HETEROGENEITY OF PHOSPHOLIPID SYNTHESIS IN RAT LIVER ENDOPLASMIC RETICULUM DURING PROLIFERATION OF SMOOTH MEMBRANES

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SUMMARY

During proliferation of smooth endoplasmic reticulum (SER) induced by phenobarbital, the specific activity of acyltransferases of the smooth microsomes increases, there is a transient rise in the phospholipid/protein ratio of these membranes, and an increased incorporation of \(^{14}\text{C}\)glycerol into smooth-membrane phospholipid. Microsomes separated into subfractions on 2 gradients exhibited a heterogeneous distribution of these characteristics, indicating a non-uniform distribution of the site of phospholipid synthesis in the ER under these conditions. Cytochemical localization of acyltransferases on whole liver and smooth and rough microsomes confirmed this heterogeneity, and indicated that the distribution of this activity was not restricted to any morphologically distinct site in the ER of the intact cell. After 4 days of phenobarbital treatment the increased membrane is restricted to lighter subfractions and is similar in distribution to that of increased acyltransferase activity. These results indicate that the synthesis of membrane phospholipid and the growth of the SER in response to phenobarbital is not uniform but occurs at randomly dispersed sites in the SER while proteins may be added preferentially at these sites resulting in a final uniform distribution.

INTRODUCTION

Previous investigations reported from this laboratory have indicated that during phenobarbital-induced proliferation of smooth endoplasmic reticulum membranes in rat hepatocytes, the phospholipid components of the proliferating membranes are synthesized in situ (Higgins & Barnett, 1972; Higgins, 1974). Both cytochemical and biochemical studies of the sn glycerol-3-phosphate, palmitoyl CoA acyltransferases indicated that in the untreated rat liver these enzymes are active in both the rough and smooth endoplasmic reticulum (RER and SER). During phenobarbital-induced proliferation of SER, however, there was an increase in the specific activity of acyltransferases in the smooth membranes and a decrease in their activity in the rough membranes. Incorporation of \(^{14}\text{C}\)glycerol into liver smooth microsomal phospholipid in vivo was increased by treatment of the animals with phenobarbital to a greater extent than incorporation into rough microsomal phospholipid, and the small rise in the latter case could be accounted for by exchange of phospholipid molecules between the microsomal membrane fractions. Consistent with these observations, the phospholipid/protein ratio of the smooth membranes exhibited a transient rise after treatment of the animal with phenobarbital, while there was no such rise in the rough membranes.
Cytochemical studies on whole liver suggested that there may be a heterogeneous distribution of acyltransferases within the endoplasmic reticulum membranes (Higgins & Barnett, 1972). As these enzymes initiate and may be rate limiting in the synthesis of membrane phospholipid such an heterogeneity is of fundamental significance in considering a mechanism for the formation of smooth membranes. However, cytochemical experiments on whole tissue suffer from a number of criticisms, which prevent these observations from being immediately accepted. The major criticisms are the need to use fixed tissue and the possibility of permeability barriers either against substrates or against capture reagents within the tissue blocks incubated. To overcome these objections we have now investigated the distribution of acyltransferase activity by localization of these enzymes in microsomal fractions incubated in suspension without prior fixation and by determining the effect of phenobarbital on acyltransferase activity and phospholipid-synthesizing activity of subfractions of microsomes.

METHODS

General methods

Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) using bovine serum albumin as a standard; phospholipid phosphorus was determined by the method of Fiske & Subbarow (1925) on aliquots of lipids extracted by the method of Folch, Lees & Sloane-Stanley (1956). Incorporation of $[^{14}C]$glycerol (New England Nuclear) into phospholipids of rat liver was determined as described previously (Higgins, 1974) except that 5 $\mu$Ci/100 g body weight were used in place of 2.5 $\mu$Ci. In investigations of the effect of phenobarbital, the drug was administered by intraperitoneal injection (10 $\mu$g/100 g body weight). Male Sprague-Dawley rats (150–250 g) were used in all experiments.

Enzyme assays

Glucose 6 phosphatase was determined by incubation of enzyme source in media containing Tris-maleate buffer, 0.1 M, pH 6.6, and glucose-6-phosphate (Sigma Chemical Co.), 0.2 M (Dalner, Siekevitz & Palade, 1966).

$5'$ nucleotidase was assayed using media containing Tris-Cl buffer, 0.05 M, pH 7.4, MgCl$_2$, 5 mM, and $5'$-adenosine monophosphate (Sigma Chemical Co.), 5 mM (Touster, Arenson, Dulaney & Hendrickson, 1970).

Acid phosphatase was assayed by incubation of fractions, which had been frozen and thawed twice, in media containing sodium acetate–acetic acid buffer, 0.1 M, pH 5.0, and $\beta$-glycerophosphate, 5 mg/ml (Barrett, 1972).

In all cases the final volume was 1.0 ml, the protein concentration 0.1–1.0 mg, and the time of incubation 0–20 min. Reactions were stopped by addition of 1.0 ml of 1 N perchloric acid or 10% trichloroacetic acid (TCA) and phosphate release was determined on 1.0 ml of supernatants after centrifugation (Fiske & Subbarow, 1925; Widnell, 1974).

Succinic dehydrogenase was assayed by spectrophotometric determination of the appearance of reduced cytochrome c at 550 nm. The incubation medium contained phosphate buffer, 0.05 M, pH 7.5, sodium succinate, 3 mM, cytochrome c, 0.1 mM, and potassium cyanide, 0.3 mM (Sottoscasa, Kuylenstierna, Ernster & Bergstrand, 1967).

