STUDIES ON THE BIOGENESIS OF SMOOTH ENDOPLASMIC RETICULUM MEMBRANES IN LIVERS OF PHENOBARBITAL-TREATED RATS

I. The Site of Activity of Acyltransferases Involved in Synthesis of the Membrane Phospholipid

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ABSTRACT

The localization of acyltransferases involved in acylation of α-glycerophosphate, during phenobarbital induced proliferation of smooth endoplasmic reticulum (ser) membranes, has been investigated using cytochemical and cell fractionation techniques. In cytochemical studies of normal rat liver, reaction product marking acyltransferase activity was associated to the greatest extent with the rough endoplasmic reticulum (rer) membranes and to a lesser extent with ser membranes. In liver from phenobarbital-treated rats, reaction product was largely restricted to ser membranes. The specific activity of the acyltransferases of rough microsomes from normal rat liver was higher than that of the smooth microsomes. On injection of phenobarbital, this fell rapidly after three injections to a low level, at which it remained during subsequent treatment. The specific activity of the smooth microsomes, on injection of phenobarbital, rose to a peak 12 hr after the first injection, after which it fell to a level at an activity above that of smooth microsomes of normal liver. A mechanism is postulated for the biogenesis of smooth membranes in which the phospholipid is synthesized *in situ* and the protein is synthesized in the rer and moves to the site of newly synthesized phospholipid, where it is inserted to produce a whole membrane.

INTRODUCTION

In order to investigate the biogenesis of cellular membranes, the initial problem lies in the choice of a suitable system in which proliferation of one membranous species occurs with no change in other organelles. In eukaryotic cells this ideal system is not attainable because even in the resting state there is a continual synthesis and breakdown of the components of all membranes. However, apart from this inherent drawback, one system which partially fulfills this requirement is the liver of the phenobarbital-treated rat. In these animals there is a marked proliferation of the smooth endoplasmic reticulum (ser) membrane of hepatocytes (1–5). Accompanying this morphological change, there is an increased accumulation of liver phospholipids (2, 4, 7) which appears to be due mainly to increased phosphatidylcholine content (6). There is also an increase in the level of microsomal enzymes, notably those involved in detoxification of drugs (2, 4, 7–10). It has been established that newly synthesized membrane proteins appear in the rough endoplasmic reticulum (rer) and sub-

sequently in the smooth membranes (4, 7). However, there have been no satisfactory studies of the site, in the cell, at which synthesis of the phospholipid component occurs although such knowledge would be of value in gaining an understanding of the way in which these membranes are assembled. The phospholipid of ser is mainly glycerophospholipid, of which phosphatidylcholine is the major constituent (11, 12). Phosphatidylcholine is synthesized by a well-known pathway, the first two steps of which involve transfer of a long chain fatty acid from acyl CoA to α-glycerophosphate to yield phosphatidic acid, with release of CoA. There is some variation in the final steps in the biosynthetic pathways for different glycerophospholipids, but all these have in common are the initial steps, catalyzed by acyltransferases.

We have recently developed methods for the fine structural localization of acyltransferases based on the formation of a heavy metal-containing precipitate at the site of free CoA release from acyl CoA (13, 14). These methods have been used to demonstrate the site at which the initial steps in the synthesis of phospholipid occur during phenobarbital-induced proliferation of ser. In addition, the site of acyltransferase activity has been determined under the same conditions in rough and smooth microsomes.

MATERIALS AND METHODS

Animals and Tissues

Male albino rats (150–200 g) were used either untreated or after one to five daily injections of phenobarbital (11 mg/100 g body weight). For studies on unfixed liver, the animals were lightly anesthetized with ether and the liver was removed and used immediately. For studies on fixed liver, the animals were anesthetized with chloral hydrate (3.1%, 1 ml/100 g body weight) and perfused with ice-cold glutaraldehyde (0.5% or 1.0%) or freshly prepared formaldehyde (1.0%) with glutaraldehyde (0.25%) in cacodylate buffer, pH 7.4, 0.05 M containing 4.5% dextrose. The liver was removed, sliced (1–2 mm thick), and washed by immersion in ice-cold cacodylate buffer for periods of 1–2 hr or overnight.

Preparation of Microsomal Fractions

Total, rough, and smooth microsomes were prepared by a modification of the method of Dallner et al. (15) from livers of rats either untreated or treated with one to five injections of phenobarbital. Livers were removed and homogenized with a Potter-Elvejhem homogenizer in ice-cold 0.25 M RNAse-free sucrose to yield a 20% (w/v) homogenate. The homogenate was centrifuged at 10,300 *g* for 20 min (Spinco 40 rotor 12,500 rpm, Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) and the supernatant was removed and recentrifuged at the same speed. To each 9.85 ml of supernatant, 0.15 ml of 1 M $CsCl_2$ was added and 12 ml of the mixture were layered on 15 ml of 1.3 M sucrose containing 15 mM $CsCl_2$ in Spinco SW 25.1 centrifuge tubes. These were centrifuged at 53,500 *g* for 150 min (Spinco SW 25.1 rotor, 24,000 rpm). The smooth microsomes which floated at the interface were removed and diluted with distilled water (1:10, approximately), and the rough microsomal pellet was resuspended in 0.25 M sucrose. Both fractions were isolated by centrifugation at 105,000 *g* for 20 min (Spinco 40 rotor, 40,000 rpm). Total microsomes were prepared by centrifugation of the original supernatant, after addition of $CsCl_2$, at 105,000 *g* for 20 min. All microsomal fractions were washed by resuspending in 0.25 M RNAse-free sucrose and recentrifuging at 105,000 *g* for 20 min. The pellets were resuspended in buffer for enzyme assays.

Biochemical Studies

Microsomal suspensions, homogenates of unfixed liver, or homogenates or slices of fixed liver were incubated in media containing L-α-glycerol-^{14}C (UL) phosphate (International Chemical and Nuclear Corporation, Burbank, Calif.) (approximately 40,000 cpm/μmole) or unlabeled α-glycerophosphate, *S*-palmityl-1-^{14}C coenzyme A (International Chemical and Nuclear Corporation) (approximately 2×10^6 cpm/μmole) or unlabeled palmityl CoA (Sigma Chemical Co., St. Louis, Mo.) in cacodylate buffer, pH 7.2, 0.05 M containing 4.5% dextrose at 37°C. Control experiments were performed as indicated in the text. At the end of the incubation period, the reaction was stopped by addition of ice-cold chloroform:methanol (2:1) to extract the lipids (16). Samples of the total lipid extract were separated on thin layers of silica gel into the major lipid classes, using the solvent system isopropyl ether:heptane:glacial acetic acid (40:60:2, v:v:v) (17). The phospholipids were separated using the method of Skipski et al. (18), consisting of a two-step, one-dimensional development of the chromatogram, using acetone:petrol (1:3, v:v) as the first system, followed by chloroform:methanol:glacial acetic acid:water (80:13:8:0.3, v:v:v:v) as the second system. The lipids were visualized with iodine vapor, which was allowed to sublime, before the lipid-containing bands were scraped directly into counting vials and counted in a Packard Tri-Carb scintillation counter (Packard Instrument Co., Downers Grove, Ill.). In experiments using slices, the tissue present was expressed in terms of the lipid-free dry weight (14).

Cytochemical Studies

Of the methods for the localization of acyltransferases described previously (13, 14), the manganous chloride and potassium ferricyanide capture reagent system was used (14). The complete incubation medium for cytochemical experiments contained manganous chloride (0.5 mg/ml; 2.5 mM), potassium ferricyanide (0.15 mg/ml; 0.45 mM), palmityl CoA (0.2 mg/ml; 0.2 mM), and α-glycerophosphate (0.5 mg/ml; 1.8 mM) in cacodylate buffer, pH 7.2, 0.05 M containing 4.5% dextrose. This buffer was used throughout these studies. Chopper slices of fixed liver (100 μ) were preincubated in potassium ferricyanide (0.15 mg/ml) in buffer for 30 min to remove endogenous oxidizable material (13), rinsed in buffer, and preincubated in 10^{-3} M diisopropyl fluorophosphate (DFP) in buffer for 30 min to inhibit palmityl CoA hydrolase (19, 20). The slices were finally rinsed in buffer and incubated in the medium described above. In control experiments, substrates were omitted. Incubations were performed at room temperature for 2 hr. The slices were removed from the incubation media, rinsed in buffer, and refixed in buffered 1% osmium tetroxide for 30–60 min, dehydrated through a graded series of ethyl alcohol, propylene oxide, and embedded in Epon. Thin sections were cut on an LKB ultramicrotome (LKB Instruments, Rockville, Md.), stained with lead nitrate for 30 sec, and viewed in an Hitachi 11B electron microscope.

