatase activity. Puromycin,[2] however, blocked the decrease in glucose-6-phosphatase activity as well as the increase in oxidative demethylation.

Repeated injections of phenobarbital into rats are known to give rise to an accumulation of smooth-surfaced endoplasmic reticulum membranes highly active in drug metabolism (Fig. 2) (5–7). When the effects of phenobarbital treatment on the specific glucose-6-phosphatase and ATPase activities were tested in the isolated microsomal subfractions, i.e. the rough-surfaced and smooth-surfaced vesicle fractions; equal decreases in the specific activities of these enzymes were found in both fractions (Fig. 2). The decrease was maximal after three injections of the drug. The rough-surfaced microsomes isolated from untreated rats displayed somewhat higher glucose-6-phosphatase and ATPase activities than the smooth-surfaced ones.

A series of biochemical and histochemical experiments was performed to find out whether the decrease in glucose-6-phosphatase activity accompanying the increase in the amount of endoplasmic reticulum membranes with high rate of drug hydroxylation could possibly be due to a phenobarbital-induced formation of specific membranes exhibiting drug hydroxylation but no glucose-6-phosphatase activity.

For the biochemical studies smooth-surfaced microsomes isolated by density gradient centrifugation in the presence of CsCl from rats treated with three injections of phenobarbital were layered on top of a continuous sucrose density gradient and were centrifuged overnight. Fig. 3 shows that the various microsomal fractions collected from the gradient displayed high oxidative demethylation activities and low glucose-6-phosphatase activities. However, there was no fraction exhibiting only one of these metabolic activities, and the fractions exhibiting the highest oxidative demethylation activity also exhibited high glucose-6-phosphatase activity. Similar results were obtained for the patterns of distribution of the ATPase and DPNH-cytochrome *c* reductase activities.

Histochemistry

A. Light Microscopy

There were no definitive differences in the amount or distribution of reaction product between control and experimental animals. In

[2] Simultaneously with each one of the three phenobarbital injections, 12.5 mg of puromycin per 100 gm of body weight were injected intraperitoneally.

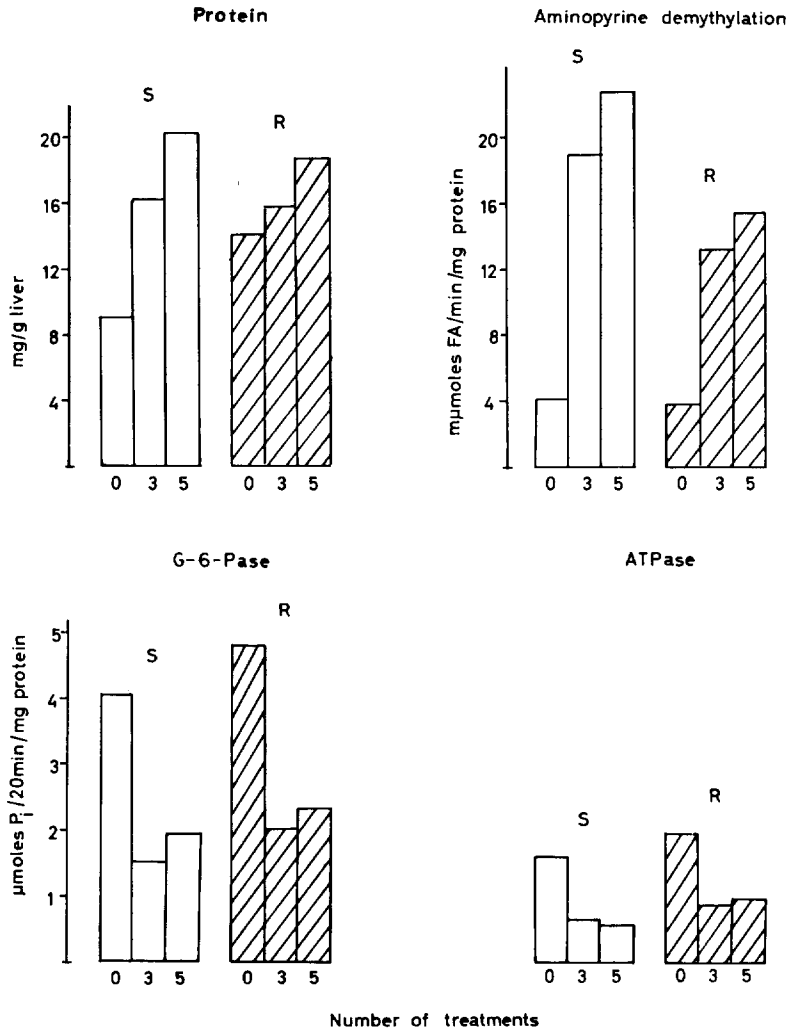


FIGURE 2 Effects of administration to rats of phenobarbital in vivo on the amount of protein and on the aminopyrine demethylation, glucose-6-phosphatase, and ATPase activities in the smooth- and rough-surfaced liver-microsomal subfractions. *S*, smooth-surfaced microsomes; *R*; rough-surfaced microsomes.

both groups delicate strands and granules of black lead sulfide deposits were present in the cytoplasm of hepatic parenchymal cells. In immersion-fixed tissues a well-defined nonreactive peripheral rim was always present. Below this zone, gradually increasing deposition of final product was observed. In the poorly fixed, central portions of the blocks, the entire cytoplasm appeared black. Sections incubated in a medium lacking substrate did not show deposition of reaction product.