UDP galactose galactosyltransferase was assayed by determination of the transfer of $[^{14}C]$galactose from UDP galactose to ovalbumin by a method based on that described by Beaufay et al. (1974). The complete incubation medium contained 25–100 $\mu$g protein; 0.1 M cacodylate buffer, pH 6.6; 10 mM ATP; 10 mM MgCl$_2$; 30 mM $\beta$-mercaptoethanol; 0.2% Triton X 100; 5% ovalbumin and uridine diphospho$[^{14}C]$galactose, 1.25 mM, specific activity 200 cpm/nmol, in a total volume of 0.1 ml. Aliquots were removed after incubation at 37°C and pipetted onto filter paper disks, which were dropped immediately into ice-cold 10% trichloroacetic acid.
These were allowed to stand for 1-15 h, were washed by 15-30 min immersion in distilled water for three changes, dried and placed in counting vials; 5 ml of scintillation fluid (Econofluor - New England Nuclear) were added and the vials were counted in a Packard TriCarb scintillation counter. Under these conditions transfer of [14C]galactose to TCA-insoluble material was linear for 30 min and up to 500 µg of microsomal protein.

**Electron microscopy**

Microsomes were fixed as pellets for 15-18 h in 1-0 % osmium tetroxide buffered to pH 7.4 with 0-05 M cacodylate nitrate buffer containing 4-5 % dextrose. Pellets were dehydrated through a series of graded solutions of ethanol, propylene oxide, and embedded in Epon. Orientation was controlled so that sections could be taken through the thickness of the pellet.

**Cell fractionation**

Rat livers were removed and homogenized using the Potter Elvehjem homogenizer in ice-cold 0.25 M RNase free sucrose to yield 20 % w/v homogenates, which were centrifuged at 12,500 rev/min (10,000 g av) for 20 min in the 40 rotor of the Beckman L2-65B centrifuge for the initial removal of large granules. The supernatants were recentrifuged at the same speed and the pellets from each step combined. Total microsomes were isolated from the supernatants by centrifugation at 40,000 rev/min for 45 min in the 40 rotor. Alternatively, supernatants were separated into subfractions on discontinuous gradients.

**Subfractionation.** Microsomes were separated into subfractions by modification of methods previously used for the separation of rough and smooth microsomes on discontinuous gradients containing CsCl (Higgins & Barrnett, 1972; Higgins, 1974). Two discontinuous gradients were used. In gradient one, 0.5 ml each of 1.3 M sucrose containing 15 mM CsCl, 0.975 M sucrose containing 11.25 mM CsCl and 0.68 M sucrose containing 7.5 mM CsCl were layered in an SW 27 rotor centrifuge tube. In gradient two, a layer of 0.78 M sucrose containing 0.05 mM CsCl was substituted for the 0.68 M sucrose layer of gradient one. CsCl was added to the supernatants after removal of LG to give a final concentration of 15 mM (0.15 ml of 1 M CsCl per 9.85 ml of supernatant); 10 ml of each supernatant were layered on discontinuous gradients and these were centrifuged at 26,000 rev/min (88,000 g av) for 100 min in the SW 27 rotor. Fractions were removed from centrifuge tubes using either a tube slicer or a Pasteur pipette. These were diluted to approximately 0.25 M sucrose with distilled water and particulate material was isolated by centrifugation at 40,000 rev/min (105,000 g) for 45 min. In experiments in which the distribution of protein through the gradient was determined, 100 ml fractions were taken from the bottom of the tubes using a tube piercer.

**Cytochemical experiments**

Cacodylate buffer pH 7.2, 0.05 M containing 4.5 % dextrose was used throughout. Rough and smooth microsomes (Higgins & Barrnett, 1972; Higgins, 1974) were resuspended in buffer containing diisopropyl fluorophosphate (DFP) (10^-5 M) and potassium ferricyanide (0.3 mg/ml) at 0 °C for 30 min. This preincubation step was used to remove endogenous oxidizable material (Higgins & Barrnett, 1970) and to inhibit palmityl CoA hydrolase (Benes, Higgins & Barrnett, 1972; Lands & Hart, 1965). Microsomes were isolated from the preincubation media by centrifugation (80,000 g av, 30 min) and washed by resuspension in buffer and recentrifugation. The pellets were resuspended in buffer and aliquots incubated in media containing palmityl CoA (Sigma Chemical Co.) (0.075 mg/ml, 0.075 mM), sn glycerol-3-phosphate (Calbiochem., San Diego, California) (0.5 mg/ml, 1.8 mM), potassium ferricyanide (0.15 mg/ml, 0.45 mM), and manganous chloride (0.5 mg/ml, 2.5 mM) in buffer for 30 min at 37 °C. In some experiments sn [14C]glycerol-3-phosphate or [14C]palmityl-CoA (New England Nuclear) diluted with unlabelled substrate was used and the incorporation of label into lipids determined at the end of the incubation period, as described previously (Higgins & Barrnett, 1972; Higgins, 1974). In cytochemical experiments the microsomes were isolated by centrifugation after incubation, washed by resuspension in buffer and recentrifugation, and the pellets fixed for 15-18 h in 1 % OsO₄ in cacodylate buffer. These were embedded in Epon, sections cut on an LKB
ultramicrotome, stained for 1-2 min with lead citrate and examined in an Hitachi 11B electron microscope. In some experiments microsomal pellets were cut into pieces after the preincubation step and were passed through the above procedure, as blocks. This eliminated the repeated centrifugation steps and gave similar cytochemical results to those obtained with suspensions. Control experiments were performed in which either one or both substrates were omitted from the incubation medium.

Assay of acyltransferases

The specific activity of the acyltransferases was determined by a microassay based on that described by Goldfine (1966) for the determination of incorporation of labelled precursors into lipids. The enzymes were assayed under fixed conditions in all cases, in media containing microsomal protein (250 μg) sn [14C]glycerol-3-phosphate, diluted to a specific activity of approximately 2000 cpm per nmol with cold substrate (1.8 μl), and palmityl CoA (0.018 μl) in a final volume of 250 μl of cacodylate buffer pH 7.2 containing 4.5% dextrose. Microsomes were warmed to 37 °C in buffer for approximately 15 s and the reaction was started by addition of substrates. Aliquots (50 μl) were removed at 15, 30, 60 and 120 s. These were pipetted on to filter paper disks (Whatman 3 MM) and dropped immediately into ice-cold 10% TCA. The disks were left for 1-18 h in TCA, washed for 30 min in 5% TCA, washed with 3 changes of distilled water for 15-30 min, dried, placed in counting vials, and 5 ml of scintillation solution added (Econofluor, New England Nuclear). The vials were counted in a Packard Tri-Carb scintillation counter. Incorporation of label was linear under these conditions for only short periods of time, and results are expressed as nmol glycerol-3-phosphate incorporated into lipid per min per mg of protein during this linear period. In preliminary investigations, acyltransferase activity was determined in parallel experiments in which one aliquot was pipetted on to a filter paper disk and an equal aliquot into chloroform/methanol (2/1) and the lipids extracted and counted as described previously (Higgins & Barnett, 1972; Higgins, 1974). Both methods gave similar results; however, the filter paper assay was rapid, less tedious, and allowed analysis of a greater number of samples.

