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

II. The Site of Phospholipid Synthesis in the Initial Phase of Membrane Proliferation

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ABSTRACT

The specific activity of the acyltransferases of smooth microsomes of rat liver rose threefold by 12 h after injection of phenobarbital, while the activity of the acyltransferases of the rough microsomes rose slightly to peak at 3–4 h, and subsequently fell. The latter rise was abolished by treatment of the animal with actinomycin D or puromycin, while that of the smooth microsomes was unaffected. Incorporation of [^{14}C]glycerol into phospholipid of smooth microsomes was elevated 100% by phenobarbital, while that of the rough microsomes was elevated 15%, and this could be accounted for by exchange between the microsomal phospholipids. The phospholipid/protein ratio of the smooth microsomes rose 1.5 times 3–4 h after injection of phenobarbital, while that of the rough microsomes fell slightly. The specific activity of NADPH cytochrome *c* reductase and NADPH diaphorase rose first in the rough microsomes, and subsequently in the smooth microsomes at a time coinciding with the return of the phospholipid/protein ratio to the control level. The rise in phospholipid/protein ratio was unaffected by actinomycin D or puromycin. These results indicate that the proliferating smooth membranes are the site of phospholipid synthesis, and that the phospholipid/protein ratio of these membranes may change independently.

INTRODUCTION

In response to intraperitoneal injections of phenobarbital, there is a marked proliferation of smooth endoplasmic reticulum membranes of rat hepatocytes (1–5). This proliferation is accompanied by an increase in the specific activity of the enzymes involved in drug detoxification (2, 4, 6–9) and by an increase in the phospholipid content of the liver, presumably due to the formation of new membranes (2, 4, 7, 10). This tissue system, therefore, presents a useful and easily controlled one in which to study the biogenesis of smooth endoplasmic reticulum membranes. Previous cytochemical studies from this laboratory have indicated that the acyltransferases involved in the first steps of synthesis of phospholipid during new membrane formation are associated with the smooth endoplasmic reticulum in the phenobarbital-treated rat, while in the untreated rat liver these enzymes are

associated mainly with the rough endoplasmic reticulum (11). Parallel investigations of the specific activity of the acyltransferases of rough and smooth microsomes confirmed the cytochemical observations. On injection of phenobarbital, the specific activity of these enzymes rose rapidly in the smooth microsomes, while that in the rough microsomes remained relatively constant for 12 h and then fell to a low level. These results were interpreted as indicating that the phospholipid components of the newly synthesized smooth endoplasmic reticulum membrane are synthesized *in situ* and that the protein component, which is probably synthesized by bound ribosomes (7, 12–14), is incorporated into the smooth membranes after phospholipid synthesis occurs. On the basis of this conclusion, it appeared probable that the early times after injection of phenobarbital, when the specific activity of the acyltransferases rises most rapidly in the smooth membranes, might be appropriate for a detailed investigation of the rate of incorporation of membrane components. If components are added separately, or out of synchrony, these differences possibly may be detected during the first 12–18 h after injection of phenobarbital, before membrane proliferation becomes well established. In the present report, lipid, protein, and enzymic components of the membranes have been investigated.

MATERIALS AND METHODS

Isolation of Liver Microsomes

Total, rough, and smooth microsomes were prepared as described previously (11) from livers of adult male albino rats (200–300 g body wt), either untreated, or after a single intraperitoneal injection of phenobarbital (11 mg per 100 g body wt). As diurnal rhythms have been demonstrated in the enzymes of the smooth endoplasmic reticulum and in the quantities of these membranes (15), rats were sacrificed during the quiescent period from 9:00 to 4:00 pm and given injections of phenobarbital at appropriate times before this.

For acyltransferase assay, microsomes removed from the gradient were washed once by resuspension in 0.25 M sucrose and recentrifugation (105,000 *g*, 45 min) in the case of rough microsomes, and by centrifugation (105,000 *g*, 45 min) of the fraction removed from the gradient after dilution with distilled water 1:5, vol/vol, in the case of smooth microsomes. For phospholipid/protein determinations, the total, rough, and smooth microsomes were washed for a second time by resuspension in 0.25 M sucrose and recentrifugation (105,000 *g*, 45 min).

Assay of Acyltransferases

Unless otherwise stated in the text, microsomes (0.3–3.0 mg protein) were incubated in media containing *sn* [^{14}C]glycerol-3-phosphate (1.8 mM) (International Chemical and Nuclear Corporation, Burbank, Calif.) and palmityl CoA (75 μM) (Sigma Chemical Co., St. Louis, Mo.) in cacodylate buffer, pH 7.2, containing 4.5% dextrose, at a final volume of 1.0 ml, at 37°C for a range of times. Cacodylate buffer was used in these experiments to maintain the conditions of parallel cytochemical investigations (11). However, preliminary experiments were performed to compare this buffer with Tris-HCl and phosphate buffers at the same pH. The same results were obtained in all cases. At the end of the incubation period, the reaction was stopped by addition of ice-cold chloroform-methanol (2:1, vol/vol) and the lipids were extracted by the method of Folch, Lees, and Sloane Stanley (16). Aliquots of the total lipid extract were counted in a Packard Tricarb scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.). In some experiments the lipids were separated in thin layers of silica gel G as described previously (11).