B. Electron Microscopy

CONTROLS. Tissues fixed by perfusion with glutaraldehyde and postfixed in OsO_4 showed good preservation of fine structure throughout the blocks. In hepatic parenchymal cells two components of the cytoplasm were particularly well preserved: (*a*) the smooth-surfaced endoplasmic reticulum, which regularly appeared as branching and interconnected tubular profiles (Fig. 4), sometimes closely associated with lipid droplets (*inset*, Fig. 4), and (*b*) the fibrillar net-

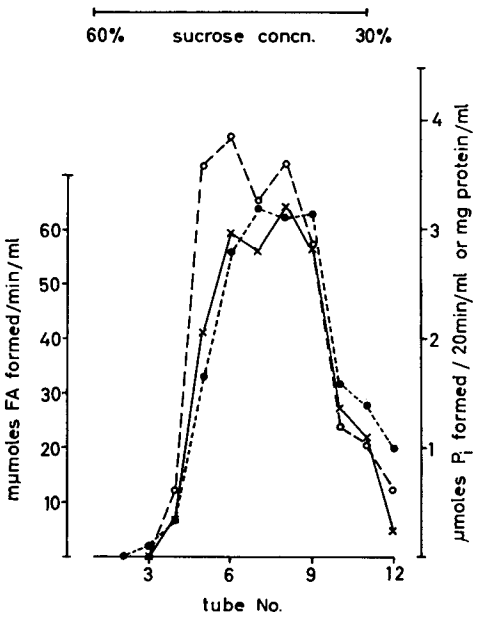


FIGURE 3 Patterns of distribution of protein, aminopyrine demethylation, and glucose-6-phosphatase activities after density gradient centrifugation of smooth-surfaced microsomes isolated from phenobarbital-treated rats. Smooth-surfaced microsomes (ca. 40 mg of microsomal protein) were isolated, from rats treated with three injections of phenobarbital, by density gradient centrifugation in the presence of CsCl (14), suspended in 3 ml of 0.25 M sucrose, and layered on top of a continuous sucrose density gradient ranging from 30 to 60% sucrose concentration. After centrifugation for 15 hr at 22,000 RPM in a Spinco SW25 rotor, the gradient was divided into 2 ml portions which were analyzed. ●-----●, protein; ×——×, aminopyrine demethylation activity (*FA*, formaldehyde); ○-----○, glucose-6-phosphatase activity.

work and the microtubules in the cytoplasmic ground substance. In tissues fixed by immersion, optimal preservation was only seen in the outermost 7 to 8 layers of parenchymal cells.

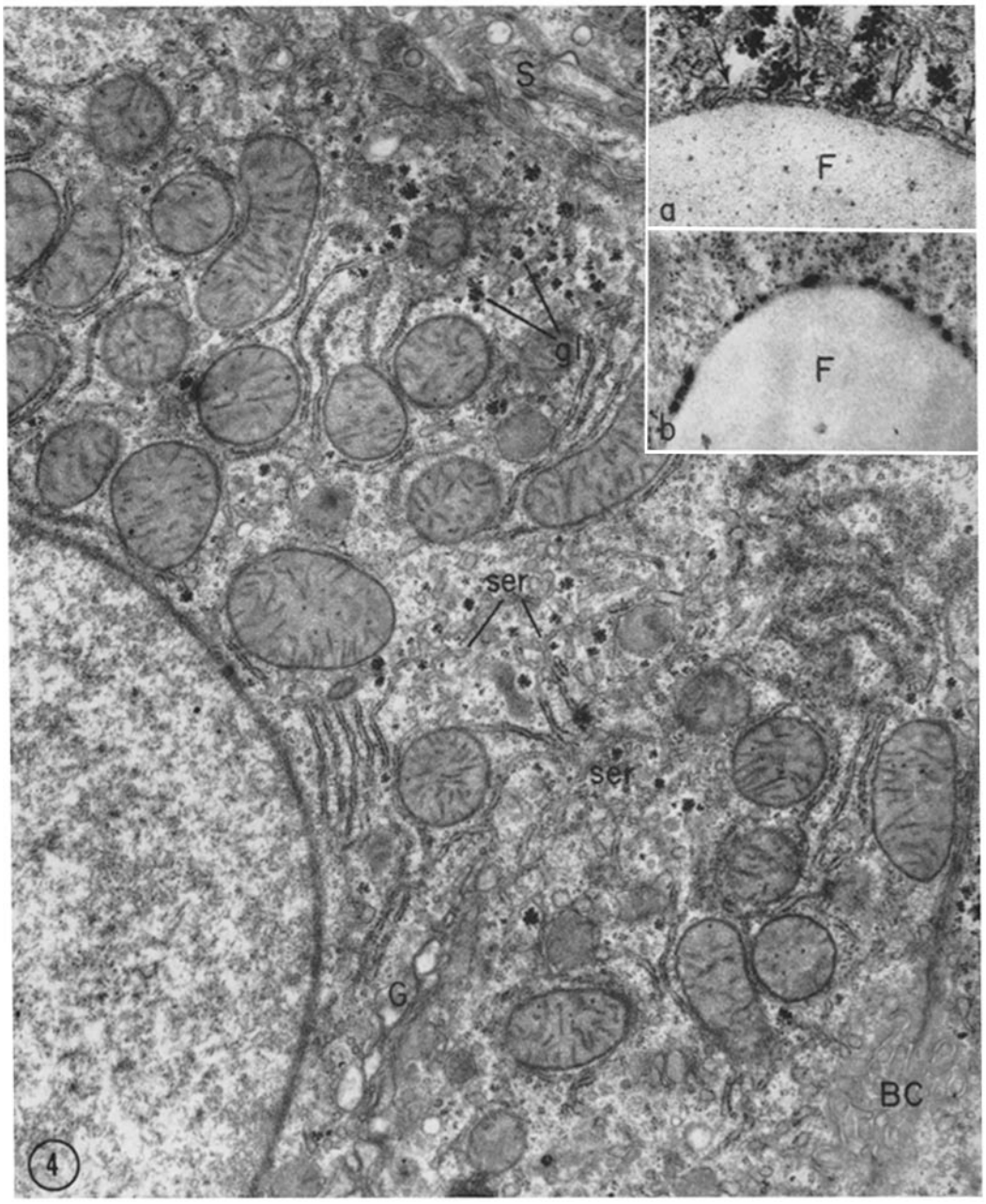
Membranes showed up as "positive" images, and membrane contrast was greatly enhanced following section staining, with lead hydroxide, of well-preserved areas of tissue. It was particularly interesting to note that the membrane contrast was very distinct even though the tissues had been directly transferred from glutaraldehyde to OsO_4 (without prior wash in buffer) (see Fig. 4).