Microsomal fractions were fixed as pellets after centrifugation by immersion in buffered 1% osmium tetroxide for 15–18 hr. The pellets were then gently detached from the centrifuge tube, dehydrated, embedded in Epon, and prepared for electron microscopy, as the tissue blocks described above.

RESULTS

The Effect of Fixation on Incorporation of α-Glycerophosphate-^{14}C into Lipids

This effect was determined using homogenates of liver from rats given five injections of phenobarbital. Incorporation was initially rapid and reached a maximum after 10–15 min incubation. Such a pattern of incorporation was found consistently in liver from untreated rats, as well as from those treated with phenobarbital, and with both fixed and unfixed tissue. Since the final level of incorporation rises with increased tissue concentration, the major cause of cessation of incorporation of α-glycerophosphate is probably due to inhibition of the enzymes by the product of their activity. In addition, although complete utilization of α-glycerophosphate does not occur, fairly rapid hydrolysis of palmityl CoA occurs in fixed and unfixed tissue (19) and this may be a factor in determining the final level of incorporation of α-glycerophosphate, as this is dependent on the presence of palmityl CoA (Fig. 1).

In order to determine the activity of the enzymes involved in the incorporation of α-glycerophosphate into lipid, the initial rate of the reaction was plotted against tissue concentration. Incorporation was linear with tissue concentration and in unfixed

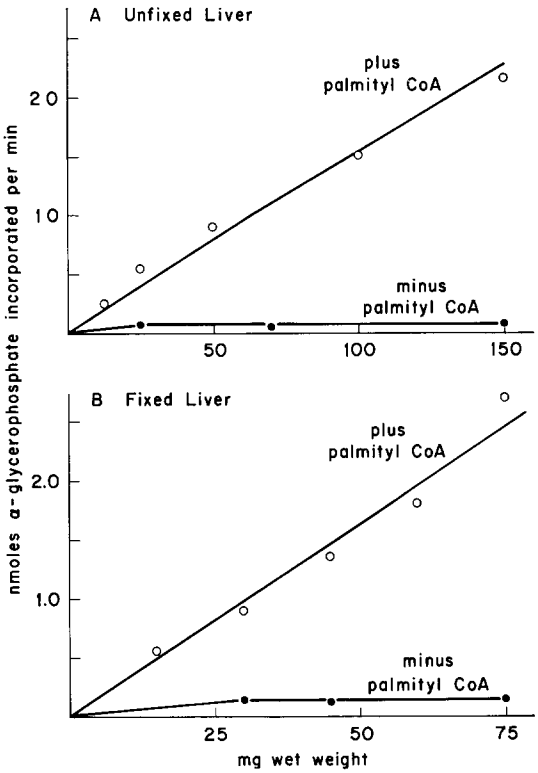


FIGURE 1 Incorporation of α-glycerophosphate-^{14}C into lipids of fixed and unfixed rat liver from rats given five injections of phenobarbital. Homogenates of unfixed and fixed rat liver from phenobarbital-treated rats (five injections) were incubated in media containing α-glycerophosphate-^{14}C (1.8 mM) with and without palmityl CoA (0.2 mM) in cacodylate buffer, pH 7.2, 0.05 M containing 4.5% dextrose (final volume 1.0 ml) at 37°C for a range of times (0–30 min). At the end of the incubation period, the reaction was stopped by addition of ice-cold chloroform:methanol (2:1) and the total lipid was extracted and counted, as described in Methods. In both A and B, the initial rate of incorporation of α-glycerophosphate-^{14}C into lipid is plotted against concentration of liver. In A, unfixed liver was used, and in B, liver fixed by perfusion with 0.5% glutaraldehyde as described in Methods. Note that the scales of A and B differ.

tissue occurred at a rate of 0.15 nmoles of α-glycerophosphate per min per mg liver (Fig. 1 A). When homogenates of liver fixed by perfusion with 0.5% glutaraldehyde and washed with buffer for 2 hr were used in similar experiments, incorporation of α-glycerophosphate was also linear with tissue concentration and occurred at a rate of 0.038 nmoles per min per mg liver (Fig. 1 B). Thus, retention of activity in fixed tissue was 22.4% of that of unfixed tissue. Similar values of 20–26% were found over a series of four experiments using this fixation and washing procedure. There is no absolute standard for relating enzyme activity of fixed liver to that of unfixed liver. Methods for determination of protein do not give accurate results when performed on fixed tissue, probably because the fixation procedure cross-links protein and hence prevents uncovering of groups measured in colorimetric assay methods. Similar problems probably exist in using DNA as a standard, and this may also be partially removed during the fixation and washing procedures. Methods based on tissue weight are also rendered unreliable because the fixation procedure causes loss of some soluble materials, and may also cause alterations in tissue volume and hence alterations in water retention. In the experiment illustrated in Fig. 1, wet weight of liver was used as a tissue standard. In addition, the trichloroacetic acid-insoluble, dry weight was determined and, using this standard retention of activity, in fixed liver was 25% of that of unfixed liver.

In order to determine the distribution of α-glycerophosphate-^{14}C incorporated by fixed or unfixed liver, the lipids were separated into the major fractions. At all incubation times and at all tissue concentrations, over 90% of the label was in the phospholipid fraction, with no significant incorporation into other esterified lipids. When the phospholipids were separated, the label was found to be mainly associated with the phosphatidic acid fraction, with smaller amounts remaining at the origin and probably corresponding to lysophosphatidic acid. In the absence of other substrates, therefore, incorporation of α-glycerophosphate into phospholipid proceeds only to phosphatidic acid and only involves the first two steps in the over-all pathway.

In addition to the fixation and washing procedures used above, other procedures were investigated. When liver was fixed by perfusion with 1.0% glutaraldehyde followed by washing for 2 hr, a retention similar to that with 0.5% glutaraldehyde was found; however, this was more variable. Since perfusion of rats with 0.5% glutaraldehyde gave adequate morphological preservation of the liver, this was selected for the cytochemical studies. When liver fixed with 1.0% glutaraldehyde was washed for 15 hr, acyltransferase activity fell to less than 10% of that of unfixed tissue. The washing time after fixation is therefore a critical factor in retaining this activity. In other tissues it has been found that acyltransferase activity survives fixation with mixtures of formaldehyde and glutaraldehyde; however, in liver this fixation completely destroyed activity.

Metabolism of α-Glycerophosphate-^{14}C by Slices of Fixed Liver: Time Course of Metabolism

Liver homogenates were used in the above experiments to eliminate possible differences in the metabolism of α-glycerophosphate due to permeability differences between fixed and unfixed hepatocytes. However, as slices were used in the cytochemical experiments, the metabolism of α-glycerophosphate by slices of fixed liver was investigated (Fig. 2). As with homogenates, there

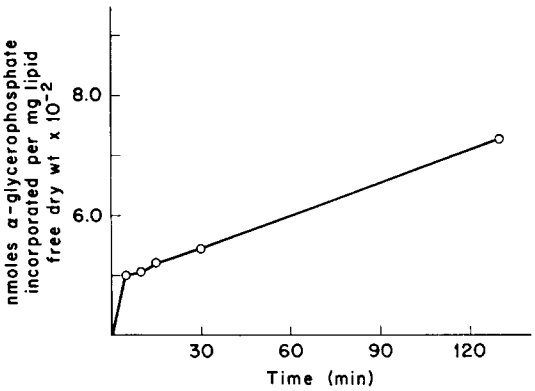


FIGURE 2 Incorporation of α-glycerophosphate-^{14}C into lipid by slices of fixed liver from rats given five injections of phenobarbital. Slices of fixed liver (approximately 50 mg wet weight) were incubated in media containing α-glycerophosphate-^{14}C (1.8 mM) and palmityl CoA (0.2 mM) in cacodylate buffer, pH 7.2, 0.05 M containing 4.5% dextrose (final volume 1.0 ml) at 37°C for a range of times. The reaction was stopped by addition of ice-cold chloroform: methanol (2:1) and the total lipid was extracted and counted, as described in Methods. Incorporation of α-glycerophosphate-^{14}C into lipids per mg lipid-free dry weight is plotted against time of incubation.

