DIFFERENTIATION OF ENDOPLASMIC RETICULUM IN HEPATOCYTES

II. Glucose-6-Phosphatase in Rough Microsomes

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ABSTRACT
Electron microscope cytochemical localization of glucose-6-phosphatase in the developing hepatocytes of fetal and newborn rats indicates that the enzyme appears simultaneously in all the rough endoplasmic reticulum of a cell, although asynchronously within the hepatocyte population as a whole. To confirm that the pattern of cytochemical deposits reflects the actual distribution of enzyme sites, a method to subfractionate rough endoplasmic reticulum was developed. The procedure is based on the retention of the cytochemical reaction product (precipitated lead phosphate) within freshly prepared rough microsomes reacted in vitro with glucose-6-phosphate and lead ions. Lead phosphate increases the density of the microsomes which have glucose-6-phosphatase activity and thereby makes possible their separation from microsomes lacking the enzyme; separation is obtained by isopycnic centrifugation on a two-step density gradient. The procedure was applied to rough microsomes isolated from rats at several stages during hepatocyte differentiation and the results obtained agree with those given by cytochemical studies in situ. Before birth, when only some of the cells react positively for glucose-6-phosphatase, only a commensurate proportion of the rough microsome fraction can be rendered dense by the enzyme reaction. At the time of birth and in the adult, when all cells react positively, practically all microsomes acquire deposit and become dense after reaction. Thus, the results of the microsome subfractionation confirm the cytochemical findings; the enzyme is evenly distributed throughout all the endoplasmic reticulum of a cell and there is no regional differentiation within the rough endoplasmic reticulum with respect to glucose-6-phosphatase. These findings suggest that new components are inserted molecule-by-molecule into a pre-existing structural framework. The membranes are thus mosaics of old and new molecules and do not contain large regions of entirely "new" membrane in which all of the components are newly synthesized or newly assembled.

INTRODUCTION
Glucose-6-phosphatase (G6P) is an enzyme strongly bound to microsomal membranes in rat hepatocytes (1). In situ cytochemical tests (2, 3) suggest that the inorganic phosphate produced by enzymatic hydrolysis of G6P is released into the ER cisternal space, where it is precipitated by lead ions present in the incubation medium. At all stages of development studied, the reaction

1 Abbreviations used are: ER, endoplasmic reticulum; G6P, glucose-6-phosphate; G6Pase, glucose-6-phosphatase; EDTA, ethylenediaminetetraacetate; Pi, inorganic phosphate; RNA, ribonucleic acid; PLP, phospholipid; TCA, trichloroacetic acid; OD, optical density.
product (lead phosphate deposit) is restricted to elements of ER; within the ER system the deposit is evenly distributed throughout the cisternal content and on the inner aspect of the limiting membrane.

The well-defined localization of the reaction product argues that diffusion of inorganic phosphate to the cytoplasmic matrix or other subcellular compartments does not take place. It does not, however, rule out diffusion of the phosphate within the cisternal space. If such intracisternal diffusion occurs, the even distribution of lead phosphate deposits could result from enzyme sites either evenly or unevenly distributed. To eliminate this uncertainty, it was necessary to approach the localization problem from a second direction. We took advantage of the well-known fact that upon homogenization the rough ER fragments into closed vesicles (4), and we performed the cytochemical reaction on isolated rough microsomal vesicles. In this system, inorganic phosphate cleaved from G6P is not expected to diffuse from within one closed vesicle to another, and the insoluble precipitate formed in the presence of lead ions should remain within the vesicles. The distribution of the precipitate in the microsome population should directly reflect the spacing of enzyme sites in the microsomal membrane, and indirectly their distribution in the membrane of the intact ER. The results of these experiments show that G6Pase is indeed evenly distributed in classes of rough microsomal vesicles.