EXPERIMENTAL ANIMALS. In general, the fine structural appearance of hepatocytes in animals given phenobarbital was similar to that in exclusively OsO_4-fixed tissues, as described previously (16). Thus, newly formed membranes of endoplasmic reticulum appeared to be growing out from pre-existing rough-surfaced cisternae and tubules. At 3 and 5 days (Fig. 9), large portions of cytoplasm were occupied by a feltwork of interconnected smooth-surfaced tubular and vesicular elements. These areas of cytoplasm clearly contained many more numerous tubular and vesicular elements than the most well-developed areas of smooth-surfaced endoplasmic reticulum in control animals. While in the latter the endoplasmic reticulum formed a rather loose network (*inset*, Fig. 9), compact masses of tubular and vesicular elements were observed in the phenobarbital-treated animals. The tubular profiles were frequently found in continuity with rough-surfaced cisternae of the endoplasmic reticulum. Alterations in other cytoplasmic organelles were not observed.

In previous studies of the morphological effects of phenobarbital, dilatation and fragmentation of the endoplasmic reticulum were noted in addition to proliferation of smooth- and rough-surfaced tubular elements (5). Since, apparently, the smooth-surfaced endoplasmic reticulum tends to break up into vesicular structures as a result of even minor injury (17, 18), including that caused by suboptimal fixation (19), the possibility has been indicated that vesicular appearance of proliferating membranes might represent an artifact of fixation (6, 16). The findings in perfusion-fixed livers in the present study supported this notion. Thus dilatation of endoplasmic reticulum or occurrence of large vesicular elements was not observed. It appeared that by perfusing the liver in vivo with glutaraldehyde a uniform stabilization of the membranes forming the endoplasmic reticulum was obtained in cells throughout the liver. This was particularly evident concerning the smooth-surfaced portion of the endoplasmic reticulum.

LOCALIZATION OF GLUCOSE-6-PHOSPHATASE

GENERAL. Since preservation of fine structure in most instances was inferior to that in nonincubated tissues, it was difficult to differentiate clearly among the different portions of endoplasmic reticulum from one another, and smooth- and rough-surfaced varieties were largely identified by their topographic arrangement and



Note: All the electron micrographs illustrated in Figs. 4 to 11 show the appearance of thin sections of Epon-embedded hepatic parenchymal cells stained with lead hydroxide.

FIGURE 4 Portion of hepatic parenchymal cell from a control animal. Tissue fixed by perfusion with glutaraldehyde for 15 min followed by immersion in glutaraldehyde for 3 hr, and postfixation directly in OsO_4 (without prior wash in buffer). General preservation of fine structure is similar to that in exclusively OsO_4-fixed specimens. Note good contrast of cytoplasmic membranes. *Inset a* shows portion of a fat droplet (*F*) which is closely surrounded by smooth-surfaced endoplasmic reticulum (*arrows*). *Inset b* illustrates the appearance of reaction product in the agranular endoplasmic reticulum surrounding a fat droplet (*F*) following incubation of glutaraldehyde-fixed tissue in the Wachstein-Meisel medium (for details of preparatory techniques, see legend for Fig. 8). *BC*, bile capillary; *G*, Golgi apparatus; *gl*, glycogen "rosettes"; *S*, sinusoid; *ser*, smooth-surfaced endoplasmic reticulum. × 16,000; *inset a* × 46,000; *inset b* × 25,500.

relationship to other cytoplasmic organelles (Fig. 8). In the most well-preserved areas where the membranes were not obscured by the precipitate, reaction product was often confined to the luminal space of cisternae and tubules (Figs. 5 and 7).

The appearance and distribution of the precipitate were the same in perfusion- and immersion-fixed tissues. However, the more uniform precipitation of reaction product in tissues fixed by perfusion made these preparations more convenient for the observations. Because of the apparently high sensitivity of the enzyme to aldehyde fixation (8, 20) and the ensuing variability in staining reaction, meaningful quantitative evaluations of the results could not be performed. Depending upon the time of incubation and, in the case of immersion-fixed tissues, the location of the cells within the tissue blocks, deposits of reaction product were spotty or formed continuous masses on the reactive membrane sites. With brief incubation times (10 min; perfusion-fixed tissues) the spotty reaction made it possible in many instances to clearly identify the type of organelle related to the reaction product. With longer incubation times the continuous masses of lead phosphate precipitate tended to obscure the underlying structures. There was no formation of precipitate in tissues incubated in a medium lacking glucose-6-phosphate.

CONTROLS. Lead phosphate precipitate was confined to the endoplasmic reticulum and the nuclear envelope (Figs. 5 to 8). In rats that were not fasted prior to sacrifice, large portions of cytoplasmic matrix were occupied by masses of glycogen granules. Such "glycogen areas" were permeated by a loose network of smooth-surfaced tubular and vesicular elements showing evidence of reaction product on their membranes (Fig. 8).