TABLE I

Incorporation of α-glycerophosphate-^{14}C into Lipid by Slices of Fixed Rat Liver in the Presence of Capture Reagents

	α-Glycerophosphate-^{14}C incorporated per mg lipid-free dry weight per 120 min
	nmoles
Slices of liver plus Palmityl CoA α-Glycerophosphate	3.71 ± 1.15 (4)
Slices of liver plus Palmityl CoA α-Glycerophosphate Potassium ferricyanide Manganous chloride	4.46 ± 1.56 (4)
Slices of liver plus α-Glycerophosphate Potassium ferricyanide Manganous chloride	0.68 ± 0.147 (4)

Slices of fixed liver were incubated in media containing α-glycerophosphate (1.8 mM) in cacodylate buffer, pH 7.2, 0.05 M containing 4.5% dextrose, in the presence and absence of palmityl CoA (0.2 mM) and capture reagents (potassium ferricyanide, 0.45 mM, and manganous chloride, 2.5 mM) for 120 min at room temperature. At the end of the incubation, the slices were homogenized in chloroform:methanol (2:1) and the lipids extracted as described in Methods. Results are given as nmoles of α-glycerophosphate-^{14}C incorporated into lipids per mg lipid-free dry tissue ±SD. Number observations are in parentheses.

is a rapid initial incorporation of label into lipids. However, this did not reach a plateau but continued to rise more slowly after the initial phase. This diphasic incorporation of α-glycerophosphate into lipid may represent initial metabolism by broken cells at the edge of the slice, and a slower metabolism by cells inside the slice, the rate of which might be determined by the rate of penetration of substrates. On the basis of the above, an incubation time of 2 hr was selected for cytochemical experiments and 100-μ slices were used to facilitate penetration of substrates and capture reagents.

Effect of Capture Reagents

The metabolism of α-glycerophosphate by slices of fixed liver incubated with and without cytochemical capture reagents in the presence and absence of palmityl CoA is indicated in Table I Liver slices were preincubated in potassium ferricyanide and 10^{-3} M DFP as in the cytochemical experiments. Incorporation of α-glycerophosphate into esterified lipids was not affected by potassium ferricyanide and manganous chloride at the concentrations used in cytochemical experiments. Omission of palmityl CoA from this incubation medium reduced incorporation considerably, to a level close to background. As in the homogenate experiments, on separation of the lipids the label appeared in the phosphatidic acid fractions, with small amounts remaining at the origin.

Cytochemical Observations

Incubation of liver from untreated rats in the complete medium for acyltransferase localization resulted in a delicate deposition of reaction product associated mainly with the rer and to a lesser extent with the ser (Figs. 3–5). When associated with the ser, the reaction product frequently tended to accumulate and form deposits having a fine lamellar structure (Fig. 3 inset). These resemble the myelin figures sometimes produced in glutaraldehyde-fixed tissues. The latter, however, are considerably less electron-opaque. Such figures were not found in control tissues incubated in the presence of capture reagents but without substrates. Reaction product also tended to occur at sites of continuity of the ser and rer, especially in the smooth, membranous end bulbs of rough cisternae. The reaction product tended to line the inner surface of the membrane or occur over the membrane but was never found on the cytoplasmic side of the cisternae (Figs. 4 and 5). Rarely, reaction product was noted in relation to Golgi cisternae and occurred between the inner and outer membranes of the mitochondria. The latter localization, however, also occurred in liver incubated without substrates and probably represents incomplete oxidation of endogenous substrates by preincubation of the tissue with ferricyanide (13). Apart from this reaction associated with mitochondria, control tissues incubated in media with capture reagents but without α-glycerophosphate or palmityl CoA showed no reaction product. Tissues incubated in media containing capture reagents and palmityl CoA showed very few deposits of reaction product associated with the endoplasmic reticulum (er). In this case some endogenous α-glycerophosphate or other acceptor molecule may be retained in the fixed tissue.

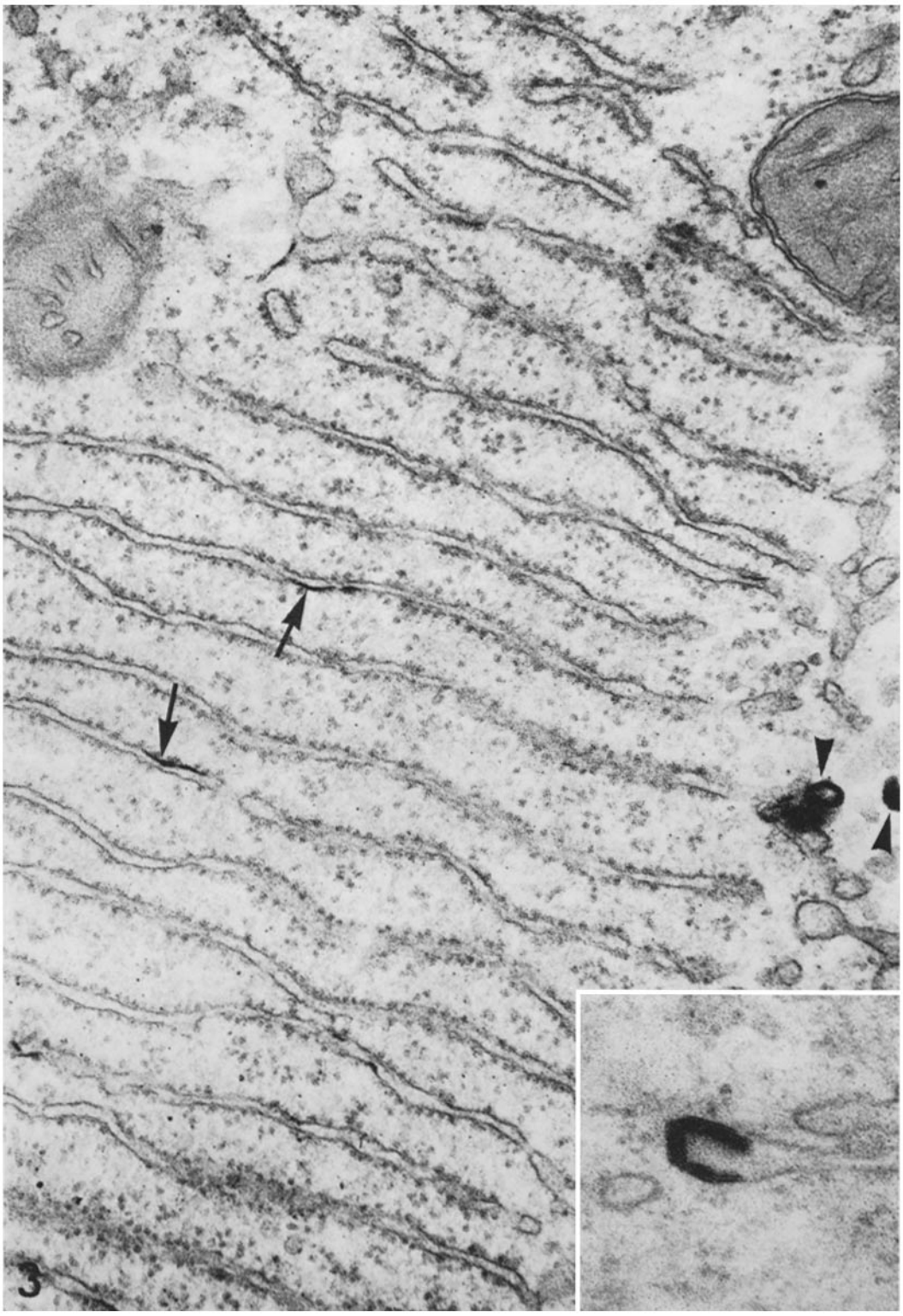
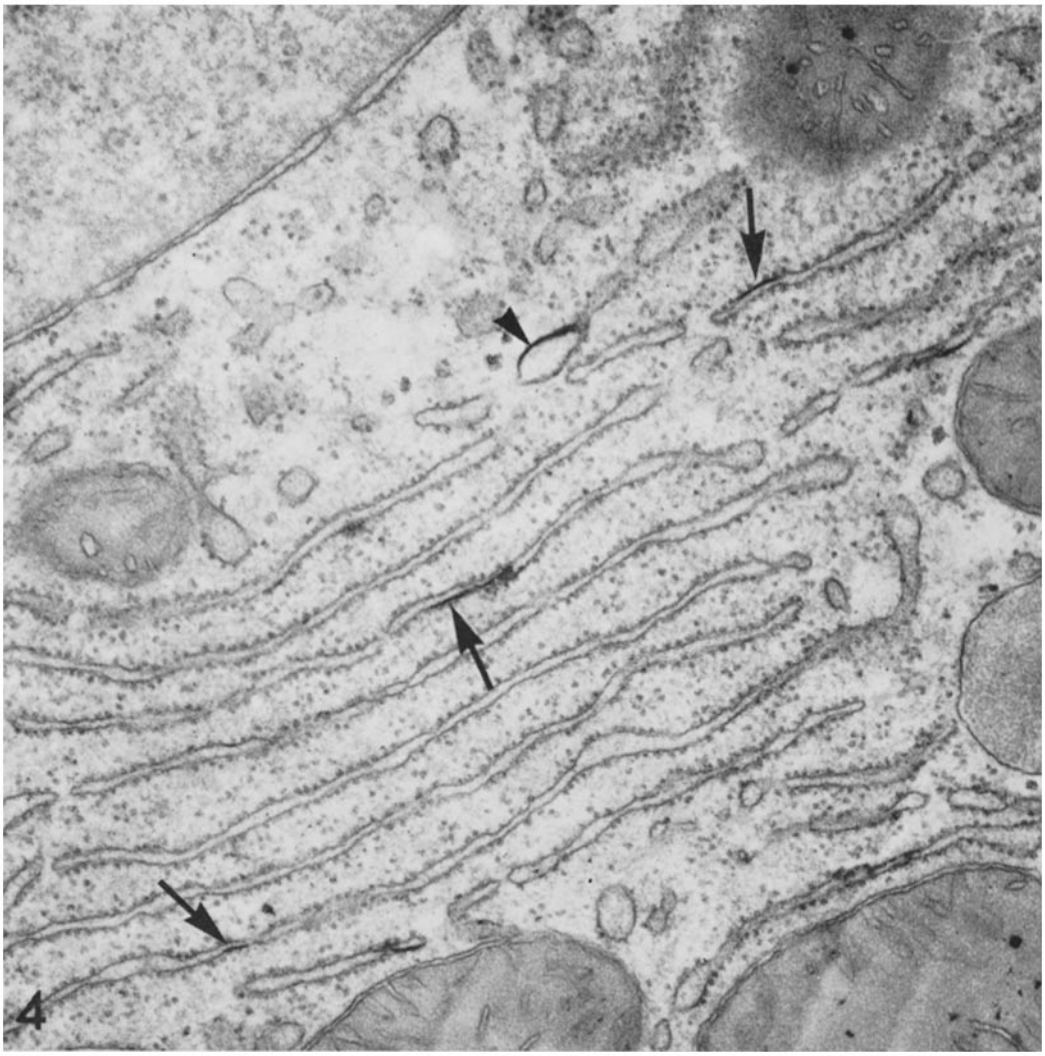