In the course of these studies, a method was developed to separate subclasses of microsomal vesicles on the basis of their enzyme activity. Microsomes which possess G6Pase are rendered dense by the enclosed lead phosphate deposit they acquire during the enzyme reaction; microsomes lacking the enzyme remain light. After the reaction, the two types of vesicles can be separated by isopycnic centrifugation. The method allows a quantitative determination of the proportion of microsomes which possess G6Pase activity at different stages of development.

**MATERIALS AND METHODS**

**Preparation of Rough Microsome Fractions**

Rough microsomes were prepared by a modification of Rothchild's procedure (5). Adult male rats (Sprague-Dawley strain) were anesthetized with ether, newborn rats (1-2 hr old) were decapitated, and fetal rats were decapitated after being removed from anesthetized mothers. The livers were homogenized at 4°C in four volumes of 0.88 M sucrose using a motor-driven Potter-Elvehjem homogenizer (6). 9-12 ml of the homogenate were centrifuged for 15 min at 10,000 g_{sp} in the Spinco S 40 rotor. The pellet was resuspended in 0.88 M sucrose and recentrifuged, and the wash was added to the original supernate to form the postmitochondrial supernate. This fraction contained microsomes and free ribosomes. The pellet contained whole cells, nuclei, mitochondria, and some microsomes.

Since hepatocytes contain large amounts of glycogen during certain stages in development, and since the glycogen appears to promote the aggregation of rough microsomes, the postmitochondrial supernatant was incubated for 60 min at 4°C with approximately 10,000 units of hog pancreas alpha amylase (Worthington Biochemical Corp., Freehold, N.J.) per gram wet weight of tissue. During incubation the solution was gently homogenized three times. After incubation, 5 ml of the suspension were layered onto a step gradient consisting of 5 ml of 2.0 M sucrose overlayed with 13 ml of 1.32 M sucrose, and the remainder of the tube was filled with 0.01 M cacodylate buffer, pH 6.6. The gradient was spun in the Spinco S 30 rotor for 12-13 hr at 78,000 g_{av} and was collected through an Instrumentation Specialties Co. (Lincoln, Neb.) density gradient fractionator. Optical density was monitored at 550 m\u00b4 and 1-ml fractions were collected into precooled tubes in an ice bath. The fractions containing the rough and smooth microsomes were separately pooled and kept at 4°C. In this gradient smooth microsomes remain at the interface between 1.32 M sucrose and the load zone, rough microsomes collect at the interface between 1.32 and 2.0 M sucrose, while free glycogen particles, if present, and glycogen particles associated with membranes are pelleted. Postmitochondrial supernates previously treated with amylase yield only a very slight reddish pellet. Polysomes and free ribosomes are in the 2.0 M sucrose layer or in the pellet. The pooled rough microsome fraction was gently sonicated in an Acoustica ultrasonic bath (American Process Equipment Corp., Panama City, Fla.) for 30 or 60 sec at 50 ma in order to break up any aggregates which might have formed during centrifugation.

**Cytochemical Reaction and Subfractionation of Rough Microsomes**

Samples of the rough microsome fraction were incubated for 60 min at 25°C in the same medium used for the in situ cytochemical experiments (3): 1 mM G6P (dipotassium salt converted from the barium salt), 2 mM lead nitrate, and 0.05 M cacodylate.
buffer, pH 6.6. The final volume (20-60 ml) depended upon the yield of rough microsomes, and contained between 1.2 and 3.2 mg rough microsomal protein/10 ml incubation mixture. The incubation was stopped by transferring the solutions to 4°C. Controls were incubated without G6P.

Despite amylase treatment, aggregation of the microsomes still occurred during incubation, probably owing to the presence of excess lead ions. To reverse this aggregation, 10 ml samples of the reacted microsomal suspension were dialyzed overnight (approx. 18 hr) at 4°C against three changes of 100 volumes of 0.01 M cacodylate buffer, pH 7.7, containing 0.1 mM ethylenediaminetetraacetate (EDTA). The dialysis bags were previously washed with 10 mM EDTA, 2% Na₂CO₃ and distilled water, since dialysis in unwashed bags caused fragmentation of the microsomes. During dialysis the bags containing the microsomal suspension were allowed to spin freely (long axis horizontal) and were gently sonicated three times for 9-10 min at 50 mA in the ultrasonic bath. The exact procedure (including amylase treatment of the postmitochondrial supernate, three changes of the dialysis medium, gentle sonication of the dialysis bags, and constant agitation of the bags) is very critical; slight variations prevent the vesicles from disaggregating.