Assay of NADPH Cytochrome c Reductase and NADPH Diaphorase

The specific activities of NADPH cytochrome *c* reductase and NADPH diaphorase of microsome fractions were determined by the method of Ernster, Siekevitz, and Palade (17), by the spectrophotometric determination of the appearance of reduced cytochrome *c* at 550 nm, and the disappearance of the absorption of 2-6 dichloroindophenol at 600 nm, respectively.

Inhibition of Protein Synthesis

Protein synthesis was inhibited in vivo by intraperitoneal injection of actinomycin D (Sigma Chemical Co.) or puromycin (Sigma Chemical Co., or Nutritional Biochemicals Corp., Cleveland, Ohio), and the extent of inhibition expressed as inhibition of incorporation of [^{3}H]leucine (2 mM sp act 50 Ci/mmol, International Chemical and Nuclear Corporation) into trichloroacetic acid-insoluble material. In one series of experiments, the inhibitor (actinomycin D, 20 μg/100 g body wt or puromycin 2 mg/100 g body wt) was injected at hourly intervals for 2 h before and 3 h after injection of phenobarbital (11 mg/100 g body wt) together with [^{3}H]leucine (5 μCi, 0.1 nmol). The rats were then sacrificed at 4.5 h after phenobarbital injection. In a second series of experiments the inhibitor (actinomycin D, 60 μg/100 g body wt, or puromycin 10 mg/100 body wt) was injected at the same time as phenobarbital (11 mg/100 body wt) and [^{3}H]leucine. These rats were sacrificed 12 h after injection. In both series of experiments, the livers were removed and homogenized in distilled water. Aliquots were taken for protein determi-

nation, and other aliquots were precipitated with trichloroacetic acid (15%) and the precipitate was isolated by centrifugation, washed twice with trichloroacetic acid (15%), once with ether, solubilized in Protosol (New England Nuclear), and counted in a Packard TriCarb scintillation counter.

In experiments in which the effects of inhibitors on the acyltransferase activity or the phospholipid/protein ratios of microsomal fractions were investigated, injection of [^{3}H]leucine was omitted and microsomal fractions were isolated from the livers, as described above, at the end of the treatment with protein inhibitor and phenobarbital.

Incorporation of [^{14}C]Glycerol into Phospholipids

[^{14}C]Glycerol (2.5 μCi/100 g body wt) was injected intraperitoneally into animals either untreated or treated with phenobarbital as above. After a predetermined time interval the livers were removed and pieces (approximately 1 g) were homogenized immediately in chloroform-methanol, 2:1, to extract the lipids, or the whole liver was homogenized in ice-cold 0.25 M sucrose and the total, rough, and smooth microsomes were isolated. The lipids were extracted from all fractions and separated into the major lipid classes as described previously (11), and the specific activities of the phospholipid and the triglyceride were determined.

Assays

Protein was assayed by the method of Lowry et al. (18) using crystalline bovine serum albumin as a standard. Phospholipid was determined as organic phosphorus by the method of Fiske and Subbarow (19), and triglyceride by the method of Snyder and Stephens (20).

RESULTS

Kinetics of Acyltransferase Activity

Incorporation of [^{14}C]glycerol-3-phosphate into lipid by microsomes was linear for short periods of time (1–5 min) and this was dependent on the microsomal protein concentration. A plateau of incorporation was reached more rapidly with higher protein concentration. Optimum activity occurred at a 75 μM palmityl CoA with 1 mg of microsomal protein; concentrations of palmityl CoA above this caused inhibition. This has been reported by others in the case of a number of enzymes in addition to acyltransferases (21–26), and appears to be related to the detergent action of palmityl CoA above its critical micellar concentration. Because of the complex kinetic characteristics acyltransferase activity was determined in each microsomal fraction under fixed conditions. Three microsomal protein concentrations about 1 mg were used and at least three incubation times (1–5 min). In this way the incorporation of glycerol-3-phosphate into lipid at concentrations of microsomal protein of 1 mg and 75 μM palmityl CoA was determined under conditions in which incorporation was linear with time.

Under all conditions, when the labeled lipids were separated into the major lipid fractions, all of the label was in the phospholipid fraction at all times and all tissue concentrations used, and on separation of the phospholipids, over 90% of the label was in the phosphatidic acid fraction while the remainder was at the origin in the thin-layer chromatography system used and possibly corresponds to lysophosphatidic acid. Two acylation steps occur under these conditions, therefore, and the first acylation is apparently rate limiting, as lysophosphatidic acid does not accumulate to any significant extent. This has been found by others in the case of partially purified solubilized enzymes of rat brain (27), rat liver microsomes (21, 28, 29), and pigeon liver microsomes (30). Lands and Hart (29) demonstrated that the acylation of 1-monoacyl glycerol-3-phosphate is more rapid than that of glycerol-3-phosphate, supporting the conclusion that the first acylation step is rate limiting.