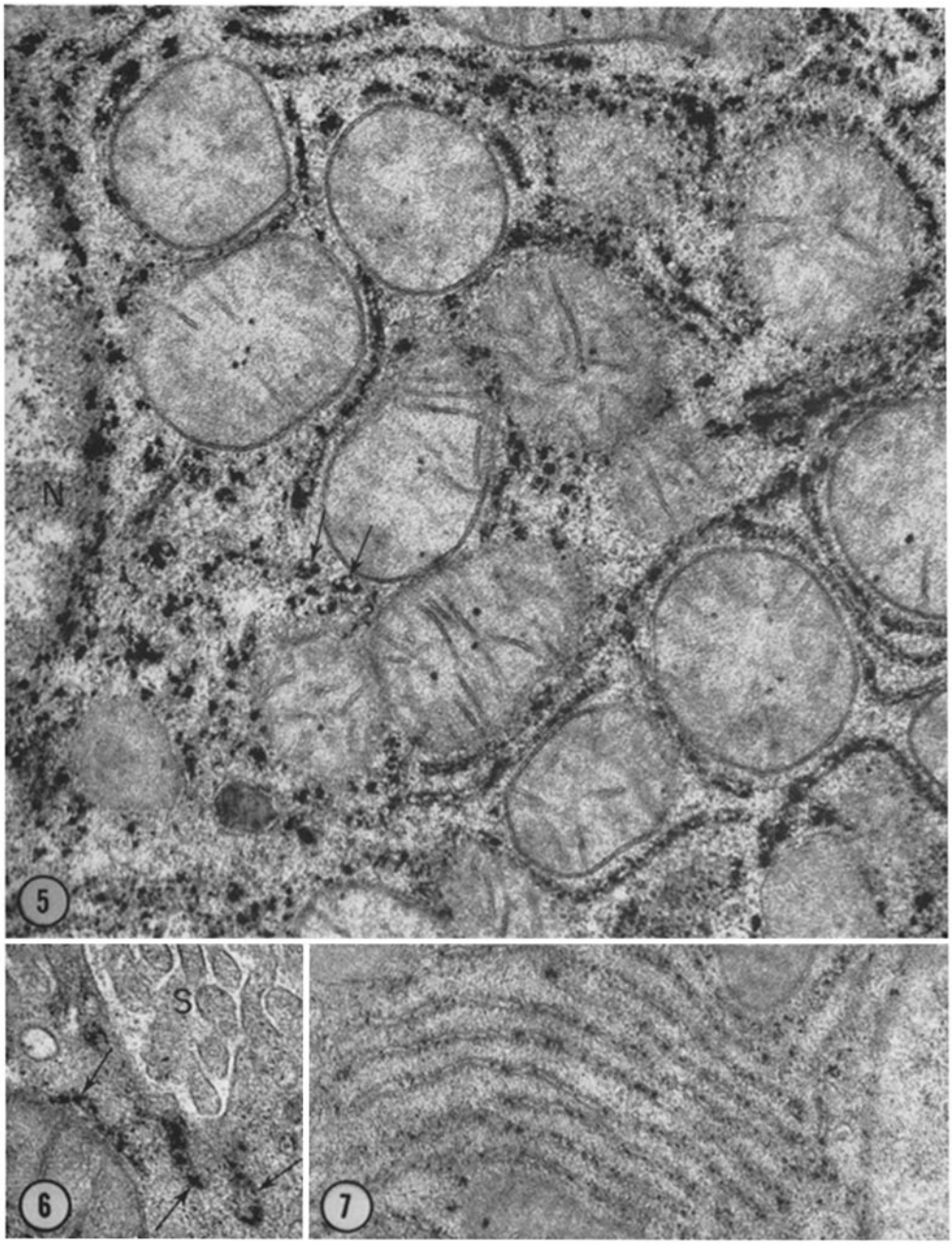
Lead phosphate precipitate appeared to be formed in relation to all portions of both the rough- and the smooth-surfaced endoplasmic reticulum (Figs. 5 to 8). Thus it was associated with smooth-surfaced endoplasmic reticulum curving around lipid droplets (Fig. 4), with the "paramembranous" smooth-surfaced cisternae along the lateral plasma membrane (21) (Fig. 8), and with the smooth-surfaced endoplasmic reticulum located immediately below the plasma membrane in sinusoidal areas of the cytoplasm (Fig. 6).

EXPERIMENTAL ANIMALS. With long incubation times and in improperly fixed areas of immersion-fixed tissues, large amounts of precipitate occurred in portions of the cells which appeared to correspond to areas with proliferating membranes. However, the precipitate tended to obscure the underlying structures, making positive identification impossible. With shorter incubation times a spotty precipitate of reaction product was noted in all areas with smooth-surfaced proliferates (Figs. 10 and 11). Specificity of the reaction was suggested by the absence of precipitate in other portions of the cytoplasm. The spotty distribution of the reaction product within the proliferates, and the close spacing of individual tubular and vesicular elements within the latter made it difficult in most instances to clearly relate the precipitate to a particular membrane, tubule, or vesicle. However, in fortuitous sections it was possible to demonstrate that reaction product was localized within the lumen of smooth-surfaced tubules or vesicles (Fig. 10). In addition, precipitate was present in the same areas that were reactive in untreated animals, *i.e.*, rough-surfaced endoplasmic reticulum, the paramembranous cisternal or tubular system (21), and the nuclear envelope.

DISCUSSION

The phenobarbital-induced enhancement of the drug-hydroxylating enzyme system and proliferation of endoplasmic reticulum membranes have proved to be constantly attended by a decrease in specific activities of glucose-6-phosphatase, ATPase, and DPNH–cytochrome *c* reductase (5). This phenomenon has been investigated to obtain further information on the enzyme-membrane relationship in liver microsomes. In the present study attention has been focused mainly on the response of glucose-6-phosphatase to phenobarbital administration in vivo. This enzyme has the advantage that it is relatively stable, and is also readily demonstrable histochemically (20).

The decrease in the specific activities of glucose-6-phosphatase, ATPase, and DPNH–cytochrome *c* reductase roughly equalled the increase in microsomal protein due to phenobarbital treatment. This finding raised the question whether drug treatment in vivo induces the formation of specific endoplasmic membranes exhibiting high drug-hydroxylating activity but lacking glucose-6-phosphatase, ATPase, and



Note: Figs. 5 to 8 show sections of *normal* hepatic parenchymal cells fixed by perfusion with glutaraldehyde for 1 min; incubated in the Wachstein-Meisel medium for 10 (Figs. 6 and 7) or 15 (Figs. 5 and 8) min; and postfixed in OsO_4. Figs. 5 to 7 show the appearance of cells from an animal that was fasted for 16 hr prior to sacrifice; Fig. 8 is from an animal that was not fasted.