After the last sonication, 20 ml of the microsomal suspension were layered over 9 ml of 2.0 M sucrose and the material was spun at 63,600 × g ave for 60 min in the Spinco SW 25.1 rotor. Under these conditions, microsomes containing lead phosphate deposit sediment to the bottom of the tube, while those lacking deposit collect at the interface between the load zone and 2.0 M sucrose. The gradients were collected with the gradient fractionator, and absorbance was monitored at 254 nm. 2-ml fractions were collected into precooled tubes in an ice bath and adjacent fractions were pooled in pairs. For biochemical analysis the pellets were resuspended in 0.88 M sucrose. Samples from the pooled fractions were assayed for G6Pase activity, RNA, protein, and phospholipid. A schematic representation of the subfraction is shown in Fig. 1.

![Scheme for subfractionation of rough microsomes based on G6Pase activity.](image-url)
Electron Microscopy of Fractions

Samples of the material at the interfaces were diluted with cacodylate buffer and centrifuged for 45 min in the Spinco SW 39 rotor at 125,000×g, as were samples of unreacted rough and smooth microsomes. The resulting pellets, as well as the pellets from the final gradients, were fixed overnight in situ at 4°C in 1% OsO₄ in 0.1 M cacodylate buffer, pH 6.6 or 7.4, then stained for 1 hr in 0.5% uranyl acetate (7) before dehydration through graded alcohols. While in 95% alcohol, they were cut into sectors which included both the top and the bottom of the pellet. During embedding, these sectors were oriented so that a thin section would include the total thickness of the pellet in the direction of centrifugation. For further details see Leskes et al. (3).

Chemical Assays

G6Pase was assayed as described previously (3). Protein was measured by the method of Lowry et al. (8) with bovine albumin as standard. Phospholipid was extracted by the procedure of Folch et al. (9) using 0.73% NaCl as the aqueous phase. Inorganic and lipid phosphorus was assayed by the method of Ames and Dubin (10), and micromoles of phosphorus were multiplied by 775 to obtain micrograms of phospholipid. RNA was extracted from a perchloric acid precipitate by heating in 0.3 N KOH for 60 min at 37°C, and the OD₂₆₀ of the extract was determined (11).

RESULTS

Morphological and Biochemical Characteristics of the Rough Microsome Fraction

In hepatocytes, G6Pase occurs in both rough and smooth endoplasmic ventricular (ER) but not in the plasma membrane or in the elements of the Golgi complex (3). Our study was limited to the distribution of G6Pase within the population of rough microsomes (derived essentially from the rough ER) (4), because the amount of smooth ER shows marked variations during development, and because the smooth microsome fraction is heterogeneous: it contains many vesicles derived from the Golgi complex and the plasma membrane which are expected to be G6Pase negative. The contamination of the rough microsomes by vesicles of such origin is minimal.

The appearance of a rough microsome fraction prepared from an animal ~2 hr after birth is shown in Fig. 2. It consists primarily of microsomal vesicles, most of which are intact and covered with ribosomes on their outer surfaces. It contains also some free ribosomes, and occasional contaminants such as lysosomes and mitochondria.

Assays of G6Pase activity and RNA, protein, and phospholipid content showed that the fraction isolated from adult rat liver has the usual features of rat liver rough microsomes (12). Calculations of the distribution of RNA and G6Pase activity indicate that ~65% of the rough ER is recovered in the fraction.