Changes in Specific Activities of Acyltransferases of Liver Microsomes in Response to Treatment with Phenobarbital

The specific activity of the acyltransferases of rough microsomes of untreated rats was slightly higher than that of smooth microsomes (Fig. 1). After injection of phenobarbital, the specific activity of the smooth microsomes rose rapidly to peak at 12 h after injection at a level approximately three times that of smooth microsomes of untreated rats. The specific activity of the rough microsomes rose slightly between 1.5 and 4.5 h after injection and then fell to a level below that of the untreated microsomes. This rise, although small, was statistically significantly ($p < 1\%$ at 1.5 h). The specific activity of total microsomes rose, paralleling that of smooth microsomes with values intermediate between those of rough and smooth, but closer to the latter in most cases. However, the method used for isolation of rough and smooth microsomes does not result in a complete yield of

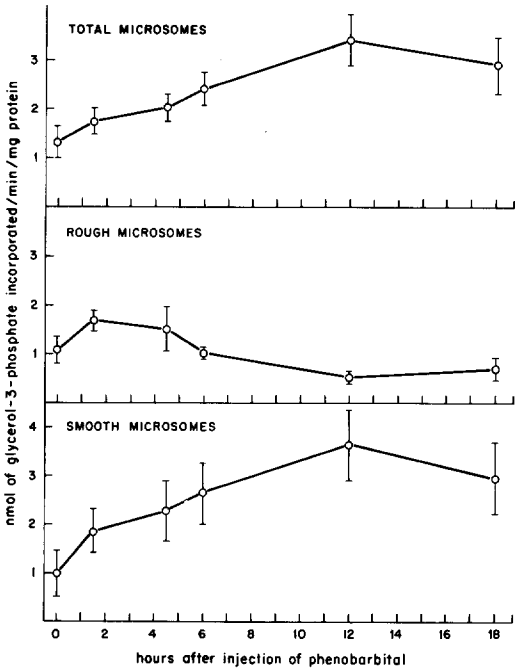

FIGURE 1 Changes in specific activity of acyltransferases of total, rough, and smooth liver microsomes in response to treatment of rats with phenobarbital. Total, rough, and smooth microsomes (0.75–3.0 mg protein) prepared from normal and phenobarbital-treated rats (11 mg/100 g injected at 0 h) as described in Materials and Methods were incubated in media containing [^{14}C]glycerol-3-phosphate (1.8 mM) and palmityl CoA (75 μM) in cacodylate buffer at 37°C for 1, 2, and 3 min. The reaction was stopped and the lipid extracted as described in Materials and Methods. At least three protein concentrations were used for each assay, and the acyltransferase specific activity at 1.0 mg protein was determined as described in the text. The specific activity (nmol glycerol-3-phosphate incorporated/min/mg protein) is plotted against time after injection of phenobarbital. Each point is the average of determinations on microsomes from at least four animals. Standard deviations are indicated by bars.

the microsomal fraction and the total microsomes prepared in this way are probably not composed of rough and smooth endoplasmic reticulum in the proportions found in the intact cell. In all preparations, the yield of smooth microsomes per liver exceeded the yield of rough microsomes, although in the untreated rat liver, rough endoplasmic reticulum accounts for some two-thirds of the total endoplasmic reticulum (5). It is probable, therefore, that in the initial centrifugation of the liver homogenates to remove mitochondria, a large proportion of the rough microsomal fraction is lost compared with the smooth microsomal fraction.

The specific activity of acyltransferases is expressed conventionally in terms of protein. However, in such a system in which the microsomal components are changing it is difficult to select a standard for the expression of enzyme activity. One alternative to protein is phospholipid. The phospholipid/protein ratio of smooth microsomes rises to peak at 3.0–4.5 h after injection of phenobarbital, while that of the rough shows a slight fall (Fig. 5). Therefore acyltransferase activity expressed in terms of phospholipid shows essentially the same pattern as that in terms of protein in the rough microsomes, while the activity of the smooth microsomes rises more slowly for 0–4.5 h but reaches a similar peak at 12–18 h after injection of phenobarbital.

Effect of Phenobarbital on the Incorporation of [^{14}C]Glycerol into Lipids of Rat Liver and Liver Microsomal Fractions

Acyltransferases catalyze the first steps in the synthesis of glycerophospholipids and triglycerides. However, the site of the increased activity in response to phenobarbital is not necessarily the site of phospholipid synthesis, although this is probable. In order to examine this more directly we investigated the incorporation of [^{14}C]glycerol into rat liver and liver microsomal lipids. Total liver phospholipids became labeled very rapidly after intraperitoneal injection of [^{14}C]glycerol (Fig. 2). In rats treated with phenobarbital, the final specific activity reached was elevated over the controls, indicating that under our experimental conditions there is an increased synthesis of total liver phospholipid in response to phenobarbital.

Exchange of Phospholipids between Rough and Smooth Liver Microsomes

It has been demonstrated by a number of investigators that exchange of phospholipids occurs between cell fractions, when these are incubated in vitro. This presents a major problem in experiments in which incorporation of isotopes into phospholipid of different fractions is determined. If this exchange is rapid, then any differences between rough and smooth microsomal fractions at the time of sacrifice of the animal would be eliminated during the fairly long times required to separate rough and smooth mi-