RESULTS

Cytochemical localization of acyltransferases on microsomal subfractions

Incorporation of sn [14C]glycerol-3-phosphate or [14C]palmityl CoA into microsomal lipid incubated in the complete cytochemical incubation medium was linear for 20-30 min. In a typical cytochemical experiment, incorporation of glycerol-3-phosphate into lipid was 5-8 nmol per min in the case of the rough microsomes and 4-33 nmol per min in that of the smooth microsomes. In the absence of palmityl CoA incorporation was 1.4 and 1.1 nmol per min respectively. Palmityl CoA increases activity therefore but is not obligatory. This is probably due to endogenous substrate which may remain tightly associated with the microsomal membrane (Sumper & Trauble, 1973). Under the same conditions incorporation of [14C]palmityl CoA into lipid was 9-58 nmol per min in the case of the rough microsomes and 10-85 nmol per min in that of the smooth microsomes. Glycerol-3-phosphate was necessary for this incorporation. Controls of cytochemical experiments were consistent with the biochemical results. Microsomes incubated in the absence of both substrates or glycerol-3-phosphate had no detectable reaction product, while those incubated in the absence of palmityl CoA showed some reaction product, but less than those incubated in complete media.

Smooth microsomes incubated in the complete medium for localization of acyltransferases for 30 min exhibited reaction product associated with only a limited
Fig. 1. Smooth microsomes incubated in the complete medium for acyltransferase localization. Reaction product is limited to a few vesicles and forms a layer lining the vesicle membrane, and is occasionally seen associated with flat sheets of membrane (arrows) which may be broken vesicles. × 51000. A−E, single reactive vesicles illustrating the forms of deposition of reaction product from a distorted vesicle having reaction product over the whole surface (A) to a vesicle having only a little reaction product (E). × 60000−100000.
Fig. 2. Rough microsomes incubated in the complete medium for localization of acyltransferases. Reaction product is associated with a limited number of vesicles and forms a separate deposit within the vesicles but associated with the membrane at one or more points. × 42,000. A–E, single reactive vesicles illustrating the forms of deposition of reaction product from an *en face* view of a sheet of reaction product (A) to a transverse section of an irregular sheet of reaction product (E). × 60,000–90,000.
Heterogeneity of phospholipid synthesis

number of vesicles, with the majority of vesicles having no activity (Fig. 1). Reaction product tended to line the membrane and occurred to varying degrees from lining or overlaying a part of the total surface seen in section, to covering the whole surface. In the latter case, distortion of the vesicle shape frequently occurred. Rough microsomes incubated in the same medium also exhibited reaction product associated with only a limited number of vesicles (Fig. 2). In this case reaction product formed a separate deposit within the vesicle frequently attached to the membrane at 2 or more points. Examination of a large number of micrographs indicated that the deposit occurred in the form of a sheet sometimes seen en face, but was most frequently seen either in cross-section or oblique section. This pattern of deposition may reflect a difference in the disposition of the acyltransferases in the rough membrane compared with the smooth membrane. The most obvious possibility is that the acyltransferases associated with the rough membranes exist as lipoproteins within the vesicle or loosely attached to the membrane. We have attempted to investigate this by treatment of microsomes with low concentrations of deoxycholate (Kreibich, Debeay & Sabatini, 1973) or by osmotic shock (Glauerman & Dallner, 1968) followed by washing to release microsomal content. Both procedures, however, resulted in complete inhibition of acyltransferase activity in both rough and smooth microsomes.

The appearance of reaction product in rough and smooth microsomes was similar in preparations from animals treated with phenobarbital and from untreated animals. However, the amount of reaction product varied. This was especially marked in the case of rough microsomes isolated from animals treated with 3 daily injections of phenobarbital in which reaction product was considerably reduced compared with rough microsomes from normal animals. In the case of smooth microsomes reaction product was always present, but to the greatest extent in microsomes from livers of phenobarbital-treated rats. When morphologically recognizable elements of Golgi membranes were observed in smooth microsomes, these did not have acyltransferase activity, which was restricted to small vesicles.

The association of reaction product marking acyltransferase activity with only a limited number of vesicles under optimal conditions for the cytochemical experiments suggests a heterogeneity in the distribution of these enzymes within the endoplasmic reticulum consistent with the cytochemical observations on whole liver (Higgins & Barrnett, 1972). As microsomes were incubated in suspension without fixation, artificial localization due to gross penetration barriers and selective inhibition by fixation are excluded. The possibility remains that some of the microsomal vesicles are impermeable to the substrates, palmitoyl CoA or glycerol-3-phosphate, or the capture reagents, potassium ferricyanide or manganous chloride. However, there is no apparent difference between reactive vesicles compared with unreactive vesicles except for a distortion of the smooth vesicles when reaction product is associated with the whole membrane surface. In addition, smooth vesicles occur in which reaction product is associated with only a part of the membrane surface, indicating that both substrates and capture reagents have access to the vesicle, but that the whole surface is not active.

The association of reaction product with only the inner surface of the rough microsomal membrane vesicle suggests that the acyltransferases are active only on one side
of the membrane. This raises questions concerning the biogenesis of membrane phospholipids, as movement of phospholipid molecules from one side of the membrane to the other is a very slow process (Scandella, Devaux & McConnell, 1972). If phosphatidic acid is synthesized by enzymes having a heterogeneous distribution across the bi-layer, as is suggested by our results, some mechanism must be postulated for the movement of this and possibly other phospholipids from one side of the bi-layer to the other during membrane formation. This is currently under investigation in this laboratory.