FIGURE 3 Section of liver from normal rat incubated in the complete medium for acyltransferase localization. Reaction product is associated with the rer (arrows) and nearby ser (arrow heads). × 53,000.



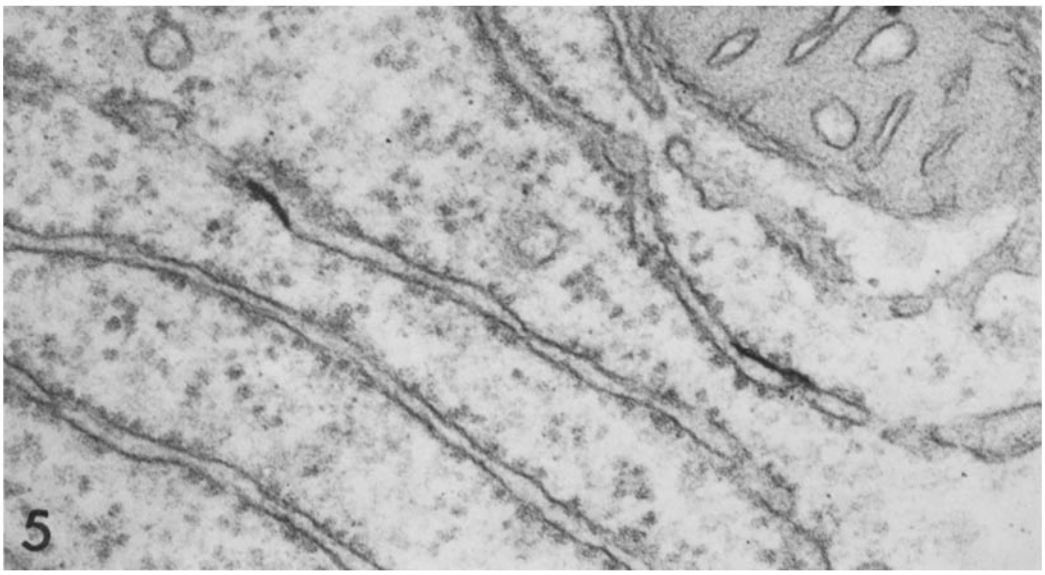


FIGURE 4 Section of liver (as Fig. 3) shows distribution of reaction product within rer (arrows) and ser (arrow heads). × 44,000.

FIGURE 5 Higher magnification of rer. Reaction product occurs over the membrane or lining the inner surface. × 79,000.

Liver from rats given five injections of phenobarbital, incubated in the complete medium for acyltransferase under the same conditions as normal liver, showed greater accumulation of reaction product, frequently having a lamellar structure. In contrast to the normal liver, reaction product was associated with the ser (Figs. 6–8) and drastically reduced in the rer. In areas of proliferated smooth membranes, reaction product appeared randomly dispersed. As with the normal liver, controls incubated in the absence of substrates showed no reaction product except for rare, presumably endogenous reaction associated with the mitochondria.

Studies on Microsomal Fractions

Rough and smooth microsomes prepared from livers of rats treated with phenobarbital and from normal rats were apparently homogeneous and free from contamination with other membranous organelles (Figs. 9, 10).

Specific Activity of Acyltransferases of Microsomes

Incorporation of α-glycerophosphate-^{14}C into lipids by microsomes showed a characteristic pattern in relation to concentration of microsomes and duration of incubations (Fig. 11). This pattern was found in all studies with rough, smooth, or total microsomes from rats treated with phenobarbital and from control rats. At low concentrations of tissue the incorporation was linear with time. At intermediate tissue concentrations, incorporation was linear initially but reached a plateau rapidly. At higher concentrations of tissue the incorporation reached a high level and then declined rapidly. Under the experimental conditions used, the incorporation of α-glycerophosphate into esterified lipid was linear with respect to microsomal protein. However, the specific activity of the acyltransferases did vary with wide ranges of protein concentration. In all experiments, therefore, at least two concentrations of microsomal protein were used, and these were in the same range for all determinations. Any variation in specific activity due to large differences in protein concentration was thus ruled out. Palmityl CoA was obligatory for the incorporation of α-glycerophosphate into lipids, and on separation of the lipids the major labeled fraction was phosphatidic acid. These results probably reflect a combination of the action of palmityl CoA hydrolase and acyltransferase activity. As palmityl CoA is obligatory for esterification of α-glycerophosphate, removal of this by hydrolysis (Table II) would cause a plateau in this metabolism. The fall in incorporation of α-glycerophosphate with higher microsomal concentrations possibly represents a turnover of newly synthesized phosphatidic acid; in the absence of palmityl CoA, resynthesis would not occur, although hydrolysis of phosphatidic acid may occur. In addition to these factors, the acyltransferases may be inhibited by accumulation of the product of their activity, in this case phosphatidic acid, which is not metabolized further.

In view of the complex kinetics indicated above, incorporation of α-glycerophosphate into phosphatidic acid by microsome fractions was measured at a number of time intervals from 0 to 30 min and with at least two tissue concentrations. Only the initial rate of the reaction was determined, and in each case the results were expressed as nmoles of α-glycerophosphate incorporated into lipids per min per mg of protein. The results of an investigation of acyltransferase activity of rough, smooth, and total microsomes from rats treated with phenobarbital are shown in Fig. 12 A. In the untreated rat the acyltransferase activity was highest in the rough microsomes. After injection of phenobarbital, this remained fairly constant for 12 hr and then fell to a low level, after three injections, at which it remained for the remainder of the phenobarbital treatment. The activity in the smooth microsomes was initially lower than that in the rough microsomes. On injection of phenobarbital, however, this rose rapidly to reach a peak at 12 hr after the first injection. The activity then fell slowly to level after three injections. The activity of the total microsomes followed a pattern similar to that of the smooth membranes, with values intermediate between those of smooth and rough microsomes.