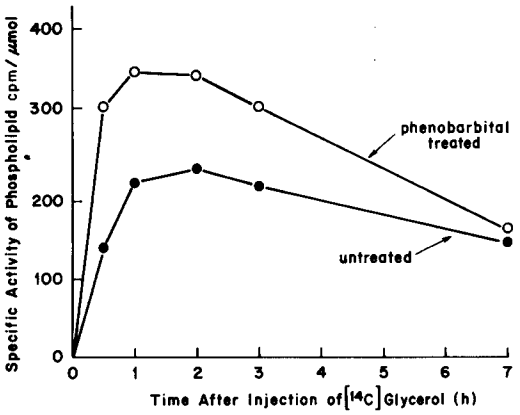

FIGURE 2 Incorporation of [^{14}C]glycerol into phospholipid of rat liver: effect of phenobarbital. Rats were injected with [^{14}C]glycerol with and without phenobarbital and were sacrificed at a series of time intervals after injection. Approximately 1 g of liver was removed from the same lobe in each animal, homogenized in distilled water, and extracted with chloroform-methanol (2:1). The lipids were separated and the specific activity of the phospholipid was determined as described in Materials and Methods. Points plotted are averages of at least three determinations on three animals.

crosomes. We therefore investigated the extent of exchange between microsomal subfractions during our experimental procedures. Both rough and smooth microsomes containing [^{14}C]phospholipids were prepared and added separately to homogenates of liver. Rough and smooth microsome fractions were isolated in each case and the specific activity of the phospholipids of these fractions was determined (Table I). In both cases the specific activity of the added microsomes was considerably reduced and recoveries were low, indicating considerable exchange between microsomes and other fractions, as well as low yields of microsomal fractions. When the label was initially in the smooth microsomes the specific activity of the phospholipids of the isolated rough microsomes was approximately 50% that of the smooth. Similarly, when the label was initially in the rough microsomes the specific activity of the phospholipids of the smooth microsomes was 25% that of the rough. Complete exchange between fractions does not occur therefore, but a significant amount of label moves from one fraction to another and this must be taken into account when considering the results of incorporation of [^{14}C]glycerol into microsome fractions.

TABLE I

Exchange of Phospholipids Between Microsomal Fractions

Fraction	Specific activity of phospholipid
	cpm/μmol
Original rough microsomes	375
Fractions isolated from homogenates to which labeled rough microsomes were added	
Rough microsomes	28.3
Smooth microsomes	9.6
Original smooth microsomes	362
Fractions isolated from homogenates to which labeled smooth microsomes were added	
Rough microsomes	23.3
Smooth microsomes	46.2

Rats were injected with [^{14}C]glycerol (2.5 μCi/100 g body weight) and sacrificed 1 h later, and rough and smooth microsomes were isolated. Lipids were extracted from aliquots of these and the remainder added separately to homogenates of liver from untreated rats. Rough and smooth microsomes were isolated from these and the lipids extracted. The specific activity of the phospholipids of all fractions was determined as described in Materials and Methods.

Incorporation of [^{14}C]Glycerol into Lipids of Rough and Smooth Microsomes

[^{14}C]Glycerol was incorporated rapidly into the phospholipid of both rough and smooth microsomes. In both phenobarbital-treated and untreated rats, incorporation peaked at approximately 1 h after injection. In microsomes from untreated animals the specific activity of the rough microsomes was higher than that of the smooth microsomes at all times studied, whereas in microsomes from phenobarbital-treated rats the specific activity of the smooth microsome phospholipid was higher than that of the rough microsomes (Fig. 3). At 30 min after injection of phenobarbital the specific activity of the smooth microsomal phospholipid was 197% that of the same fraction from untreated rat liver, while the specific activity of the phospholipid of the rough microsomes of phenobarbital-treated rats was 113% that of the rough microsomes of the untreated rats. This difference was maintained at later times, although this was less marked. At 120 min after phenobarbi-

tal injection there was a fall in the specific activity of the phospholipids of both rough and smooth microsomes compared with the same fractions from livers of untreated rats. If exchange occurs between the phospholipids of these fractions at a rate comparable to that in the experiments indicated above, then the differences between rough and smooth microsomes would be greater at the time of sacrifice of the animal. However, it is extremely difficult to assess the exchange between fractions, as this occurs continuously, and both fractions were labeled initially at the time of sacrifice. Nevertheless, these results do indicate that on treatment with phenobarbital there is a shift in the predominant site of incorporation of [^{14}C]glycerol into phospholipid from rough microsomes to smooth.

The specific activity of the triglycerides of rough, smooth, and total microsomes did not show any increase in response to phenobarbital but rather showed a small decrease at 60 and 120 min. The specific activity of the triglyceride of the total microsomes was greater than that of both rough and smooth microsome fractions, suggesting that there may be more than one pool of triglyceride in the liver, one of which was lost from the total microsomes on subfractionation (Fig. 4). This pool probably consisted of lipoprotein particles or lipid droplets, many of which floated during the separation of rough and smooth microsomes. These could remain trapped in the total microsomes, which were subjected to less washing than the subfractions. The triglyceride content of rough and smooth microsomes was low, and this may represent largely a structural component of the membrane (32).