Disaggregation of Reacted Rough Microsomes

Rough microsomes incubated in the presence of lead ions (with or without G6P) aggregate and become dense enough to pellet through 2.0 M sucrose, presumably because of lead binding to the membranes. Hence, after a G6Pase reaction, the aggregation must be reversed as a prerequisite for separating reacting from nonreacting vesicles. Disaggregation was achieved at the end of the reaction by removing excess lead ions in the incubation medium by dialysis against 0.1 mM EDTA.

Sonication and the extended period of dialysis against EDTA might result, however, in loss of lead phosphate deposit from within reacted vesicles, due to possible lead chelation by EDTA. To examine the possibility that the vesicles which float above 2.0 M sucrose had lost the lead phosphate they contained at the end of the reaction, samples of reacted microsomes were taken before and after dialysis, treated with 5% trichloroacetic acid (TCA) (to solubilize the lead precipitates contained within the vesicles) and assayed for Pᵢ. Table I shows that there was no loss of Pᵢ during sonication and dialysis. In fact, in all cases there was a moderate Pᵢ increase after dialysis, probably because the enzymatic reaction is not completely stopped at the end of the 60 min incubation period. Dialysis at 4°C only slowly removes G6P from the microsomal suspension and further reaction probably proceeds slowly for some time.

Subfractionation of Reacted Rough Microsomes

Fig. 3 shows the results of experiments in which rough microsomes isolated from animals at several stages of development were incubated with lead and G6P, dialyzed as described, and sedimented on step gradients to determine the proportion of vesicles which contain reaction product...
**TABLE I**

*Retention of Inorganic Phosphate during Dialysis against EDTA*

Rough microsomes were incubated with G6P and lead nitrate and then dialyzed and sonicated as described in Materials and Methods. Samples were removed before and after dialysis, the tissue was precipitated with 5% TCA, and the inorganic phosphate in the supernatant was measured.

<table>
<thead>
<tr>
<th>Age</th>
<th>End of reaction</th>
<th>After dialysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>-2 days</td>
<td>0.017</td>
<td>0.028</td>
</tr>
<tr>
<td>Birth</td>
<td>0.101</td>
<td>0.121</td>
</tr>
<tr>
<td>Birth</td>
<td>0.061</td>
<td>0.088</td>
</tr>
<tr>
<td>Adult</td>
<td>0.067</td>
<td>0.076</td>
</tr>
</tbody>
</table>

*Values are averages of two determinations. Essentially no change occurs during dialysis in volume and in micrograms of protein per milliliter.*

Essentially no change occurs during dialysis in volume and in micrograms of protein per milliliter.

The absolute amount of material in the pellet at this stage varies slightly (cf. Figs. 3 and 4), probably owing to the difficulty in determining the exact age of the fetus. By 2 days before birth (Fig. 3 B) a small proportion of the material is found in the pellet, although the majority of the vesicles still lack deposit and remain at the interface. At the time of birth the vast majority of the microsomes are found in the pellet (Fig. 3 C), indicating that at this stage most of the vesicles contain lead phosphate deposit, and hence have G6Pase activity. The pattern in the adult is similar to that at birth; all of the rough microsomal vesicles become dense after reaction.

These findings are in good agreement with

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Figure 2. Rough microsome pellet (middle layer) prepared from livers of 1-2-hr old animals. The pellet consists of rounded vesicles and flattened cisternae (C). Arrows mark area where ribosomes are clearly visible on the outer surface of the microsomal vesicles. X 40,000.
both in situ cytochemical observations (3) and measurements of G6Pase specific activity in developing rat liver. 3 days before birth only a small number of hepatocytes contain cytochemical deposit. By 2 days before birth more cells react positively for the enzyme, although they are still in the minority. At birth and in the adult, virtually all cells react cytochemically and the deposit is seen throughout all of their ER. Table II compares the G6Pase specific activity of the unreacted rough microsome fraction with the proportion of microsomes (as phospholipid) found in the pellet after reaction and sedimentation. Comparison of columns three and five reveals that the relative specific activity of the enzyme in the microsomes (compared to that in the adult) correlates well with the relative proportion of dense lead phosphate–carrying vesicles after reaction. This correlation further supports our contention that vesicles which contain G6Pase acquire deposit, become dense, and pellet through 2.0 M sucrose. In each of the gradients shown in Fig. 3, the interface and pellet combined account for 60–85% of the total phospholipid recovered in the gradients but only for 40–75% of the total protein. This discrepancy is probably due to the detachment of ribosomes and ribosomal subunits by EDTA. The released ribosomal protein and RNA do not sediment but remain at the top of the gradient in the load zone. Phospholipid, therefore, a better marker for locating microsomal vesicles.