The acyltransferase activity of the microsomal fractions prepared from livers of rats recovering from a series of five injections of phenobarbital was also investigated (Fig. 12 B). The activity of the rough microsomes rose slowly while that of the smooth membranes fell slowly to reach the normal levels at approximately 4 wk after cessation of the phenobarbital treatment.

In order to determine whether palmityl CoA hydrolase may have contributed to the changes in acyltransferase activity, the level of this enzyme in

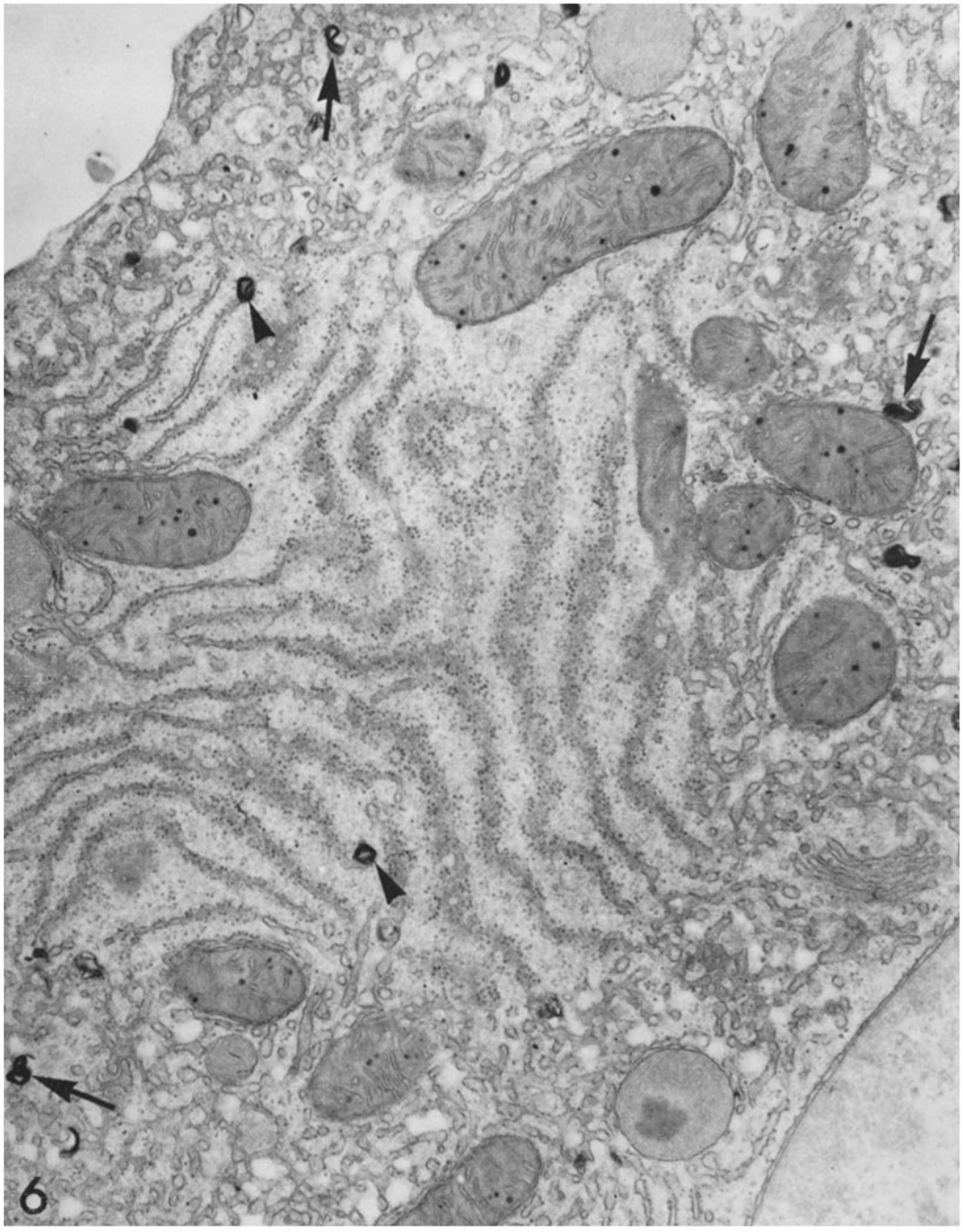
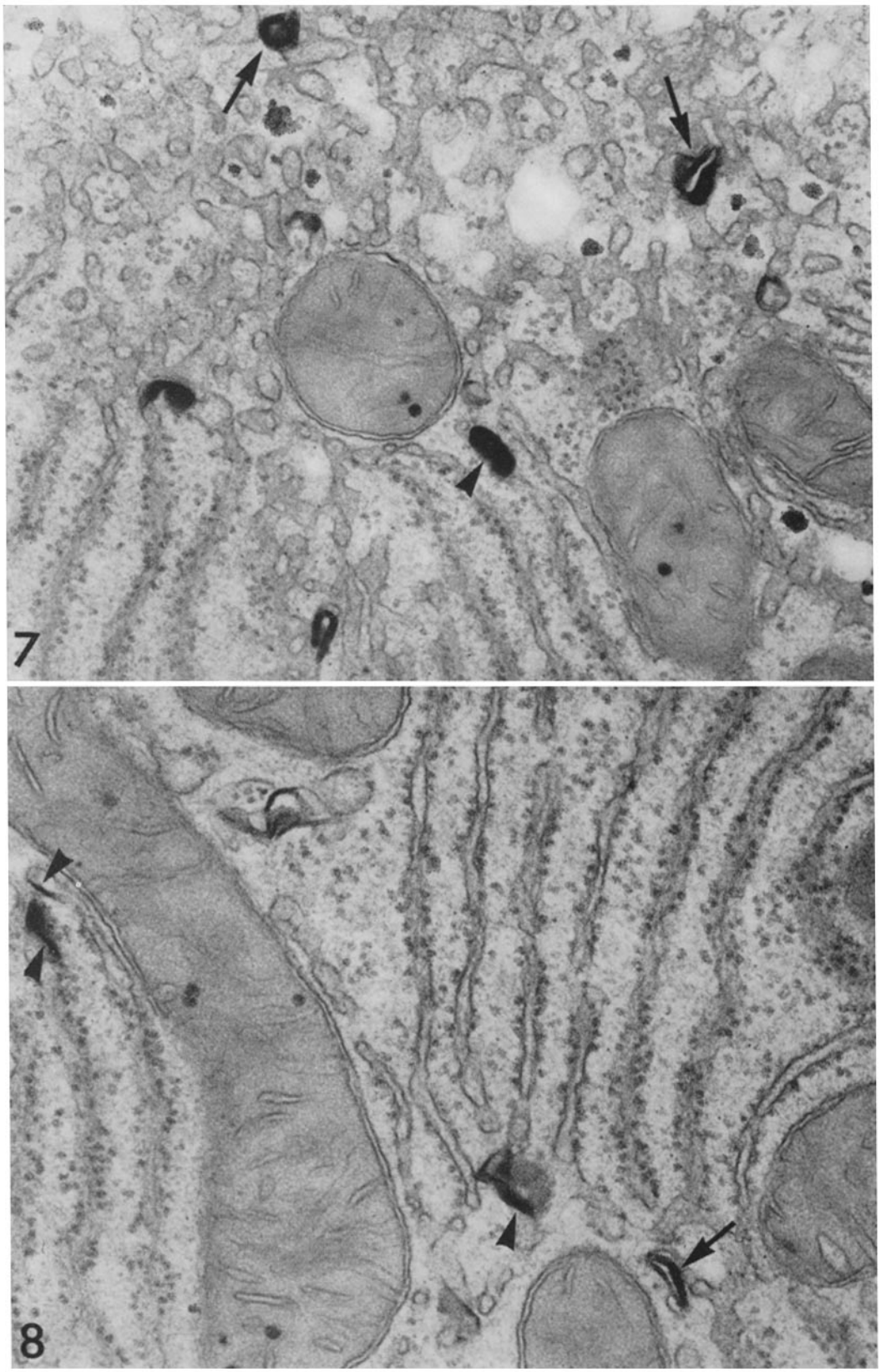


FIGURE 6 Section of liver from rat given five injections of phenobarbital incubated in the complete medium for acyltransferase localization. Note reaction product, heavier than in untreated rats, associated primarily with the ser (arrows), often close to sites of continuity with rer (arrow heads). × 57,000.