Changes in Phospholipid/Protein Ratios of Liver Microsomes in Response to Treatment with Phenobarbital

The previous results indicate that the phospholipids of phenobarbital-induced membranes are synthesized *in situ*. Reports from a number of laboratories indicate that protein is synthesized by bound ribosomes. As the major components are synthesized at separate sites, it is possible that transient differences exist in the rate of incorporation of these two major components of smooth microsomes. The phospholipid/protein ratios of the microsomal fractions were therefore investigated. This ratio was similar in both rough and smooth microsomes prepared from livers of untreated rats (Fig. 5). After injection of phenobarbital, however, there was a rapid rise in the phospholipid/protein ratio of the smooth microsomes

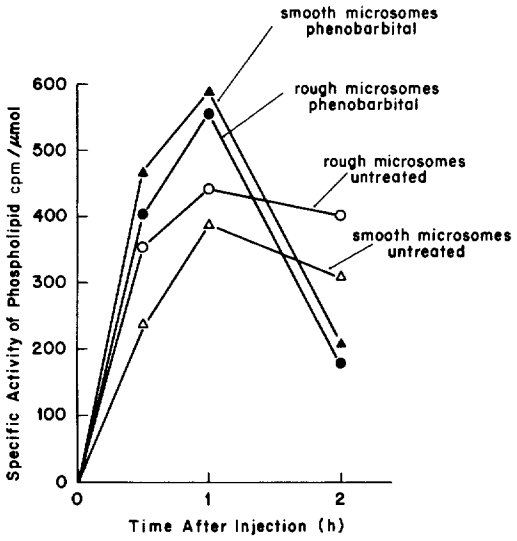

FIGURE 3 Specific activity of phospholipid.

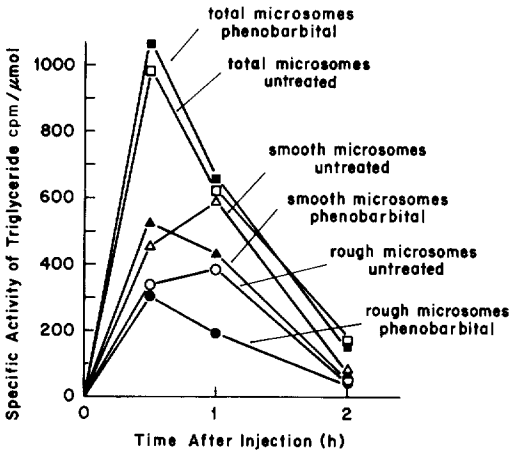

FIGURE 4 Specific activity of triglyceride.

FIGURES 3 and 4 Incorporation of [^{14}C]glycerol into phospholipid and triglyceride of rough, smooth, and total microsomes of rat liver; effect of phenobarbital. Rats were injected with [^{14}C]glycerol with and without phenobarbital and were sacrificed at a series of time intervals after injection. Livers were removed and rough, smooth and total microsomes isolated. Lipids were extracted and separated and the specific activities of the phospholipid and triglyceride were determined. Points plotted are averages of at least three determinations on at least three animals.

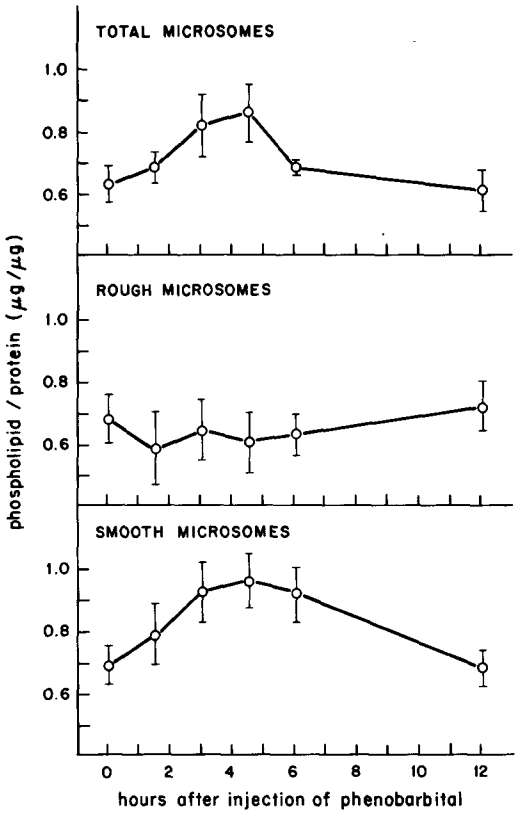

FIGURE 5 Changes in phospholipid/protein ratios of total, rough, and smooth liver microsomes in response to treatment of rats with phenobarbital. Total, rough, and smooth microsomes prepared as described in Materials and Methods were suspended in cacodylate buffer, and aliquots were used for assay of protein and for extraction of lipids and assay of phospholipid as described in Materials and Methods. Phospholipid/protein ratios are plotted against time after injection of phenobarbital. Each point is the average of determinations on microsomes from at least four animals. Standard deviations are indicated by bars.

to peak at 3.0–4.5 h after injection at a level approximately 150% that of the untreated smooth microsomes ($p < 0.02\%$). The phospholipid/protein ratio of the rough microsomes fell slightly during the 12 h of the experiment, while that of the total microsomes rose, paralleling the smooth microsomes, with values intermediate between that of rough and that of smooth.

Microsomal fractions potentially may contain secretory or soluble proteins trapped within the vesicles. The existence of such proteins is of considerable significance in studies of phospholipid/protein ratios of membranes. In control experiments, therefore, both rough and smooth microsomes from livers of normal and phenobarbital-treated rats were subjected to osmotic shock as described by Glaumann and Dallner (32). Phospholipid/protein ratios in this case were not significantly different from those of microsomes washed in 0.25 M sucrose shown in Fig. 5. Since the completion of this work, an investigation has been reported in which rough microsomes were subjected to low concentrations of deoxycholate to release adsorbed and trapped materials (44). Similar procedures were applied to rough and smooth microsomal fractions in our studies. These caused an elevation in the phospholipid/protein ratios of fractions, but the relative differences between rough and smooth microsomes from livers of normal rats and rats treated with phenobarbital for 4.5 h and 18 h were maintained.