Subfractionation of microsomes

Microsomal supernatants after removal of large granules were separated on 2 discontinuous gradients, as described in Methods. In both gradients 3 bands formed one at each interface and a pellet at the bottom of the tube. Although the bulk of the protein was concentrated at each interface, protein also occurred between bands. Therefore in removal of subfractions material was taken from above and below the interface so that the total gradients were divided. The 0.25 M sucrose layer contained no particulate matter on centrifugation; however, this had a high concentration of soluble protein. The floating bands from gradient one were designated Sα, Sb and Sc in order of increasing density, and the pellet R', and from gradient two S1, S2 and S3 in order of increasing density, and the pellet R. Sα was small and frequently not present and was, therefore, only used for morphological examination, while S1 was present to varied degrees, but usually sufficient for analysis and enzyme assay.

Morphology of subfractions. Both pellets R and R' were similar morphologically and consisted predominantly of membrane vesicles having bound ribosomes (Fig. 3). These fractions were comparable with the rough microsomes isolated previously. The heaviest floating fractions Sc and S3 were also comparable morphologically and consisted mainly of smooth-membraned vesicles, some having a few bound ribosomes (Fig. 4). These may be derived from rough vesicles which have lost ribosomes during isolation, or may be derived from areas of continuity between rough and smooth endoplasmic reticulum. The bulk of the pellets derived from S2 and Sb were also similar morphologically and consisted of smooth-membraned vesicles, some of which contained one or more lipoprotein or lipid droplets (Fig. 5). These vesicles may be derived from the Golgi complex in the case of fairly large membranous envelopes containing multiple lipid droplets or, as is more frequently found, the vesicles containing single lipid droplets may be derived from the SER. Although the bulk of the pellet of Sb was as illustrated in Fig. 4, the surface of the pellet differed in morphology. At the extreme surface were many large vesicles containing lipid droplets and similar to the Golgi preparations described by others (Fig. 6) (Ehrenreich, Bergeron, Siekevitz & Palade, 1973). Moving towards the centre of the pellet the Golgi-like vesicles became less apparent and were succeeded by flattened lamellae without lipid droplets (Fig. 7). These are also characteristic of Golgi membrane preparations. Throughout both areas were smooth-membrane vesicles similar to those making up the bulk of the fraction. The apparent contamination of the total Sb fraction with Golgi-derived vesicles is probably small and was not found in all preparations; however, the possibility of this
Fig. 3. Subfraction R from gradient two. This consists of vesicles having bound ribosomes. × 34,500.
Fig. 4. Subfraction S3 from gradient two. Note vesicles having some bound ribosomes (arrows) although smooth vesicles predominate. $\times 34,500$. 
Fig. 5. Subfraction S2 from gradient two. Note vesicles having lipid droplets as contents (arrows) although smooth vesicles predominate. × 34 500.
contamination led to the choice of gradient two as preferable for further studies. The S2 fraction from this gradient did not contain these obviously Golgi-derived vesicles and was similar to Fig. 5 in all preparations examined.

The lightest fraction Sa and Sb from both gradients showed the greatest morphological variation between experiments. In some preparations this fraction consisted of large vesicles similar to those illustrated in Fig. 6, and in others flattened lamellae predominated, as in Fig. 7. In both cases, many smaller smooth vesicles were also observed.

Thus, subfractions from the total microsomal fraction, when examined morphologically, exhibited patterns in which vesicles having bound ribosomes tended to occur in the heavier fractions, those containing lipid or lipoprotein droplets tended to occur in the lighter fractions, while smooth-membraned vesicles tended to be at a higher concentration in the floating bands, especially from the middle of the gradient.

**Distribution of marker enzymes.** Under the homogenization and centrifugation conditions used, the yield of glucose-6-phosphate in the total microsomal fraction was 37-47% of the activity in the homogenate (Table 1). The remainder was recovered in large granules. The microsomal fraction prepared in this way had no detectable succinic dehydrogenase activity or acid phosphatase activity. It did, however, have 33% of the galactosyltransferase activity and 18% of the 5'-nucleotidase. The specific activity of the latter was not enriched compared with the homogenate, and that of the galactosyltransferase was enriched approximately 2-fold. This proportion of contamination could not be reduced by slower centrifugation, and it is probable that in order completely to remove vesicles derived from the Golgi membranes and plasma membrane from total microsomes some alternative physical method will be necessary.

Using representative specific activities reported by others for 5'-nucleotidase of isolated plasma membranes (Dorling & LePage, 1973; Jones, Staton & Kiesow, 1973; Ray, 1970; Touster et al. 1970) and galactosyl transferase of isolated Golgi membranes (Ehrenreich et al. 1973; Mörre, Cheetham & Nyquist, 1972) in cases where these are compared with the specific activity of the original homogenate (25-fold in the case of 5'-nucleotidase, 100-fold in the case of galactosyltransferase), it can be calculated that the protein contribution is 4% from plasma membrane and 2% from Golgi membranes. These calculations are based on the assumption that neither 5'-nucleotidase nor galactosyltransferase are present in the ER membranes. Although this appears to be true in the case of the latter, there is evidence that at least a part of the 5'-nucleotidase of whole homogenates may be components of ER, Golgi membranes and outer mitochondrial membranes (Widnell, 1972, 1974). It is also true that heterogeneity exists in the distribution of 5'-nucleotidase in plasma membranes of hepatocytes. In
fractions prepared by zonal centrifugation, plasma membrane vesicles in a 'light' fraction have a high 5'-nucleotidase activity (enriched 40-fold compared with the homogenate) while a 'heavy' fraction has low 5'-nucleotidase (enriched 17-fold compared with the homogenate) (Evans, 1970; Evans & Gurd, 1971; Evans, Bergeron & Geschwild, 1973). The heavy fraction, which is isolated from the 'nuclear' fraction of a homogenate, is also more active in binding insulin and is believed to be derived from both lateral junctional complexes and the sinusoidal surface of the hepatocyte. Thus the vesicles of the plasma membrane, which occur in the microsomal fraction, have a high 5'-nucleotidase activity and are believed to be largely derived from the bile canicular membranes (Evans et al. 1973). The microsomal fraction is therefore probably contaminated with plasma membrane protein to a smaller degree than calculated above.