FIGURES 7 and 8 Higher magnification of tissue as in Fig. 6. Note reaction product within the cisternae of the ser (arrows) frequently close to sites of continuity with the rough membranes (arrow heads). The reaction product occurs both as laminated heavy deposits and fine linear deposits. × 53,000.

microsomes from rats treated with five injections of phenobarbital and from untreated rats was determined. This activity was the same in all fractions from both groups of animals (Table II).

DISCUSSION

As cytochemical methods for the localization of acyltransferase activity are based on the formation of heavy metal precipitates at the site of release of free CoA, hydrolysis of palmityl CoA which occurs in fixed liver, even after preincubation with DFP, presents a problem in the interpretation of our results. However, omission of α-glycerophosphate from the incubation media resulted in almost complete absence of reaction product. In addition, other studies have shown that palmityl CoA hydrolase is removed from the fixed liver homogenates by washing and does not interfere with the present observations on transferases (19).

Loss of acyltransferase activity during fixation (approximately 70–75%) may be due to either random loss from all sites of activity and/or selective loss from some sites. Evidence favors the former possibility since distribution of reaction product was similar in liver fixed with 0.5% or 1.0% glutaraldehyde, although activity was three times higher with the lower concentration of fixative. In addition, there was a shift in the site of deposition of reaction product as well as in specific activity after phenobarbital treatment although the experimental conditions were the same. Thus, selective loss of activity from either rough or smooth membranes does not occur, and this concurs with other studies which have indicated that acyltransferases are present in the microsomal fraction for which they may be considered as markers (21).

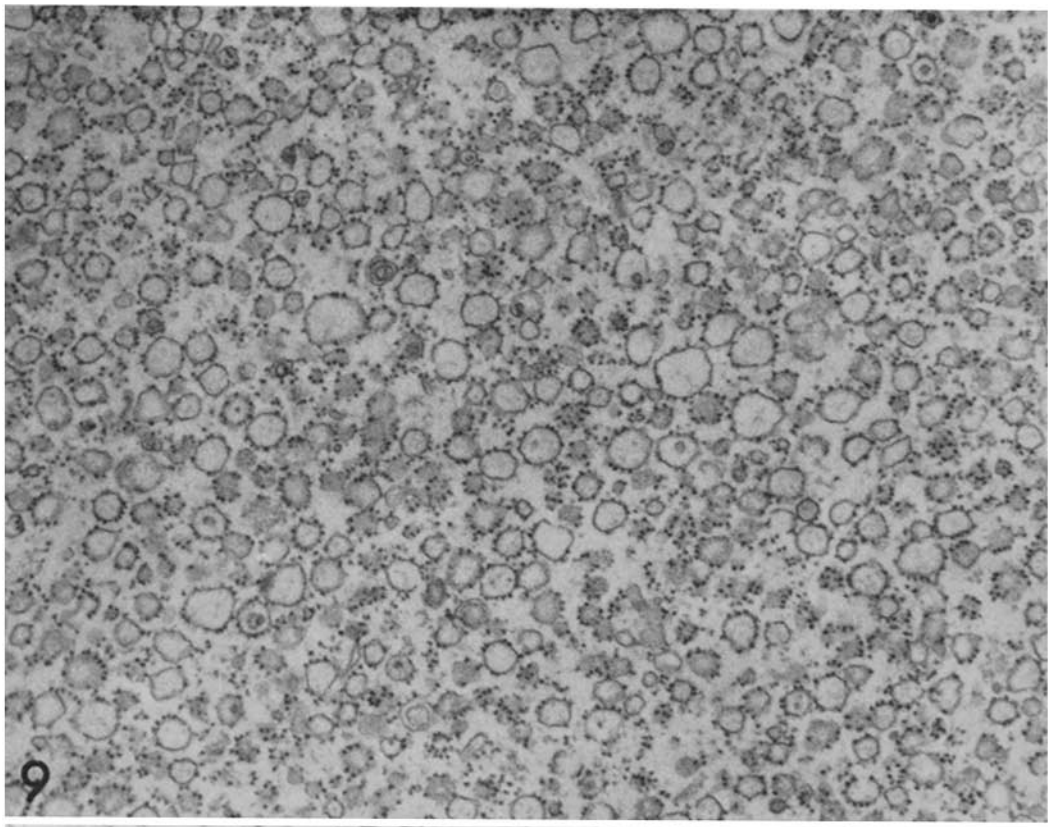
It has been demonstrated that in the liver of phenobarbital-treated rats increased synthesis of phosphatidylcholine occurs by a usually secondary pathway in which methylation of phosphatidylethanolamine takes place (6). Although other enzymes involved in the biosynthesis of glycerophospholipid have not been studied in the present investigation, the acyltransferases catalyze the first steps of these pathways. In addition, the enzymes of these synthetic pathways appear to be closely organized so that the site of acyltransferase activity probably represents the site of the complete synthetic pathway for glycerophospholipids, at least at an organelle level. The methods used in the present study cannot differentiate between the acyltransferases involved in synthesis of phospholipid or triglyceride. However, previous results have indicated that the synthesis of triglyceride proceeds to completion in fixed tissue (14, 19). For this reason, we believe that the acyltransferases under investigation are involved in phospholipid synthesis rather than increased triglyceride synthesis.

In the present study, phosphatidic acid was the major product of the metabolism of α-glycerophosphate. Two acylation steps are therefore involved. Naturally occurring phospholipids tend to have saturated fatty acids at the one position of glycerol and unsaturated fatty acids at the two position. This may be determined by the specificity of the acyltransferases involved in their synthesis (22–24). As palmityl CoA was the only substrate used in addition to α-glycerophosphate in our studies, the acylation of both one and two positions of α-glycerophosphate may not occur under optimal conditions. In addition, a number of studies have indicated that high concentrations of palmityl CoA are inhibitory to these enzymes (21, 25, 26). The concentrations used in the present study were selected to facilitate penetration of substrates into fixed tissue blocks for cytochemical experiments and were also used for biochemical studies to maintain standard conditions. Maximum acyltransferase activity may not have been achieved therefore. However, the same conditions were maintained throughout all experiments, and changes in acyltransferase activity, while not necessarily absolute values, reflect changes in the enzyme level of the tissue.

Previous studies of the site of synthesis and turnover of the lipid constituents of the smooth and/or rough microsomes during phenobarbital-induced membrane proliferation have been re-

FIGURE 9 Section of rough microsomes preparation, which consists of membranous vesicles studded with ribosomes with no evidence of contamination with other organelles. × 35,000.

FIGURE 10 Micrograph of smooth microsome preparation, which consists of smooth membranous vesicles with no evidence of contamination with other membranous organelles. × 45,000.



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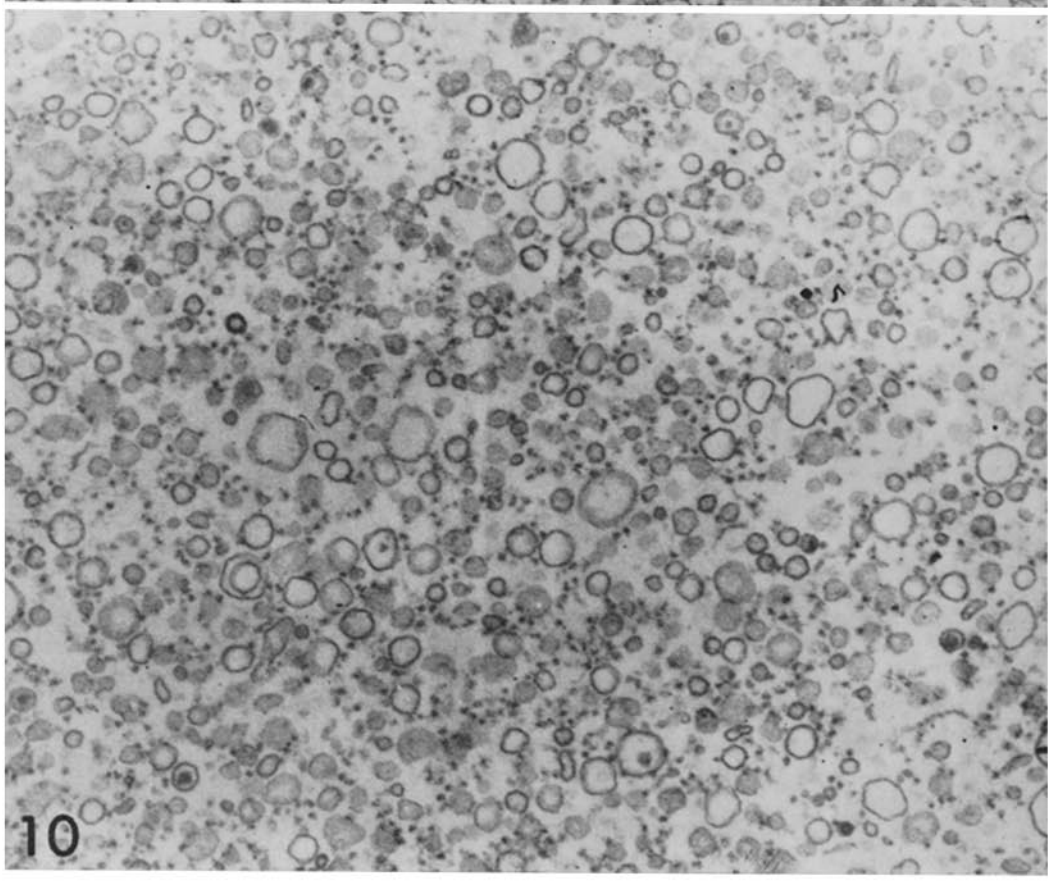