The rise in phospholipid/protein ratio of the microsomal membranes was followed by a fall 4.5 h after injection of phenobarbital, while the rise in specific activity of the acyltransferases peaked 12 h after injection. Increased phospholipid synthesis occurs for a considerable time after injection of phenobarbital, as smooth membrane proliferation is not marked until after two daily injections of phenobarbital and continues during repeated daily injections. However, synthesis of new smooth membrane enzymes occurs at the same time as the membrane proliferation, and the incorporation of these proteins into smooth membranes is responsible for the fall in the phospholipid/protein ratio, and also for the later fall in specific activity of the acyltransferases.

Changes in the Specific Activity of NADPH Cytochrome c Reductase and NADPH Diaphorase of Microsomes in Response to Phenobarbital

The enzymes which rise in liver microsomes in response to phenobarbital are those involved in the detoxification of drugs. Orrenius (7) has demonstrated that in the early times after injection of phenobarbital, the specific activities of a number of these enzymes rise in parallel first in the rough microsomes, then in the smooth. Of these enzymes, NADPH cytochrome *c* reductase and NADPH diaphorase were selected as markers of increased protein synthesis under our experimental conditions. In experiments parallel to those

in which the specific activity of the acyltransferases and the phospholipid/protein ratios were determined, the specific activities of the NADPH cytochrome *c* reductase rose rapidly in the rough microsomes in response to phenobarbital and subsequently rose in the smooth microsomes (Fig. 6). Similar changes, although less marked, also occurred in the case of NADPH diaphorase. The time sequence of these changes is consistent with increased enzyme incorporation into the smooth membranes, accounting for the fall in phospholipid/protein ratio in these membranes at 4.5 h after injection of phenobarbital.

Effect of Inhibitors of Protein Synthesis on the Acyltransferase Activity and the Phospholipid/Protein Ratios of Microsome Fractions of Livers of Phenobarbital-Treated Rats

Administration of actinomycin D or puromycin at the same time as phenobarbital resulted in over 60% inhibition of protein synthesis during the 12 h after treatment. When the inhibitors were administered at hourly intervals and the animals sacrificed at 4.5 h after injections of phenobarbital, inhibition of protein synthesized was greater than 80%.

The rise in specific activity of the acyltransferases of smooth microsomes 12 h after injection of phenobarbital was unaffected by inhibition of protein synthesis by either actinomycin D or puromycin (Table III). The specific activity of these enzymes in rough microsomes was also unaffected by these inhibitors, although at this time point the activity was low compared with the untreated control. At 4.5 h after phenobarbital, the specific activity of the acyltransferases of the rough microsomes was elevated compared with the untreated control ($p < 1.0\%$) (Fig. 1, Table II). Administration of actinomycin D or puromycin resulted in a fall in this activity ($p < 0.2\%$ in each case), indicating that this rise is due to new protein synthesis and may be blocked at the level of transcription or translation. In contrast to this, at 4.5 h after phenobarbital, the specific activity of the acyltransferases of the smooth microsomes from rats treated with actinomycin D or puromycin was elevated compared with that of the control treated with phenobarbital alone ($p < 0.2\%$ in each case) (Table II). The rise in acyltransferase activity of the smooth microsomes at both 4.5 and

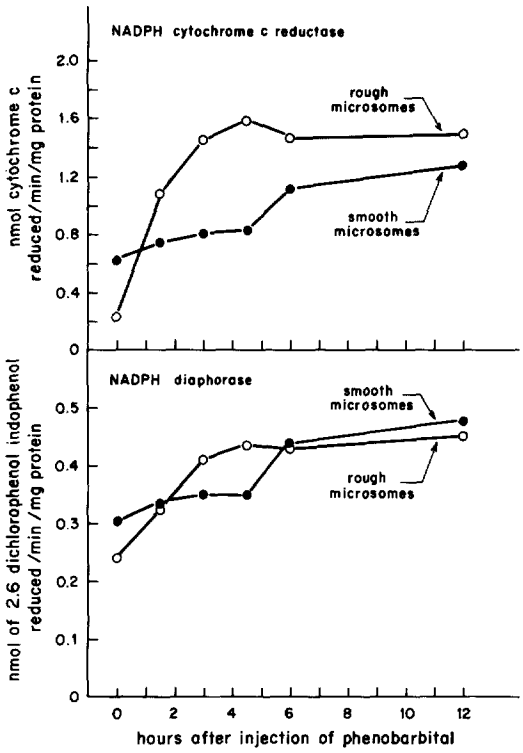

FIGURE 6 Changes in NADPH cytochrome reductase and NADPH diaphorase activity of rough and smooth liver microsomes in response to treatment of rats with phenobarbital. Rough and smooth microsomes were prepared from livers of normal and phenobarbital-treated rats, and the specific activities of NADPH cytochrome *c* reductase and NADPH diaphorase were determined as described in Materials and Methods. The specific activity of these enzymes is plotted against the time after injection of phenobarbital. Each point is the average of determinations on microsomes of three animals.