FIGURE 5 Meandering tubular profiles of rough-surfaced endoplasmic reticulum partly curving around mitochondria show presence of precipitate. A similar precipitate is located within smooth-surfaced vesicular elements (*arrows*) and the nuclear envelope. Note that the final product appears to be confined to the lumens of the endoplasmic cisternae and tubules. *N*, nucleus. × 34,000.

FIGURE 6 Micrograph illustrating presence of reaction product in smooth-surfaced tubular elements (*arrows*) closely associated with the sinusoidal border of the cell. *S*, sinusoid. × 40,000.

FIGURE 7 Reaction product is located on the nuclear membrane and the cisternae of rough-surfaced endoplasmic reticulum. In the latter, most of the lead phosphate precipitate is deposited inside the cisternae. × 28,000.

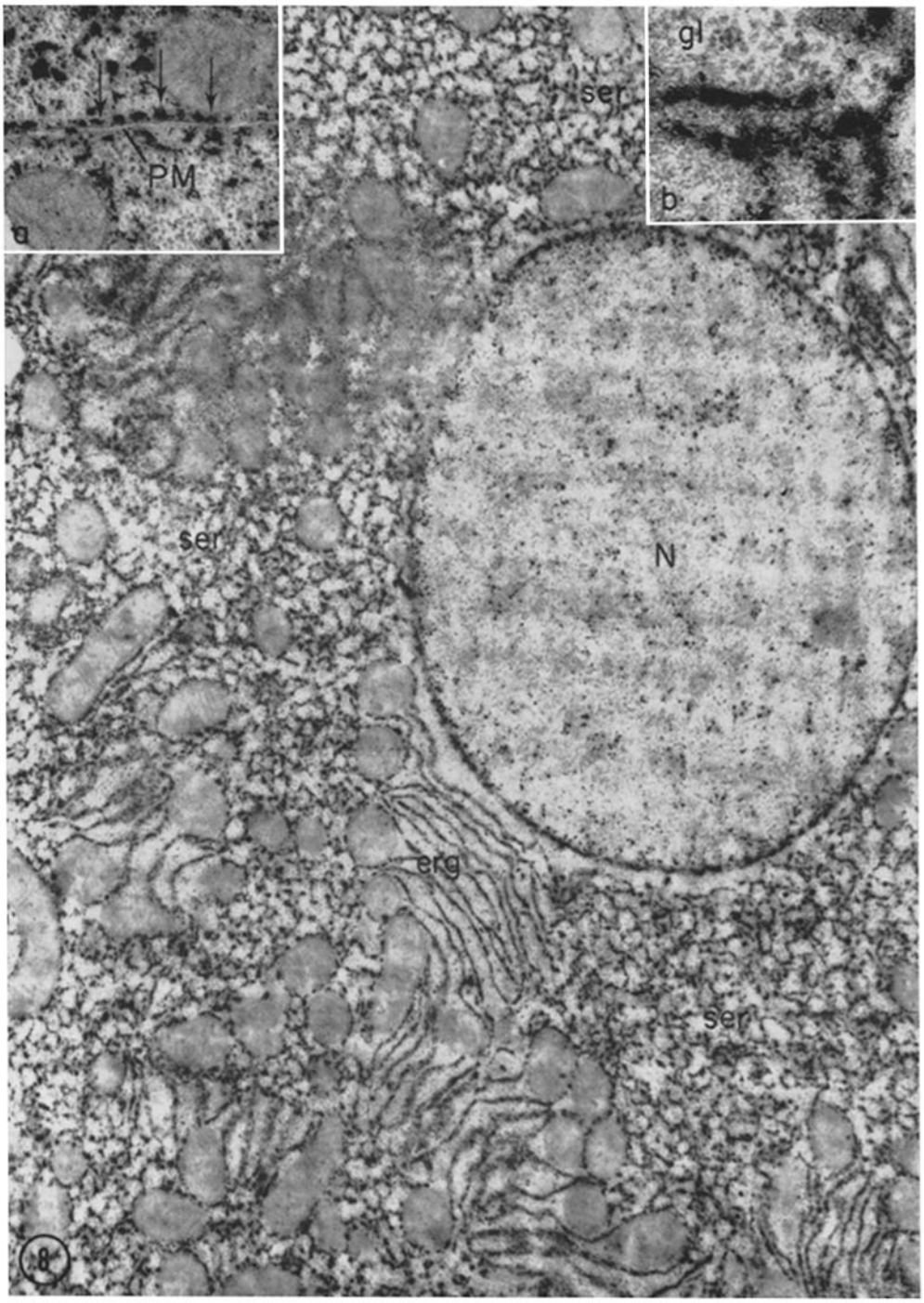


FIGURE 8 Survey micrograph showing part of a hepatic parenchymal cell which contained abundant glycogen (nonfasted animal). Reaction product is confined to long, slender arrays of ergastoplasm (*erg*), cf. also Fig. 7, short smooth-surfaced tubular or vesicular, partly interconnected elements (*ser*) in "glycogen areas" (cf. *inset b*), and the nuclear envelope. In addition there is some spurious reaction product within the nucleus (*N*). *Inset a* shows deposition of reaction product on membrane system ("paramembranous cisternal system") (*arrows*) located along the plasma membrane (*PM*) and believed to represent a specialized part of the endoplasmic reticulum. *Inset b* is a high magnification of a portion of a glycogen area to illustrate the appearance of the smooth-surfaced tubular profiles containing reaction product in these areas *gl*, glycogen. × 12,000; *inset a* × 23,000; *inset b* × 59,000.