**Controls**

If irreversible aggregation of vesicles occurred during or subsequent to the incubation with G6P and lead, the centrifugal patterns observed (Fig. 3)
TABLE II
Correlation of G6Pase Specific Activity in Unreacted Rough Microsomes with Phospholipid Pelleted after G6Pase Cytochemical Reaction

The G6Pase specific activity was measured on a sample of the same rough microsomes used for incubation with G6P and lead ions.

Biochemical assay conditions: 30 mM G6P, 0.03 M cacodylate buffer, pH 6.6, 37° C. Cytochemical assay conditions: 1 mM G6P, 2 mM lead nitrate, 0.05 M cacodylate buffer, pH 6.6, 25°C.

The specific activities are average of two determinations.

The total recovery of phospholipid in the gradients was between 79 and 115%.

<table>
<thead>
<tr>
<th>Age</th>
<th>Specific activity of G6Pase</th>
<th>PLP recovered in pellet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µmoles P_i/mg protein/20 min</td>
<td>% total</td>
</tr>
<tr>
<td>-3 days</td>
<td>0.10</td>
<td>3</td>
</tr>
<tr>
<td>-3 days</td>
<td>0.20</td>
<td>6</td>
</tr>
<tr>
<td>-2 days</td>
<td>0.61</td>
<td>19</td>
</tr>
<tr>
<td>Birth</td>
<td>2.94</td>
<td>90</td>
</tr>
<tr>
<td>Birth</td>
<td>3.24</td>
<td>90</td>
</tr>
<tr>
<td>Adult</td>
<td>3.26</td>
<td>100</td>
</tr>
</tbody>
</table>

would not reflect the true distribution of reacted and unreacted vesicles. To exclude this possibility, two types of control experiments were performed. In the first, equal quantities of isolated rough microsomes from animals at two stages of development, birth and 3 days before birth, were mixed before reaction in the histochemical medium. After reaction, the mixture was dialyzed and analyzed on a step gradient. Equivalent amounts of microsomes from each stage were also reacted separately and analyzed. The results of this experiment are shown in Fig. 4. The majority of rough microsomes from animals 3 days before birth (Fig. 4 A) float while only a small fraction pellets. Almost all of the microsomes from newborn animals (Fig. 4 B) pellet, as is also shown in Fig. 3. In the mixture (½ A + ½ B) one-half of the membranes float and one-half pellet through the dense sucrose; this is just what is expected in the absence of aggregation. The percent phospholipid expected at the interface and in the pellet of the mixture were 32% and 43% respectively; the actual recoveries were 35% and 44%.

In a second type of control a sample of rough microsomes was incubated with lead in the absence of substrate, and was then dialyzed and sonicated. In all cases these unreacted microsomes

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**Figure 4** Separation of a mixture of reacted rough microsomes from -3-day and new-born rats; distribution of phospholipid and protein on step gradients. In the mixture, equal samples of rough microsomes prepared from rats of the two ages were mixed before incubation with lead and G6P. All of the microsomes were incubated and dialyzed as described in Materials and Methods. After dialysis the suspensions were loaded above 2.0 M sucrose and were spun for 60 min at 63,000 g_avg. 4-ml fractions and the pellets were assayed for phospholipid and protein. Fraction 6 (interface) and the pellet in each gradient were assayed in duplicate. All of the gradients are from the same experiment. Total PLP recoveries on the gradients were between 70 and 115%.