12 h after phenobarbital is not due to new protein synthesis. At the earlier time point, inhibition of other membrane protein synthesis results in an apparent increased specific activity of these enzymes. This may be due to an inhibition of increased synthesis of smooth membrane protein, which normally dilutes the acyltransferases, or possibly to some complex control mechanism, in which synthesis of a controlling protein may be abolished.

The rise in phospholipid/protein ratio of the smooth microsomes at 4.5 h after phenobarbital (Fig. 9) was not affected by administration of actinomycin D or puromycin (Table III). There was a slight increase in the phospholipid/protein

TABLE II

Effect of Actinomycin D or Puromycin on the Specific Activity of the Acyltransferases of Microsomal Fractions of Phenobarbital-Treated Rat Liver

Treatment	Specific activity of acyltransferases	
	Rough microsomes	Smooth microsomes
None	1.07 ± 0.310 (6)	0.994 ± 0.4613 (6)
12 h after phenobarbital	0.52 ± 0.0176 (4)	3.63 ± 1.2 (5)
12 h after phenobarbital plus puromycin	0.772 ± 0.443 (6)	3.31 ± 0.255 (6)
12 h after phenobarbital plus actinomycin D	0.87 ± 0.62 (4)	3.26 ± 0.327 (4)
4.5 h after phenobarbital	1.51 ± 0.456 (5)	2.26 ± 0.63 (5)
4.5 h after phenobarbital plus puromycin	0.207 ± 0.023 (4)	3.95 ± 1.2 (4)
4.5 h after phenobarbital plus actinomycin D	0.595 ± 0.084 (5)	3.616 ± 0.93 (5)

Rats were treated with phenobarbital and actinomycin D or puromycin as described in Materials and Methods, and the rough and smooth microsomes were isolated at the end of the treatment. Acyltransferase activity was determined as nmol of [^{14}C]glycerol-3-phosphate incorporated into lipid per min per mg protein as described in Materials and Methods. The results are expressed as average specific activity ± standard deviation (obs.).

TABLE III

Effect of Actinomycin D or Puromycin on the Phospholipid/Protein Ratio of Microsomal Fractions Prepared from Phenobarbital-Treated Rat Liver

Treatment	Phospholipid/protein ratio	
	Rough microsomes	Smooth microsomes
None	0.686 ± 0.0741 (6)	0.688 ± 0.059 (5)
4.5 h after phenobarbital	0.601 ± 0.09667 (4)	0.951 ± 0.0792 (5)
4.5 h after phenobarbital plus puromycin	0.736 ± 0.088 (4)	0.922 ± 0.126 (4)
4.5 h after phenobarbital plus actinomycin	0.6204 ± 0.08 (4)	0.901 ± 0.1175 (4)

Rats were treated with phenobarbital and actinomycin D or puromycin, rough and smooth microsomes were isolated, and the phospholipid/protein ratios were determined as described in Materials and Methods. Results are expressed as average ± standard deviation (obs.)

ratio of the rough microsomes after puromycin treatment, which may be significant (p = 5.0%); but there was no change in the phospholipid/protein ratio of the rough microsomes after actinomycin D treatment. In the case of puromycin-treated rat liver, the yield of rough microsomes was consistently low, suggesting that the treatment may modify this fraction.

DISCUSSION

Studies of membrane biogenesis in eukaryotic cells are complicated by the fact that there is a continual turnover of all components of cell membranes in these cells. In addition, in the normal hepatocyte a variety of metabolic events occurs, some of which involve synthetic processes similar to those involved in membrane formation. For example, formation of plasma lipoproteins involves synthesis of lipids and protein and packaging of these to produce a complex structure of regulated composition which is exported from the cell. In the normal hepatocyte, therefore, it is difficult if not impossible to ascertain the events in lipid synthesis related to membrane formation. Thus, a basic assumption of our studies is that the changes related to new membrane formation are superimposed on those occurring in the normal cell, when smooth endoplasmic reticulum membrane formation is induced experimentally by phenobarbital. For this reason, although both rough and smooth microsomes have the enzymic machinery for the synthesis of phospholipid, the changes induced by phenobarbital have been related to smooth membrane formation.

Previous cytochemical experiments reported from this laboratory indicated that during proliferation of smooth endoplasmic reticulum membranes induced by phenobarbital, acyltransferases were active in the smooth endoplasmic reticulum