Table 1. Distribution of marker enzymes in fractions of liver

<table>
<thead>
<tr>
<th></th>
<th>Homogenate</th>
<th>Large granules</th>
<th>Total microsomes</th>
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<tbody>
<tr>
<td><strong>Protein</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mg/g liver</td>
<td>160±31 ± 219 (3)</td>
<td>78±22 ± 189 (3)</td>
<td>28±11 ± 4.53 (3)</td>
</tr>
<tr>
<td>% distribution*</td>
<td>48±89 ± 161 (3)</td>
<td>17±5 ± 3.97 (3)</td>
<td></td>
</tr>
<tr>
<td><strong>5'-nucleotidase</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% distribution†</td>
<td>97±93 ± 3.21 (3)</td>
<td>81±57 ± 4.94 (3)</td>
<td>18±62 ± 4.77 (3)</td>
</tr>
<tr>
<td>specific activity§</td>
<td>80±91 ± 14.18 (3)</td>
<td>82±24 ± 17.24 (3)</td>
<td>60±32 ± 18.82 (3)</td>
</tr>
<tr>
<td><strong>Glucose-6-phosphatase</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% distribution†</td>
<td>92±99 ± 5.6 (3)</td>
<td>57±31 ± 4.97 (3)</td>
<td>42±68 ± 4.96 (3)</td>
</tr>
<tr>
<td>specific activity§</td>
<td>124±71 ± 2.98 (3)</td>
<td>75±50 ± 21.21 (3)</td>
<td>238±72 ± 19.23 (3)</td>
</tr>
<tr>
<td><strong>Galactosyltransferase</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% distribution†</td>
<td>94±78 ± 5.94 (3)</td>
<td>66±31 ± 5.64 (3)</td>
<td>33±40 ± 5.86 (3)</td>
</tr>
<tr>
<td>specific activity§</td>
<td>0.13±52 ± 0.048 (3)</td>
<td>0.14±60 ± 0.0614 (3)</td>
<td>0.25±4 ± 0.508 (3)</td>
</tr>
</tbody>
</table>

Rat livers were homogenized and separated into large granules, and total microsomes and homogenates and fractions analysed as described in Methods. Results are expressed as mean ± standard deviation (number of observations).

* Expressed as % of protein in original homogenate.
† Expressed as % of recovery in LG and TM.
§ nmol of phosphate released/min/mg protein or nmol galactose transferred to TCA-insoluble protein/min/mg protein.
Heterogeneity of phospholipid synthesis

Table 2. Specific activities of marker enzymes of microsomal subfractions

<table>
<thead>
<tr>
<th>Glucose-6-phosphatase*</th>
<th>5'-nucleotidase*</th>
<th>Galactosyltransferase*</th>
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</thead>
<tbody>
<tr>
<td><strong>Gradient one</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sb</td>
<td>314 ± 28 (5)</td>
<td>304 ± 19.8 (5)</td>
</tr>
<tr>
<td>Sc</td>
<td>355 ± 56 (5)</td>
<td>354 ± 17.6 (5)</td>
</tr>
<tr>
<td>R'</td>
<td>286 ± 27 (5)</td>
<td>632 ± 17.8 (5)</td>
</tr>
<tr>
<td><strong>Gradient two</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S1</td>
<td>108 ± 30 (5)</td>
<td>381 ± 14.8 (5)</td>
</tr>
<tr>
<td>S2</td>
<td>355 ± 31 (5)</td>
<td>353 ± 25.2 (5)</td>
</tr>
<tr>
<td>S3</td>
<td>374 ± 52 (5)</td>
<td>614 ± 18.9 (5)</td>
</tr>
<tr>
<td>R</td>
<td>290 ± 55 (5)</td>
<td>546 ± 14.6 (5)</td>
</tr>
</tbody>
</table>

Total microsomes were separated on gradients one and two and the subfractions analysed as described in Methods. Results are expressed as mean ± standard deviation (number of observations).

Effect of phenobarbital on subfractions

Acyltransferase activity of subfractions. Previous studies have demonstrated that in response to injections of phenobarbital there is a rise in the specific activity of acyltransferases of smooth microsomes to peak at 18 h after the first injection, while the specific activity of the rough microsomes remains relatively constant for 24 h then falls (Higgins & Barnett, 1972; Higgins, 1974). The specific activity of the acyltransferases of subfractions was therefore determined in microsomes from control rats and after 18 h and 3 days of phenobarbital treatment. All subfractions from control animals prepared on either gradient one or two exhibited acyltransferase activity (Fig. 8). This was highest in Sb from gradient one and S2 from gradient two, although the differences between fractions were not large. In microsomes from rats given one injection of phenobarbital, 18 h prior to sacrifice, the specific activity was increased approximately 3-fold in Sb, 4-fold in S2, and 2-fold in S1, with no change in S3. Similar changes in specific activity were also found in these fractions prepared from rats given 3 injections of phenobarbital, although the absolute levels were lower in this case (Fig. 8). The increased specific activity found previously is therefore not uniformly distributed in subfractions of smooth microsomes. Sb from gradient one contributes protein to 2 fractions on gradient two and, although both of these have an increased specific activity, S2 shows the greatest change. It is probably therefore the smooth vesicles of Sb, S1 and S2 in which the specific activity of the acyltransferases increases. This is also indicated by the cytochemical results in which these enzymes were localized only in the smooth vesicles even when Golgi-derived membranes were present. The specific activity of the pellet R from both gradients was low and fell slightly after 4 days of phenobarbital treatment. This is in contrast to the previous results (Higgins & Barnett, 1972) in which the activity of rough microsomes was
higher or equal to that of the total smooth microsomes in the control and fell to a low level after repeated injections of phenobarbital. This may represent operational differences between the pellets, although these appear similar morphologically.

Fig. 8. Microsomal subfractions were prepared on gradients one and two as described in Methods from livers of normal rats (N) and of rats treated 18 h and 3 days with phenobarbital. The specific activities of the acyltransferases were determined as described in Methods and stated as nmol α-glycerophosphate incorporated/min/mg protein. Results are plotted as means of 4 experiments with standard deviations indicated by bars.