294 THE JOURNAL OF CELL BIOLOGY • VOLUME 49, 1971
remained above the 2.0 M sucrose in the step gradient (although if not treated to remove the lead they sedimented through it). Another sample was incubated with lead and substrate and then dialyzed separately. Mixing equal amounts of these two samples and the centrifuging them together produced exactly the separation expected in the absence of aggregation: one-half of the microsomes floated above the dense sucrose and one-half pelleted. It is thus quite unlikely that there is any significant aggregation present at the time at which reacted microsomes are sedimented on the step gradient. Therefore, in the cases of the newborn and the adult, nearly all of the reacted rough microsomes sediment through the 2.0 M sucrose because nearly all contained dense lead phosphate.

G6Pase Activity in Microsomal Subfractions

G6Pase is a rather labile enzyme and isolated rough microsomes which are maintained at 4°C overnight (the time required to dialyze the reacted microsomes) lose 85% of their original G6Pase activity. The rest of the activity is lost if these microsomes are dialyzed against EDTA. Attempts to reactivate the enzyme by incubation with Mg++ were unsuccessful. Therefore, we were not able to substantiate further the results of our subfractionation experiments by demonstrating that the vesicles which pellet in the step gradients contain G6Pase activity, while those which remain at the interface do not.

Morphology of Subfractions

In all fractionation experiments, the material at the interface and in the pellet of the step gradients were examined by electron microscopy. Fig. 5a shows the interface fraction from a gradient containing a mixture of microsomes from a fetus 3 days before birth and a newborn animal. The microsomes were mixed before reaction. Many of the vesicles are intact, although there are some membrane fragments, broken vesicles, and free ribosomes. The vast majority of the vesicles in the fraction do not contain any lead phosphate deposit.

Fig. 5b illustrates the pellet obtained in a step gradient containing reacted microsomes from a newborn animal. Most vesicles contain an electron-opaque deposit of lead phosphate which fills them either completely or partially. In the latter case, the deposits remain closely applied to the inner surface of the limiting membrane of the vesicles. Membrane fragments, an occasional mitochondrion, and some vesicles which appear to lack deposit contaminate the pellets. Some of these apparently empty vesicles may contain deposit which is out of the plane of the section. In some areas (Fig. 6) the microsomal membrane surrounding the lead deposit is clearly seen. Considering the treatment—dialysis against EDTA and sonication—the membranes are adequately preserved. These micrographs are representative of all fractions examined. The material that pellets through 2.0 M sucrose always consists of reacted vesicles containing lead phosphate deposit, while the material that remains above 2.0 M sucrose is composed of unreacted, empty vesicles. Electron microscope examination thus confirms that the step gradient separates two types of rough microsomes: those which contain G6Pase and those which do not.

DISCUSSION

The in situ cytochemical experiments described in the previous paper (3) showed that G6Pase activity appears simultaneously within all the ER of a given hepatocyte, but asynchronously among the cells of the hepatocyte population. Within the ER, the reaction product occurred either on the inner aspect of its membrane or in the cisternal space. A similar location has been noted before (12–14) for G6Pase and other phosphatases. In our case, it was important to demonstrate that the widespread distribution of the G6Pase reaction product within the ER of a given cell either represents or closely approximates the actual distribution of enzyme sites and does not result from extensive diffusion of released Pi within the cisternal space before precipitation by lead.

At present, there is no way of ascertaining whether the lead phosphate deposits mark enzyme sites or other separate sites which act as foci of precipitation in the ER. The second alternative implies diffusion of inorganic phosphate—possibly also of lead phosphate—at least over short distances. That some diffusion occurs in the cytochemical tests is evidenced by the large size of lead phosphate deposits and by their presence in the cisternal space (3). By fragmenting the ER system into closed microsomal vesicles...
we tried to determine the spacing of enzyme sites assuming that, once this spacing is known, we will be in a better position to assess the importance of diffusion in the production of the reaction pattern.