and essentially inactive in the rough endoplasmic reticulum. These results were interpreted as indicating that the phospholipid of the newly synthesized smooth membranes is synthesized *in situ*, while the protein which is apparently synthesized by bound ribosomes may be inserted into the smooth membranes after synthesis of the phospholipid component (11). The observations reported here support this hypothesis. First, the specific activity of the acyltransferases of the smooth membranes rose rapidly after injection of phenobarbital. Acyltransferases catalyze the first steps in the synthesis of glycerolipids, and an increase in their specific activity may reflect an increased synthesis either of triglyceride or of phospholipids in the smooth membranes. Incorporation of [^{14}C]glycerol into phospholipids of smooth membranes was increased by phenobarbital, while incorporation into the phospholipid of rough microsomes was elevated only slightly and this may be accounted for by exchange occurring during the isolation procedure. Incorporation of [^{14}C]glycerol into triglyceride was not increased by phenobarbital. The rise in acyltransferase was therefore directed towards phospholipid synthesis rather than triglyceride. This is consistent with the second observation that the phospholipid/protein ratio of the smooth microsomes rose after injection of phenobarbital. There was also a slight but significant rise in the acyltransferase activity of the rough microsomes after injection of phenobarbital. This was not accompanied by a rise in the phospholipid/protein ratio of this fraction. However, increased phospholipid synthesis in these membranes may be accompanied by increased protein syntheses. The specific activity of the NADPH cytochrome *c* reductase and NADPH diaphorase of the rough microsomes rose immediately after injection of phenobarbital, indicating that a synchronized synthesis of phospholipid and protein may occur. It has been demonstrated by morphometric analysis that there is an increased surface area of rough endoplasmic reticulum the 1st day after injection of phenobarbital (5). Although this is small compared with the later proliferation of smooth membranes, an increase in rough membrane phospholipid and protein must be involved in this proliferation. It is also possible that the increased acyltransferase activity of the rough microsomes is due to increased synthesis of these enzymes for transfer to the smooth membranes for growth over several days of phenobarbital treatment. The rise in acyltransferase activity of the rough microsomes is abolished by inhibition of protein synthesis, in contrast to the acyltransferase activity of the smooth microsomes which is not altered by inhibition of protein synthesis.

Holtzmann and Gillette (31) have reported that in male rats there is an increased synthesis of phospholipid in response to phenobarbital, but that the predominant effect of this drug appears to be to decrease the breakdown of phospholipid in microsomes, resulting in its accumulation. Direct comparison between our results and those of Holtzmann and Gillette is not possible because of the difference in experimental protocol; however, the increase in incorporation of [^{14}C]glycerol into phospholipids found here is of the same order as that reported by these investigators. If the predominant effect of phenobarbital is to decrease breakdown of phospholipid, however, synthesis of phospholipids must continue at either the same or an elevated rate. Our results indicate that the site of this synthesis shifts on treatment with phenobarbital to take place predominantly in the proliferating smooth membranes. This is especially marked in the livers of rats treated for 2 or more days with phenobarbital, when the acyltransferase activity of the rough microsomes is very low (11).

It is well established that one site of synthesis of new smooth membrane enzymes is the rough endoplasmic reticulum. This has been demonstrated in the normal rat liver (12, 14, 33), the phenobarbital-treated rat liver (2, 3, 7, 12–14), and the developing rat liver, in which proliferation of smooth membrane occurs (34–37). As the enzymes investigated remain elevated in both the rough and smooth microsomes, it is not clear from these investigations whether the proteins of the rough and smooth membranes are a single pool, appearing first in the rough membranes, or whether rough and smooth membrane proteins are individual pools and incorporated into their specific membrane structure separately. The data presented here indicate that the smooth membrane phospholipid is synthesized *in situ*. The new protein synthesized at the site of the rough membranes either may be released, possibly into the cisternae, and incorporated into the smooth membrane after phospholipid synthesis, or alternatively, may be incorporated into the structure of the rough membrane and move by lateral diffusion to the smooth membranes. The possibility of a rapid lateral movement of plasma membrane proteins has been demonstrated by Frye and Edidin (38) and is an important concept in the fluid mosaic model of

membrane structure suggested by Singer and Nicolson (39). The role of free ribosomes in the biogenesis of membrane proteins has not been clarified. If these ribosomes are involved in addition to bound ribosomes, it is also necessary to postulate movement of protein from the cytoplasm to the area of new phospholipid synthesis in the smooth endoplasmic reticulum.

Our observations that the phospholipid/protein ratio and the specific activities of the acyltransferases of the smooth microsomes may change without similar changes in the rough microsomes raise questions concerning the existence of free lateral flow of phospholipid and acyltransferase molecules between the rough and smooth endoplasmic reticulum membranes. It has been clearly established that the rough and smooth membranes are continuous in hepatocytes of normal and phenobarbital-treated rat liver (2, 3, 40). It has also been demonstrated, using both model systems and sarcoplasmic reticulum vesicles, that there is a rapid lateral diffusion of spin-labeled phospholipid molecules within the plane of membranes (41, 42). The present results suggest that such a rapid flow of phospholipid molecules and acyltransferase molecules, sufficient to cause immediate randomization of membrane components, does not occur between rough and smooth membranes. This raises the possibility of barriers to free diffusion of components between morphologically continuous membranes or of controlling forces involved in maintaining the individual composition of the rough and smooth membranes with regard to both lipid and protein.

The theories concerning the structure of cellular membranes are at present in a state of flux, and there is increasing evidence that membranes are fluid structures (39). The results described here serve to emphasize this concept. The phospholipid/protein ratio of the smooth microsomes rose in response to phenobarbital, and this rise occurred when over 80% of new protein synthesis was inhibited. Transient differences in the composition of smooth endoplasmic reticulum membranes may occur, therefore, and a rigid composition is not obligatory under all physiological conditions. In addition, phospholipids of smooth endoplasmic reticulum may be synthesized without proteins. Thus, synthesis of complete lipoprotein subunits of these membranes does not take place. Similar observations were made by Mindich (43) in *Bacillus subtilis* mutants incapable of lipid synthesis. These organisms synthesized membranes having a high protein-to-lipid ratio compared with the wild strain. These observations are consistent with a membrane structure such as that suggested by Singer and Nicolson (39) in which the protein components are embedded to varying depths in a phospholipid bilayer, and in which the two components may change independently of each other.

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