**Phospholipid/protein ratios of subfractions.** After injection of phenobarbital there is a transient increase in the phospholipid/protein ratio of the smooth microsomes, which peaks at 4-5 h (Higgins, 1974). This ratio of the subfractions was therefore determined at this time after injection and compared with control rats. Microsomal fractions were washed by resuspension in 0.25 M sucrose and recentrifuged. In some experiments these were then treated with 0.049% deoxycholate (DOC) in high-salt buffer under the conditions described by Kreibich et al. (1973) to release proteins trapped within the vesicles. This treatment was found to release over 80% of the label incorporated into protein at 30 min after injection of [3H]leucine as described by Kreibich et al. (1973). The phospholipid/protein ratio of each subfraction from both gradients one and two was elevated by treatment with DOC, although the relative ratio between fractions was not changed (Fig. 9). At 4-5 h after injection of phenobarbital the phospholipid/protein ratio of Sb and S2, both with and without DOC treatment, was increased significantly compared with the same fractions from control rats ($P < 0.005$). There was some increase in the ratio of S1 ($P < 0.01$) and no significant change in other fractions ($P > 0.01$) (Fig. 4). These results are consistent with the previous observations and indicate that the transient increase in the phospholipid to protein
Heterogeneity of phospholipid synthesis shows a heterogeneous distribution within subfractions of the smooth microsomes, and this pattern is similar to that of acyltransferase activity.

Incorporation of $[^{14}C]$glycerol into subfractions. $[^{14}C]$glycerol was incorporated rapidly into phospholipids of microsomes. In control rats this incorporation was similar in subfractions S2, S3 and R, while the rate of appearance of label in the phospholipids of S1 was slower but reached the similar final level to other fractions after

![Graphs showing incorporation of $[^{14}C]$glycerol into subfractions of smooth microsomes.](image)

Fig. 9. Microsomal subfractions were prepared on gradients one and two as described in Methods from livers of control rats or rats given one injection of phenobarbital 4.5 h prior to sacrifice. These were either washed by resuspension on 0.25 M sucrose and recentrifugation (untreated, A, C) or treated for 30 min at room temperature with 0.049% deoxycholate in high-salt buffer as described by Kreibich et al. (1973) (DOC-treated, B, D). Phospholipid/protein ratios were determined as described in Methods. Results are plotted as means of 4 experiments with standard deviations indicated by bars, and in each section the left-hand set of data are from controls (C) and the right-hand set are after phenobarbital treatment (Pb).

60 min (Fig. 10). When animals were given injections of phenobarbital followed by $[^{14}C]$glycerol at time intervals such that sacrifice was at 1 h after the first injection, incorporation of the label into phospholipid was elevated at all times and in all subfractions. It has been demonstrated previously that exchange occurs between the phospholipids of rough and smooth microsomes during the isolation procedures used for these cell fractions (Higgins, 1974). While this is not complete during our experimental conditions, such an exchange would tend to mask differences in the specific activity of phospholipids of subfractions at the time of sacrifice; however, relative differences are maintained.

Incorporation of $[^{14}C]$glycerol into the phospholipid of S2 is increased to the
greatest extent by phenobarbital treatment with an increase of 3-fold that of the control at early times after injection (Fig. 10). The specific activity of phospholipids of S₁ and S₃ is also increased, but more slowly and to a lesser extent, while the specific activity of R showed the least change, with the largest increase 50% above that of the control. The latter is marginally significant and can be accounted for by exchange of phospholipid between more rapidly labelled fractions and R.

![Graph showing specific activity of phospholipids](image_url)

Fig. 10. Microsomal subfractions were prepared on gradient two, as described in Methods from livers of rats given injections of [14C]glycerol and the specific activity of the membrane phospholipids determined as described in Methods. The specific activity of the phospholipids is plotted against the time after injections of [14C]glycerol in control rats (open circles) and rats given one injection of phenobarbital 1 h prior to sacrifice in addition to the isotope (closed circles). The specific activity of the phospholipid in experiments in which rats were treated for 4 days with phenobarbital is indicated by crosses and broken lines. Results are expressed as means of at least 3 experiments with standard deviations indicated by bars, and averages of 2 experiments for rats treated for 4 days with phenobarbital.

The appearance of labelled precursors in membrane lipids may be a result of incorporation at the site of synthesis and/or the movement of newly synthesized lipid from the site of synthesis. S₂ is the first subfraction labelled, indicating that these vesicles contain one site of synthesis of phospholipid. S₁ and S₃ are labelled more slowly, and this may be a consequence of slower synthesis of phospholipid at these sites and/or movement of labelled lipid from S₂ either in vivo or after sacrifice. The subfractions produced are operational, however, and a spectral pattern in character-
Heterogeneity of phospholipid synthesis

Characteristics of these is not unexpected. Thus, elements which are concentrated in S2 might be present in S1 and S3 at a lower concentration.

After 4 days of phenobarbital treatment the incorporation of \([^{14}\text{C}]\text{glycerol}\) into phospholipid of all subfractions was not elevated compared with the control. However, there is an increase of over 2-fold in the surface of the SER membranes measured by morphometric analysis (Staubli, Hess & Weibel, 1969). This increase is restricted to the lighter subfractions of the microsomes (Figs. 11, 12) corresponding to S2 and to a lesser extent S3. It is not possible to determine accurately the dilution of the specific activity of the phospholipids due to this increased membrane, as total yields of SER have not been achieved and morphometric analyses cannot distinguish between the SER which contributes vesicles to the different subfractions. However, with a 70\% yield of SER, this is at least 2-fold in the case of S2 (Fig. 11). Taking this into account, therefore, the total incorporation of \([^{14}\text{C}]\text{glycerol}\) into phospholipid is
J. A. Higgins

elevated over the control in S2, to a lesser extent in S3, and there is little change in S1 and R.