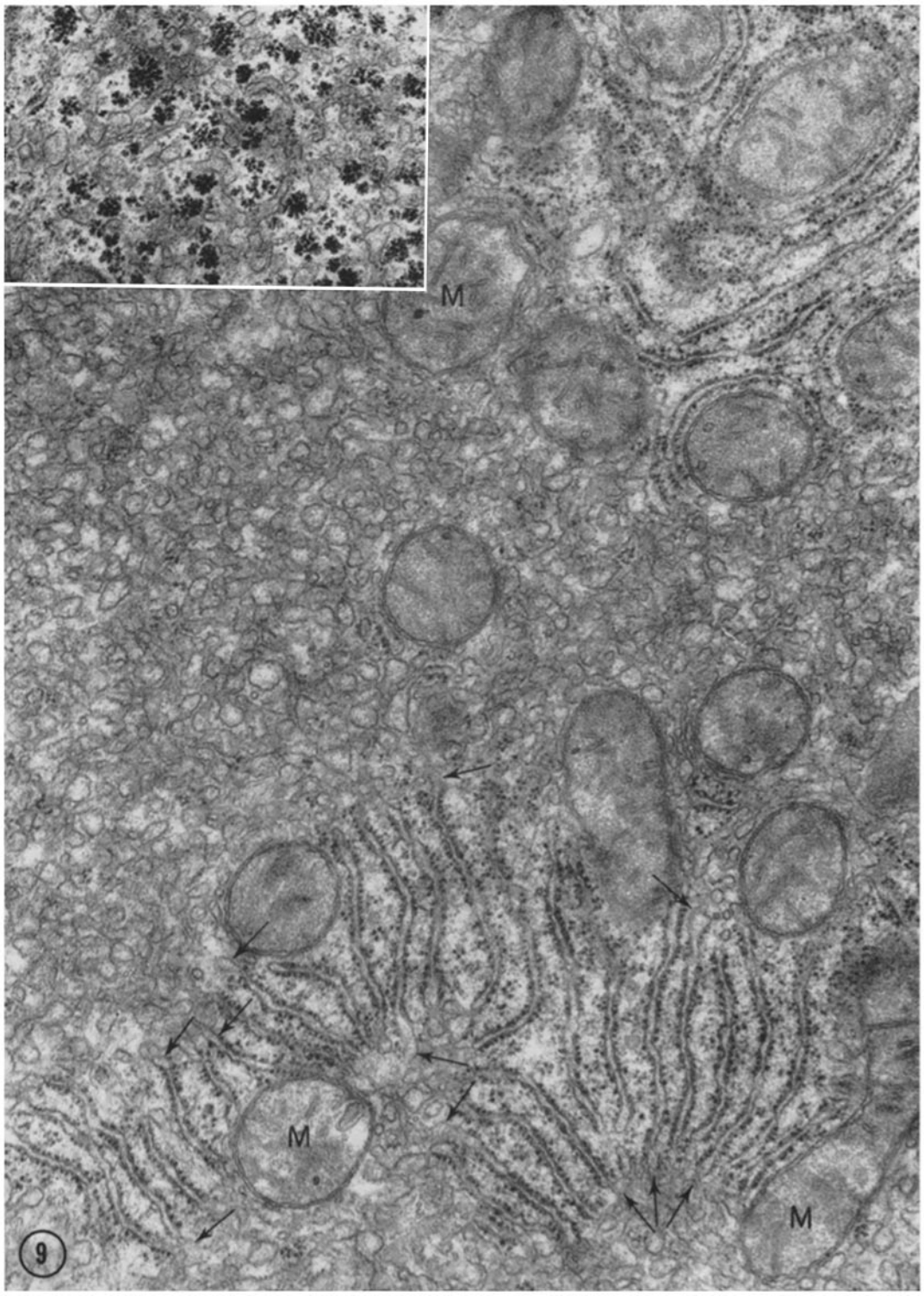


FIGURE 9 Experimental animal: 120 hr. 5 injections of phenobarbital. Specimen fixed by perfusion with glutaraldehyde for 15 min; immersion in glutaraldehyde for 3 hr; and after fixation with OsO_4 following a 24 hr wash in cold buffer. Extensive proliferation of smooth-surfaced branching tubular and vesicular elements; some of them appear to be continuous with rough-surfaced endoplasmic reticulum (*arrows*). The proliferates are much more extensive and compact than in areas with maximal abundance of smooth-surfaced endoplasmic reticulum in control animals (*inset*). Some mitochondria (*M*) show rarefaction of their matrix, possibly caused by the wash in buffer (cf. Fig. 4). × 27,000; *inset* × 30,000.