In the cell fractionation experiments described in this paper all of the rough microsomal vesicles isolated from newborn and adult rat liver contain lead phosphate deposit after reaction in vitro. It follows that each vesicle has at least one site of G6Pase activity and that the enzyme is widely distributed in closely spaced sites throughout the intact rough ER from which the rough microsomes are derived. We can assume that a comparably uniform distribution is found at the earlier stages of development (−3, −2 days) when the existence of reacting and nonreacting rough microsomes can be correlated with the heterogeneity in development observed at the cell population level (3). It can be assumed that the nonreacting microsomes come from cells which still lack G6Pase activity, while the reacting vesicles are derived from cells which have already acquired the enzyme.

Our results show, therefore, that the cytochemical pattern can be explained and accounted for by the close spacing of the enzyme molecules irrespective of the presence or absence of intracisternal diffusion. We cannot definitely rule out diffusion, but we can say that, if it occurs, it cannot alter the cytochemical pattern above a certain limit which is given by the size of the microsomes. The average diameter of the rough microsomal vesicles, determined by measuring vesicle profiles in micrographs, is 120 µm. Since every vesicle has at least one site of enzyme activity, there is at least one site per average vesicle surface area, $4 \times 10^4$ µm². If we assume that these sites are uniformly spaced along the membrane, two neighboring sites are on the average no further apart than ~200 µm. The resolution of the technique can be increased by decreasing the size of the microsomal vesicles. Dallman et al. (15) have apparently succeeded in preparing, by repeated sonication, microsomal vesicles which are 100th of their original volume. It may, therefore, be possible to obtain by their procedure a heterogeneous population of small microsomes, some of which contain G6Pase and

This method of measuring tends to underestimate sizes since the actual diameter is measured only if a vesicle is cut through its center. However, the thickness of the sections (approximately 60 µm) is of the same order of magnitude as the diameter of the vesicles. The sizes of the vesicles seen in sections should be reasonably good estimates of their actual diameters. In addition, to find the smallest maximum distance between G6Pase sites we are actually interested in the smallest vesicles in the population so the figure of 120 µm can be used as an approximation.

Assume the membrane is composed of squares having the same area as the average surface area of the vesicles, $4 \times 10^4$ µm². If enzyme sites are evenly spaced in the network, the maximum distance between neighboring sites is equal to the length of a side of the square, 200 µm.

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**Figure 5 a** Interface fraction obtained by centrifuging on a density step gradient a mixture of hepatic rough microsomes from −3-day fetuses and newborn rats, mixed before incubation with G6P and lead ions. The field taken at the top of the pellet was prepared by resedimenting the material that remained above the 2.0 M sucrose layer. The pellet consists of closed and broken (b) vesicles, membrane fragments (f), and aggregated free or detached ribosomes (long arrow). Some of the latter have penetrated ruptured vesicles (b, and short arrow). Ribosomes still attached to microsomal membranes are marked r. Note that all vesicles are free of lead phosphate deposit. Examples of such empty microsomes are marked e. X 43,000.

**Figure 5 b** Pellet fraction obtained by centrifuging on a density step gradient hepatic rough microsomes of 1–2-hr old rats after incubation with G6P and lead ions. The microsomes of this fraction sedimented through 2.0 M sucrose. The field was taken in the middle of the pellet. Most microsomal vesicles are partially (p) or completely (c) filled with electron-opaque deposit of lead phosphate. In partially filled vesicles the deposit appears in discontinuous masses in close contact with the inner aspect of the membrane. Note that the reaction product is generally located within the microsomal cavities and that no deposits are found on the outer surface of the microsomal membranes or between microsomes. A few apparently empty vesicles may contain deposits that are either light (e) or were missed (e₂) by the section. X 43,000.