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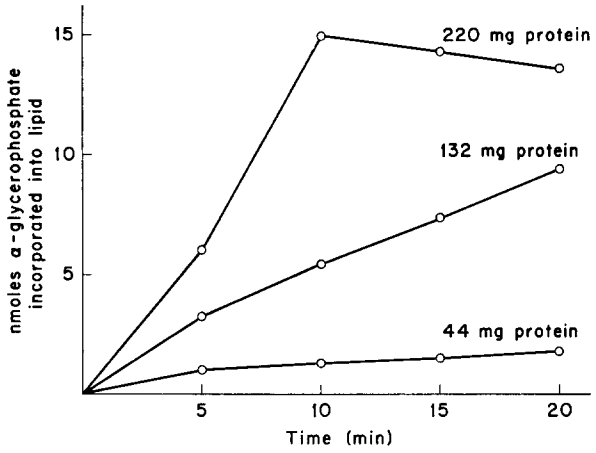

FIGURE 11 Incorporation of α-glycerophosphate-^{14}C into lipids by smooth microsomes of rat liver. Smooth microsomes were incubated in media containing α-glycerophosphate-^{14}C (1.8 mM) and palmityl CoA (0.2 mM) in cacodylate buffer, pH 7.2, 0.05 M containing 4.5% dextrose (final volume 1.0 ml) for a range of times. The reaction was stopped by addition of ice-cold chloroform:methanol (2:1) and the lipid was extracted and counted as described in Methods. Incorporation of α-glycerophosphate into lipids is plotted against time, for three microsome concentrations.

TABLE II

Palmityl CoA Hydrolase Activity of Rough and Smooth Microsomes of Liver from Untreated Rats and Phenobarbital-Treated Rats

	Palmityl CoA hydrolyzed per min per mg microsome protein
	nmoles
Untreated rats	
Rough microsomes	4.16
Smooth microsomes	3.69
Phenobarbital-treated rats	
Rough microsomes	3.31
Smooth microsomes	3.58

Rough and smooth microsomes were prepared from rats either untreated or given five injections of phenobarbital. Samples were incubated in media containing palmityl-^{14}C CoA (0.2 mM) in cacodylate buffer, pH 7.2, 0.05 M containing 4.5% dextrose (final volume 1.0 ml) at 37°C for a range of times (0–15 min). The lipids were extracted and separated as described in Methods. The results are the average of two determinations.

stricted to investigations of the fate in these membranes of labeled precursors of phospholipids administered in vivo. Glauman and Dallner (11), who studied the incorporation of glycerol-^{14}C into microsome phospholipids in the phenobarbital-treated rat, found no difference between the labels incorporated into the rough and smooth microsomes, although a subfraction of smooth membranes showed a lower level of incorporated label than the other fractions. Orrenius, using ^{32}P as a phospholipid precursor, also found similar incorporation of this label into rough and smooth microsomes in the phenobarbital-treated rat liver (7). At first examination this appears to be in conflict with our observations in which phospholipid appears to be synthesized in the smooth membranes during their rapid proliferation. However, exchange occurs between phospholipids of cell fractions both in vivo and during isolation procedures (27–31). Such an exchange would therefore mask any difference in the distribution of newly synthesized phospholipid between the microsomal fractions when the label is incorporated in vivo. For this reason, studies of the distribution of enzymes of lipid synthesis in the isolated fractions or by cytochemical techniques in whole tissue are of greater significance.

Holtzman and Gillette (47) have investigated the rates of synthesis and breakdown of microsomal phospholipid and have presented evidence that the predominant effect of phenobarbital is to decrease catabolism of phospholipids. In female rats no increased synthesis occurred, while in male rats increased synthesis peaked 18–24 hr after injection of phenobarbital, but was insufficient to account for the accumulation of phospholipid. As

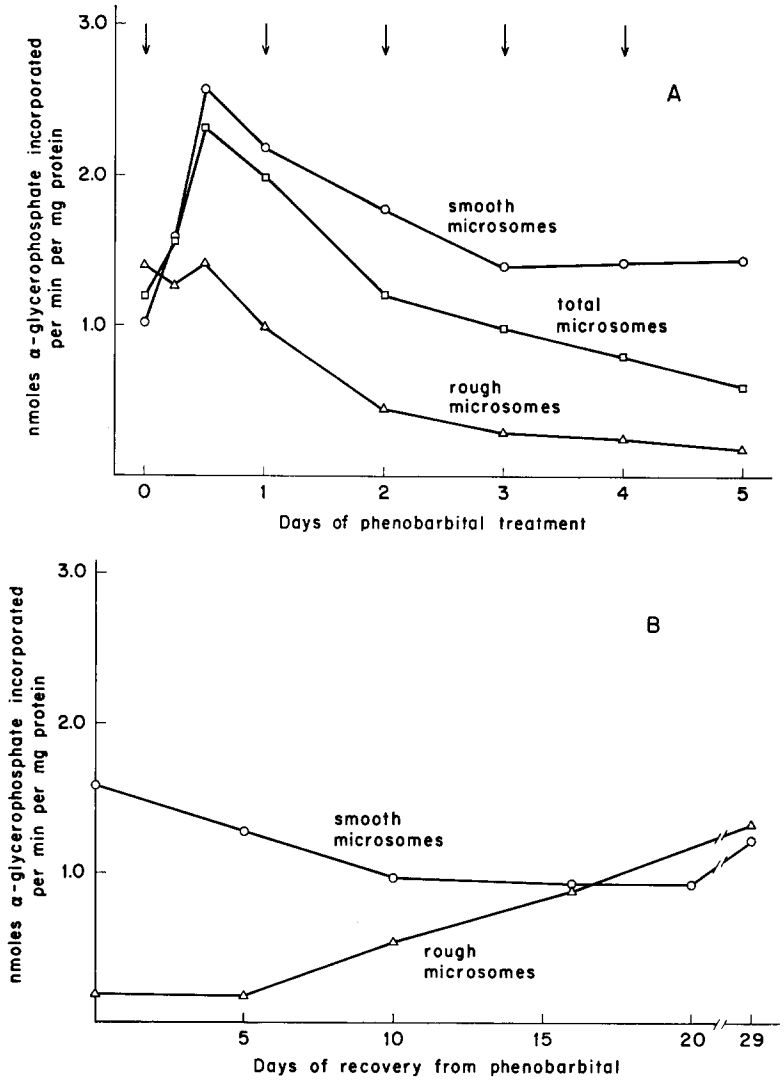

FIGURE 12 Specific activity of acyltransferase of rough, smooth, and total microsomes prepared from livers of rats treated with phenobarbital. (A) Rats were given injections of phenobarbital at 24 hr intervals (arrows). These were sacrificed 24 hr after the last injection, except for time intervals after the first injection. The points at 0 injections were from untreated rats. The livers were removed and microsomes prepared. Rough, smooth, and total microsomes (0.5–3.0 mg protein) were incubated in media containing α-glycerophosphate-^{14}C (0.5 mg/ml) and palmityl CoA (0.2 mg/ml) in cacodylate buffer, pH 7.2, 0.05 M containing 4.5% dextrose (final volume 1.0 ml), at 37°C at a range of times (0–15 min). The initial rate of incorporation of α-glycerophosphate-^{14}C into lipid (nmoles incorporated per min per mg protein) is plotted against days of injections of phenobarbital. This varied with tissue concentration. High tissue concentrations showed linear incorporation for only short time periods, 10 min, while low tissue concentrations showed linear incorporation throughout the incubation time (15 min). Each point is the average of three determinations on each of rough, smooth, and total microsomes from at least three animals. (B) Rats were given a series of five injections of phenobarbital. The livers were removed at a range of times after the end of this treatment, and the rough, smooth, and total microsomes prepared. The specific activity of the acyltransferases of the microsomes was determined as in A. nmoles of α-glycerophosphate incorporated per mg of protein per min is plotted against the days of recovery. Each point is the average of three determinations on two rats.