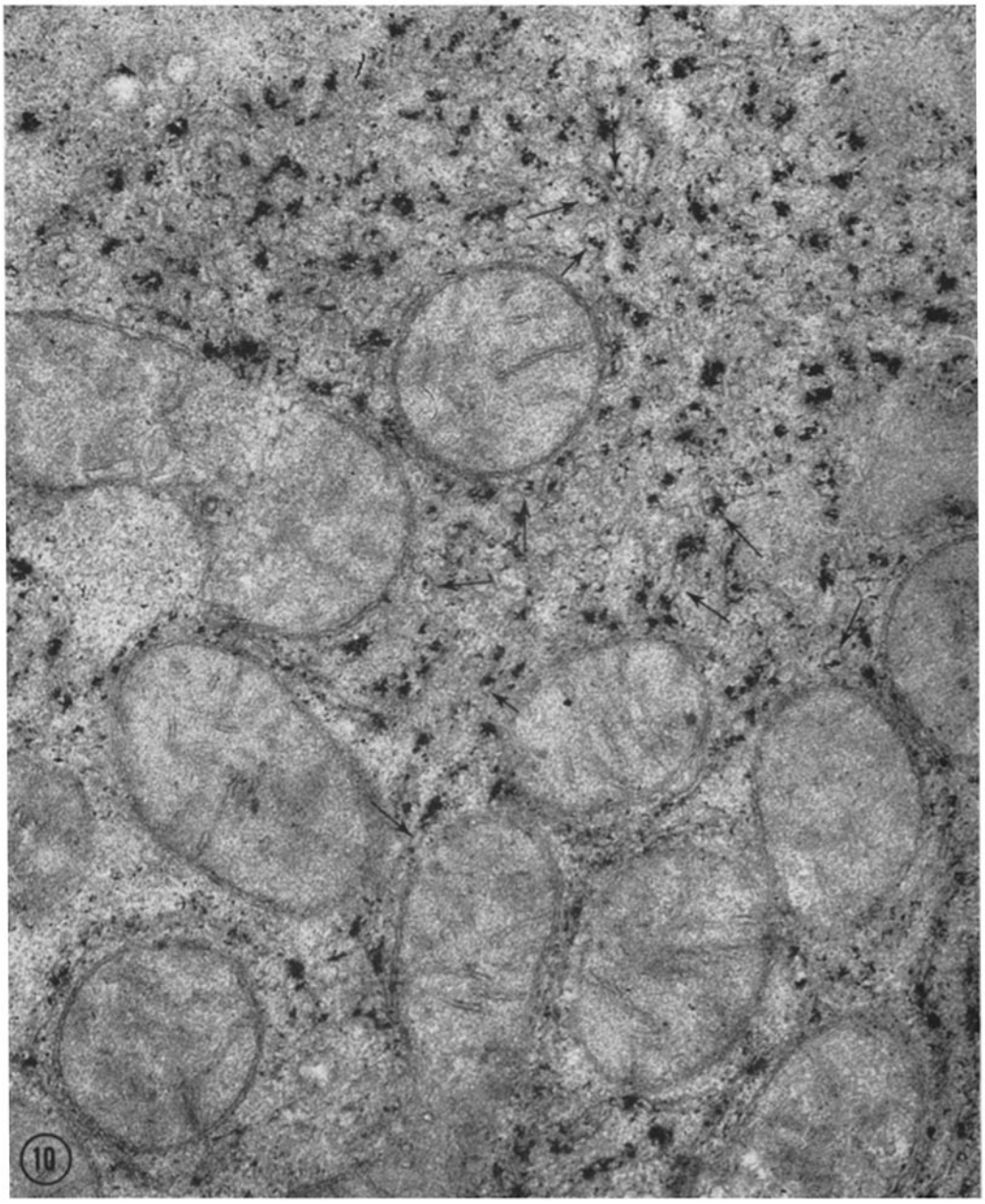


FIGURE 10 Experimental animal: 72 hr. 3 injections of phenobarbital. Incubation for demonstration of glucose-6-phosphatase (concerning preparation of specimen, see legend for Figs. 6 and 7). Lead phosphate reaction product is deposited in an area with moderate proliferation of smooth-surfaced membranes (upper portion of the micrograph). Note that in many instances the precipitate is confined to the interior of tubular and vesicular profiles (*arrows*). × 49,000.