Holtzman & Gillette (1968) have investigated the incorporation of $^{32}$P into phospholipid of total microsomes and concluded that there is no increased synthesis of phospholipid after phenobarbital treatment and that the major effect of this drug is to delay catabolism of membrane phospholipid. Their results are in conflict with ours, which indicate that there is an increased incorporation of $[^{14}$C]glycerol into smooth microsomes in response to phenobarbital treatment. There are, however, a number of differences in the experimental protocols used. Holtzman & Gillette examined incorporation of $^{32}$P after 1 h, at which time in our experiments incorporation of glycerol had peaked. In addition, the experimental animals used were fasted for up to 4 days, which may have a variety of effects on their physiological state. In short-term experiments we allowed access to food until injection of phenobarbital, at which time food was removed from both control and experimental rats. In longer-term experiments food was removed from both control and experimental animals for the duration of anaesthetic. As this was during the quiescent period it is probable that the food intake per rat was not changed significantly by this protocol. Holtzman & Gillette also used total microsomes rather than subfractions and it is possible that differences in the smooth microsomes might be masked in experiments where both forms of ER are present. The most serious difficulty, however, in interpreting their results is that they corrected incorporation of label into microsomes by the total phospholipid pool of the microsomes at different times after injection of phenobarbital. The method used for the isolation of total microsomes (9000 g for 15 min in isotonic KCl) would result in significant loss of ER membrane into the ‘mitochondrial’ pellet and therefore result in incomplete yields of microsomes, although these were not determined. Thus, using these methods it is difficult to obtain a correct measure of phospholipid content of the total ER. If the error in the determination is large then differences in the incorporation of $^{32}$P into membrane phospholipid may not be detected. As most of our investigations were conducted in the first hour after phenobarbital treatment, changes in the total phospholipid of the ER would not be a factor; however, at 4 days of phenobarbital treatment this is important. It is possible that decreased catabolism is a factor in the proliferation of SER in response to phenobarbital. However, there is also increased synthesis of phospholipid, which is restricted to the SER and is heterogeneously distributed in these membranes.

Effect of phenobarbital on distribution of protein between microsomal subfractions

In order to determine which microsomal subfraction increased in response to phenobarbital the protein distribution of the gradients of microsomes from livers of normal and phenobarbital-treated rats was determined. In these experiments it was found that the yields of microsomes using the conditions described above were insufficient or variable and reproducible differences in the patterns of protein distribution could not be obtained. The use of lower centrifugal speeds ($10000$ rev/min, $6600$ g av 20 min), resuspension of the large granule fraction and centrifugation at the same speed, and combination of the supernatants from which total microsomes were
isolated, resulted in an increased yield to 67% of ER membranes measured in terms of glucose-6-phosphatase (Table 3). When the nitrogen cavitation bomb \( (5.861 \times 10^3 \text{ kN m}^{-2} (850 \text{ lb in.}^{-2}) \), Hunter & Commerford (1961) was used to prepare the homogenate this could be increased to 85% under the same centrifugation conditions probably because this method of homogenization results in rupture of a greater number of

Table 3. Distribution of marker enzymes in fractions prepared by centrifugation at 10,000 g for 20 min

<table>
<thead>
<tr>
<th>Marker Enzyme</th>
<th>homogenate</th>
<th>large granule</th>
<th>total microsomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potter Elvehjem</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Succinic dehydrogenase</td>
<td>% distribution*</td>
<td>97.92 ± 8.2 (5)</td>
<td>96.79 ± 1.44 (5)</td>
</tr>
<tr>
<td></td>
<td>Specific activity†</td>
<td>47.04 ± 10.44 (5)</td>
<td>58.57 ± 23.79 (5)</td>
</tr>
<tr>
<td>5'-nucleotidase</td>
<td>% distribution*</td>
<td>107.9 ± 4.95 (5)</td>
<td>38.82 ± 9.33 (5)</td>
</tr>
<tr>
<td></td>
<td>Specific activity*</td>
<td>55.14 ± 17.3 (5)</td>
<td>64.34 ± 23.34 (5)</td>
</tr>
<tr>
<td>Glucose-6-phosphatase</td>
<td>% distribution*</td>
<td>109.33 ± 13.74 (5)</td>
<td>33.45 ± 4.16 (5)</td>
</tr>
<tr>
<td></td>
<td>Specific activity*</td>
<td>119.78 ± 36.94 (5)</td>
<td>73.32 ± 27.78 (5)</td>
</tr>
<tr>
<td>Galactosyltransferase</td>
<td>% distribution*</td>
<td>109.33 ± 13.74 (4)</td>
<td>33.45 ± 4.16 (4)</td>
</tr>
<tr>
<td></td>
<td>Specific activity*</td>
<td>0.08 ± 0.0088 (4)</td>
<td>0.13 ± 0.0176 (4)</td>
</tr>
<tr>
<td>Bomb</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Succinic dehydrogenase</td>
<td>% distribution*</td>
<td>95.13 ± 11.71 (4)</td>
<td>96.86 ± 0.62 (4)</td>
</tr>
<tr>
<td></td>
<td>Specific activity†</td>
<td>42.56 ± 14.08 (4)</td>
<td>65.39 ± 10.44 (4)</td>
</tr>
<tr>
<td>5'-nucleotidase</td>
<td>% distribution*</td>
<td>102.56 ± 19.56 (6)</td>
<td>33.02 ± 4.82 (6)</td>
</tr>
<tr>
<td></td>
<td>Specific activity*</td>
<td>78.98 ± 8.86 (6)</td>
<td>122.26 ± 20.66 (6)</td>
</tr>
<tr>
<td>Glucose-6-phosphatase</td>
<td>% distribution*</td>
<td>98.35 ± 6.54 (6)</td>
<td>17.74 ± 3.48 (6)</td>
</tr>
<tr>
<td></td>
<td>Specific activity*</td>
<td>115.78 ± 24.88 (6)</td>
<td>93.98 ± 3.82 (6)</td>
</tr>
<tr>
<td>Galactosyltransferase</td>
<td>% distribution*</td>
<td>101.68 ± 33.26 (5)</td>
<td>22.19 ± 3.33 (5)</td>
</tr>
<tr>
<td></td>
<td>Specific activity*</td>
<td>0.113 ± 0.0284 (5)</td>
<td>0.138 ± 0.038 (5)</td>
</tr>
</tbody>
</table>

Rat livers were homogenized and separated into large granules and total microsomes with high yield and the homogenates and fractions analysed as described in Methods. Results are expressed as mean ± standard deviation (number of observations).