we have used only male rats in our investigations, it is possible that the increased acyltransferase activity, which we have observed, occurs only in male rats. However, we found that the rise in acyltransferase specific activity is restricted to the smooth microsomes while that in the rough microsomes falls, suggesting that the synthesis of phospholipid in the rough microsomes may be reduced while that in the smooth rises. As Holtzman and Gillette only investigated total microsomal phospholipid, their results may be due to the added effect of these different events.

The cytochemical studies indicated that after three or five injections of phenobarbital acyltransferase activity was drastically reduced in the rer and increased in the ser Studies of isolated microsomes confirmed and extended these observations. In the untreated rat the specific activity of acyltransferases of rough microsomes was higher than that of the smooth microsomes. No correction was made in these studies for protein of ribosomes, so that the values of the activity of rough microsomes in terms of activity per mg membrane protein are higher than those given, which are related to the total protein. With daily injections of phenobarbital, the acyltransferase activity of the rough microsomes remained relatively constant for 12 hr and then fell to a low level after three injections. This level was maintained during subsequent treatment. The acyltransferase activity of the smooth microsomes rose to a peak 12 hr after the first injection of phenobarbital, and then fell slowly to a level of a specific activity higher than that of the smooth microsomes of untreated rat liver. The fall in specific activity of the smooth microsomes may be partially due to dilution of the enzymes with increased microsomal protein, which is established after two injections of phenobarbital (5).

The above changes in specific activity of acyltransferases in the microsome fractions may be due to synthesis of new enzyme protein, to activation of preexisting enzyme in the ser and inactivation of that in the rer, or to shift of acyltransferase from rer to ser. All of these possibilities may occur and it is not, at present, possible to distinguish between them. However, at least in the first 12 hr after injection of phenobarbital, there is an increase in the activity in the total microsomes that parallels the increase in the smooth microsomes. The changes do not therefore depend solely on shift of enzyme activity from rer to ser, although such shifts may be also involved in these early changes.

In contrast to the enzymes involved in drug detoxification, which return to a normal level rapidly after cessation of phenobarbital treatment (3), the acyltransferases in the present studies returned to the normal level slowly over a period of approximately 4 wk. At this time, the ser of the liver cells of treated rats was morphologically similar to that of normal rats.

The relationship of the ser to the rer and/or the biogenesis of these membranes has been studied in the normal rat liver (32, 33, 39), in the phenobarbital-treated rat liver (2, 3, 5, 7, 10, 32, 34, 39), and in the developing rat liver when proliferation of smooth membranes also occurs (16, 35–37). Although these are different physiological situations, it is considered that similar mechanisms for biogenesis of er membranes are involved. It is reasonably well established by these investigations that under all conditions studied the protein components of the smooth membranes are synthesized by membrane-bound ribosomes, and may be incorporated into the rer before appearing in the ser. In addition, morphological continuity is frequently seen between the rer and ser (2, 3, 16, 38). These biochemical and morphological observations led to the theory that rer is the site of membrane proliferation and that the smooth membranes arise by loss of ribosomes from the rough membranes (2, 4, 5, 7, 32, 33, 35). It has been observed that the individual enzyme components of the microsomes turn over at different rates (4, 33, 39). The above hypothesis was therefore qualified by the theory that the er membranes proliferate by synthesis of tesserae or units of single or groups of molecules, which are inserted at different rates into the preexisting membranes (33–37). The smooth membranes may then be produced by loss of ribosomes so that the two forms of membrane are in equilibrium. A major criticism of this model is that the rough and smooth membranes differ in composition (11, 12, 40, 41). In the present investigation, the changes in acyltransferase activity in response to phenobarbital and on recovery from phenobarbital were different in rough and smooth microsomes. In addition, the above mechanism does not account for the observation that phospholipid synthesis takes place in the smooth membranes during their proliferation.

An alternative mechanism, which would not require loss of ribosomes, is one in which assembly of new smooth membrane components is restricted to an area of rer close to the site of continuity with

ser. The individual components may be synthesized at this site or throughout the rer. The newly assembled smooth membranes may then move from this site as complete membranes or arise by selective loss of ribosomes from this small area of rer. This model accounts for the criticism indicated above in that the rough and smooth membranes need not have the same composition. The area of rer involved in the assembly of new smooth membrane may differ from the rest of the rough membranes. This would, however, be masked by homogenization and isolation of the total rough microsomes. However, the bulk of phospholipid synthesis, during smooth membrane proliferation in response to phenobarbital, occurs in the smooth membranes and is not restricted to sites of continuity between rough and smooth membranes. For this reason, this mechanism is probably not the major one. However, the frequent localization of acyltransferase activity close to sites of continuity between the rough and smooth membranes does suggest that it may be operative to some extent.

A third mechanism for the biogenesis of ser, based on the observations of the present study, is that the phospholipid component is synthesized *in situ* and that the protein component is synthesized in the rer. The protein either as single molecules or groups of molecules (tesserae, see references 33–37) may be released into the cisternae of the rer, move to the smooth membranes, and insert into the site of newly synthesized phospholipid. Although release of proteins into the cisternal space has not been established, there is evidence from experiments with cell fractions for a vectoral release of newly synthesized protein into the intermicrosomal compartment which would correspond to the cisternal space in the intact cell (42–45). In support of this mechanism, it has been demonstrated that during phenobarbital-induced proliferation of smooth membranes the phospholipid of the total microsomes rises before the protein (2, 5). In the latter studies, rough and smooth microsomes were not separated. However, the results of our investigation indicate that the smooth membranes are the major site of phospholipid synthesis during proliferation. It is possible therefore that this increased phospholipid to protein ratio occurs in the smooth membranes. In terms of the reported sequence of these events, the rise in acyltransferase activity in response to phenobarbital precedes the over-all rise in protein. This sequence of events fits the above model.

In the normal rat liver in which a number of events involving phospholipid synthesis are occurring in addition to the steady synthesis of new membrane, acyltransferase activity is associated mainly with the rer and to a lesser extent with the ser. These observations are not in conflict, however, with the model for synthesis of smooth membranes suggested above, which may be a general mechanism of the biogenesis of all er membranes. Thus, biogenesis of the rough membranes would involve synthesis of the phospholipid *in situ* as in the case of smooth membranes. On treatment of the rats with phenobarbital, the biosynthetic events directed towards the biogenesis of smooth membranes are superimposed on the normal, steady-state metabolism. Phenobarbital treatment, in fact, appears to modify this normal activity so that the synthesis of phospholipid in the rough membranes falls to a low level.

In the suggested model, if synthesis of phospholipids either in the rough or smooth membranes is synchronized with synthesis of individual enzymes in the rer, then these proteins may be inserted into the membrane at sites of newly synthesized phospholipid, resulting in a heterogeneous distribution of enzymes throughout the membranes. Alternatively, the composition and arrangement of newly synthesized phospholipid may provide sites having affinity for a particular protein, again producing heterogeneity. This model also allows for the turnover of protein molecules at different rates, since the phospholipid provides a basic structure into which the proteins may be inserted. Although these aspects of this model are speculative, they are supported by the cytochemical observations that phospholipid synthesis in the smooth membranes appears randomly distributed, and does not occur over the total surface at any one time.

The possible structures of biological membranes may be generally considered as based on a bilayer or a subunit structure (46). The present finding that the synthesis of the er membranes involves separate synthesis of the principal constituents favors a bilayer structure. The concept of a subunit structure of lipoprotein units is not consistent with the present observations. However, separate autonomous subunits of lipid alone and protein alone in addition to a bilayer structure are compatible with the model suggested.

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