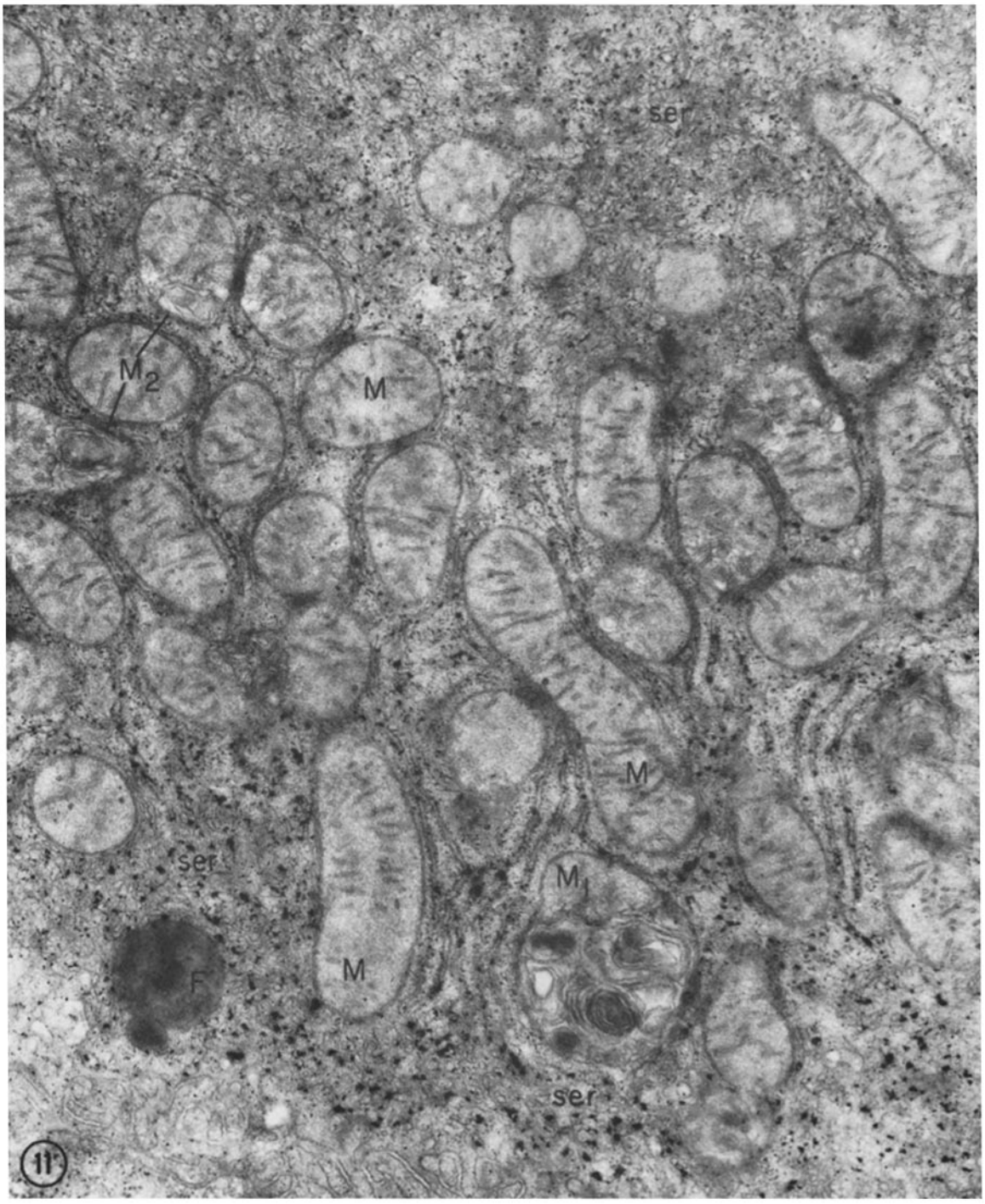


FIGURE 11 Experimental animal: 120 hr. 5 injections of phenobarbital. From an area of tissue fixed by immersion in glutaraldehyde for 1 hr and incubated in the Wachstein-Meisel medium for 15 min; and postfixed in OsO_4. Extensive proliferation of smooth-surfaced tubular and vesicular elements (*ser*) in some areas of cytoplasm. Although some mitochondria (*M*) show normal appearance, others are enlarged (M_1) and contain variable amounts of membrane whorls (M_1 and M_2). The alterations in M_1 and M_2 are believed to represent preparation artifacts (possibly partial solubilization of mitochondrial phospholipids prior to fixation in OsO_4). Fat droplet (F). × 25,000.

DPNH-oxidizing activities. This hypothesis was not supported by the present results. Both smooth- and rough-surfaced microsomes exhibited decreased glucose-6-phosphatase and ATPase activities after phenobarbital administration in vivo. Attempts to subfractionate the smooth microsomes via centrifugation on a sucrose density gradient were unsuccessful, and all the fractions recovered exhibited low but significant glucose-6-phosphatase activities. Furthermore, the pattern of distribution of glucose-6-phosphatase activity among the various membrane fractions collected from the gradient almost exactly equalled that of the oxidative demethylation activity.

Actinomycin D has earlier been reported to inhibit the induction by phenobarbital of the drug-hydroxylating enzyme system (5) as well as the decrease in the level of this system after withdrawal of the inducer (16). Simultaneous administration of actinomycin D now proved to have no effect on the decrease in glucose-6-phosphatase activity after phenobarbital treatment; this suggests that the phenomenon is not due to the DNA-dependent synthesis of a repressor. It should be added here that the phenobarbital-induced proliferation of endoplasmic reticulum membranes has been found to be only partially inhibited by actinomycin D (5, 16).

Glucose-6-phosphate is a compound which is relatively resistant to hydrolysis, and presently available evidence indicates that the enzyme responsible for the hydrolysis in the Wachstein-Meisel procedure is a specific glucose-6-phosphatase. The specificity of this histochemical procedure is supported by experiments with chemically related substrates and with various inhibitors (20, 22). Previous histochemical studies (20) have suggested that glucose-6-phosphatase might not be associated with all portions of the endoplasmic reticulum of hepatic parenchymal cells in the adult rat. Thus, it was proposed that glucose-6-phosphatase might be lacking in the endoplasmic reticulum of glycogen areas. With the improved methods utilized, in the present study, for preserving tissue fine structure and retaining enzyme activity, it appears that all portions of the endoplasmic reticulum do indeed contain glucose-6-phosphatase in the normal rat. Rosen et al. (22) recently presented evidence indicating the presence of glucose-6-phosphatase in the tubular endoplasmic reticulum of glycogen areas in neonatal mouse liver hepatocytes. Although a spotty precipitation of reaction product was noted in the smooth-surfaced proliferates of phenobarbital-treated animals, the occurrence of massive precipitates over these areas in "overincubated" sections makes it unlikely that only some of the tubular and vesicular elements carried glucose-6-phosphatase. When taken together with the biochemical results, the evidence would appear, therefore, to indicate that glucose-6-phosphatase is present in the proliferating membrane systems induced by the administration of phenobarbital.

Although it was beyond the scope of the present investigation to study in detail the effects of fixation on the activity of glucose-6-phosphatase, one point of practical importance may be worth mentioning. Since, apparently, glucose-6-phosphatase is very rapidly inactivated by glutaraldehyde (8, 20), the peripheral negative zone seen in the light microscope in sections incubated in the Wachstein-Meisel medium may be taken as an approximative measure of the depth of penetration of the fixative. Since, after 1 hr of immersion fixation, this zone is only $\sim$200 μ wide, the evidence would appear to suggest that the rate of penetration of glutaraldehyde is very low and of approximately the same order of magnitude as that of OsO_4. Preliminary studies of the fine-structural appearance of cells at various depths of glutaraldehyde immersion-fixed rat liver support the assumption that optimal preservation of fine structure is only present in the outermost 8 to 10 layers of cells (23).

In the present study the enzymes exhibiting decreased specific activities after phenobarbital treatment in vivo, *i.e.* glucose-6-phosphatase, ATPase, and DPNH-cytochrome *c* reductase, were only measured by their activities. However, since administration of puromycin in vivo inhibited the phenobarbital-induced decrease in specific enzyme activities and treatment with phenobarbital of the isolated microsomes in vitro did not affect the glucose-6-phosphatase activity, it seems reasonable to assume that the observed decrease in activity represents an equal decrease in enzyme content per unit of microsomal membrane. A decrease in the level of glucose-6-phosphatase has also been observed following administration of carcinogens (24).

The present results are compatible with the assumption that phenobarbital administration in vivo gives rise to an enhanced synthesis of the drug-hydroxylating enzyme system and of en-

doplasmic reticulum membranes, but does not appreciably affect the rate of synthesis of glucose-6-phosphatase, ATPase, and DPNH–cytochrome *c* reductase. Still another type of response to phenobarbital treatment is shown by cytochrome b_5, which reveals an increased concentration parallel to the increase in membrane constituents (5, 7). One may speculate that the relationship of this enzyme to the membrane differs from those of the other enzymes studied, but further data are necessary for elucidating this phenomenon.

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

We are also indebted to Miss Margareta Sparthan, Miss Gesa Thies, and Mrs. Hjördis Thor for valuable technical assistance.

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

Received for publication 22 March 1966.

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