* Expressed as in Table 1.
† Succinic dehydrogenase expressed as nmol cytochrome c reduced/min/mg protein.

cells. The increased yield of ER membranes, however, was at the expense of a greater contamination of the microsomal fraction with Golgi and plasma membrane-derived membranes (Table 3). In the present study this was acceptable, as the distribution of these contaminating membranes in the subfractions was not modified by treatment of the rats with phenobarbital. The specific activity of the acyltransferases of the
subfractions prepared from lower yields of microsomes showed an increase restricted to the fraction corresponding to S2.

After 1 day of treatment with phenobarbital there was no consistent change in the distribution of protein between microsomal subfractions compared with those from livers of untreated rats. However, after 4 days of treatment, when SER proliferation is well established (Staubli et al. 1969) the pattern of protein distribution changed markedly (Figs. 11, 12). It was not possible to detect differences on the gradient in fractions corresponding to S1 as this was masked by protein remaining in the 0·25 M sucrose layer. However, when removed and isolated by centrifugation, this fraction was not increased in quantity. Fractions corresponding to S2 were increased approximately 2-fold, while fractions S3 increased slightly and R was unchanged. The protein tended to be higher in the subfractions at the top of the gradient in separations of microsomes prepared using the bomb. This was also true in fractions from phenobarbital-treated rats; however, the increased protein was restricted to the same fractions.

DISCUSSION

The separation of microsomes by centrifugation on gradients results in operational fractions, rather than 'pure' fractions from a defined site in the cell. However, using this approach spectral changes in functional characteristics of subfractions of ER may be determined and related to possible heterogeneity in the behaviour of the total organelle. In the present investigation microsomes were separated on 2 gradients and, in both cases, the distribution of contaminating membranes was investigated to ensure that functional changes were not a consequence of modification of the pattern of contamination by membranes derived from sites other than ER.

The effect of phenobarbital on smooth microsomes was similar to that described previously. This fraction exhibited increased acyltransferase activity, an increased rate of incorporation of [14C]glycerol into phospholipid and a rapid transient increase in the phospholipid/protein ratio of the membranes with no similar change in the rough microsomes. These changes were interpreted as indicating that during phenobarbital-induced proliferation the SER is the site of synthesis of phospholipid, and that this synthesis precedes the incorporation of protein into these membranes. The present results indicate that all of these changes are concentrated in one subfraction of the microsomes, and therefore that the topography of phospholipid synthesis in the SER is not uniform. The cytochemical observations on whole liver (Higgins & Barrnett, 1972) and on microsomal fractions confirmed this heterogeneity, and in addition indicate that the distribution of this activity is random and is not restricted to any morphologically distinct site.

During proliferation of SER membranes only the lighter subfractions of the microsomes were found to increase. The rough microsomal pellet and the heavier subfractions which contain smooth microsomes and intermediate vesicles, possibly derived from regions of continuity of the SER and the RER, are not increased. The mechanism of vesiculation of the ER during homogenization, and the parameters which
Heterogeneity of phospholipid synthesis

dictate the movement of vesicles on the gradient are not known. However, it appears likely that the same parameters are involved in the separation of vesicles from both normal and phenobarbital-treated microsomes. It is possible that the increased activity of phospholipid-synthesizing enzymes causes small changes perhaps in density or permeability, which result in separation of these vesicles. Consistent with this, the acyltransferase activity is greatest in S2 prepared from both control and phenobarbital-treated rat livers, although the activity is greater in the latter case.

Cytochemical studies of the localization of glucose-6-phosphatase in the ER of neonatal rat liver demonstrated that this enzyme is added uniformly during SER formation (Leskes, Sickervitz & Palade, 1971a, b). In this system, therefore, there is no 'old' or 'new' membrane but insertion of this protein component through the whole membrane resulting in growth. In preliminary investigations we have found that all microsomal subfractions showed increased NADPH cytochrome c reductase after phenobarbital treatment, suggesting a similar uniform distribution of new protein. In order to correlate these observations it is necessary to postulate a mechanism for the biogenesis of SER in which protein molecules are added throughout the ER, but preferentially at sites at which phospholipid synthesis occurs, resulting in growth of the SER at these sites, but a uniform distribution of the protein. Alternatively, after assembly at the site of phospholipid synthesis the protein may diffuse in the membrane and become uniformly dispersed, although growth is at limited sites. To examine this more closely, the rates of incorporation of precursors into proteins of the subfractions of liver microsomes at different times during proliferation are under investigation in this laboratory.

There is evidence that cell membranes are fluid structures in which proteins and lipids are free to move within the plane of the membrane (Frye & Edidin, 1970; Scandella et al. 1972; Singer & Nicolson, 1972). In contrast to this there is evidence from studies of membrane biogenesis in prokaryotic cells that proteins and phospholipids synthesized simultaneously remain associated during subsequent growth of the membrane (Esfahani, Limbrick, Knutton, Oka & Wakil, 1971; Wilson & Fox, 1971). Eukaryotic cells are less suitable for manipulation of the protein and lipid composition of membranes, and similar studies have not been performed on these. However, there are many enzymes which require specific lipids for activity (Bock & Fleischer, 1974; Garland, Cori & Chang, 1974; Kimelberg & Papahadjopoulos, 1972; Omura & Sato, 1964a, b; Widnell, 1972) suggesting that an association between protein and lipid at the time of assembly may also occur in this case. While proteins may move within the plane of the membrane, therefore, it is probable that they carry with them some lipid which is not in equilibrium with the bi-layer. A mechanism for the biogenesis of membrane in which synthesis of a protein component by either free or bound ribosomes may be coordinated with synthesis of a specific phospholipid, and in which the assembly occurs at the site of phospholipid synthesis, is therefore attractive and consistent with the observations reported here.

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Heterogeneity of phospholipid synthesis


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