296 THE JOURNAL OF CELL BIOLOGY • VOLUME 49, 1971
FIGURE 6  Details in a preparation similar to that shown in Fig. 5 b. The microsomes are partly filled with lead phosphate deposit in Fig. 6 a and completely or nearly completely filled in Fig. 6 b. The arrows point to areas where the limiting membrane of the vesicles is clearly visible. Ribosomes still attached to this membrane are marked r. Fig. 6 a, X 105,000; Fig. 6 b, X 150,000.

some of which do not. In this way the spacing of G6Pase sites might become accessible to direct measurement, and possible differentiation within the ER might be investigated at a higher resolution than the one here attained. Such experiments could also indicate the limit below which intracisternal diffusion may affect the cytochemical pattern observed in situ.

Dallner et al. (16) have studied the distribution of G6Pase in hepatic rough microsomes obtained from newborn and adult rats and fractionated by sedimentation on a continuous sucrose gradient. They found G6Pase specific activity to be higher in slowly sedimenting microsomes than in more rapidly sedimenting vesicles, concluded that the rough ER may comprise specialized regions of high enzymatic activity, and suggested that newly synthesized enzymes may be inserted into localized regions of developing ER membranes. Although differences in enzyme concentration per unit area cannot accurately be detected cytochemically (17), our results suggest that qualitatively there is no regional differentiation within the ER with respect to G6Pase at the level of resolution attained. The interpretation of Dallner’s results is difficult; the findings may reflect, for instance, quantitative rather than qualitative differences between the fragments of membrane into which the ER was resolved.

Our inquiry has been restricted to rough microsomes; hence, our conclusions concerning the uniform distribution of G6Pase sites are restricted to the rough ER. In situ cytochemistry provides only limited information about the smooth ER. In adult liver, the reaction product is present in almost all smooth ER membranes, and in the liver of the newborn animal there does not appear to be any newly formed smooth ER which lacks G6Pase activity. However, we cannot conclude from the cytochemical evidence alone that the smooth ER is undifferentiated with respect to this enzyme.

In both the intact (fixed) cell and the isolated (unfixed) microsomes, lead phosphate deposit is found only on the cisternal side of the membrane. In fact, recovery of released Pi within microsomal vesicles appears to be quantitative even after dialysis and sonication, which makes it unlikely that Pi is lost from the vesicles or released to their external side. These findings could be explained by the preferential location of foci of precipitation on the cisternal side of the membrane, or by the unidirectional release into the cisternal space of Pi split from G6P by G6Pase. We favor the second alternative for there is no evidence so far of Pi release on the cytoplasmic side of the membrane. Since the permeability of the microsomal membrane to the substrate, G6P, is unknown, we cannot draw any conclusion about the exact position of the enzyme within the ER membrane.

The method we have developed alters specifically the density of enzyme-containing microsomes by sequestering a reaction product within the microsomal vesicle. Such a method may have wide application. It should be applicable to any enzyme reaction (e.g., all phosphatases) in which a product can be trapped as a heavy metal precipitate within a vesicular structure. Cellular components differing in enzyme composition could thus be separated from each other. One drawback to the procedure for G6Pase is the inability to recover enzyme activity after dialysis. The long time needed to dialyze away excess lead ions and the long exposure to EDTA appear to cause...
enzyme inactivation. Removal of free lead ions by column chromatography may eliminate the drawback.

The results presented in these two papers can be used to propose a tentative model for membrane assembly; three such models are presented in Fig. 7. In the first (Fig. 7, A), new membrane is formed de novo, completely independently of pre-existing old membranes; there is no continuity between the cisternae of the old and new ER. In such a case, after in situ cytochemical incubation, a positive reaction would be found limited to only some cisternae and would be located along the entire length of these ER elements. Cell fractionation, followed by cytochemical reaction in vitro, would produce a heterogeneous population of microsomes, some with and some without G6Pase activity.

The second model (Fig. 7, B) proposes that within areas of pre-existing membrane there is a

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**Figure 7** Models for membrane assembly. Dark areas represent regions of new membrane containing newly synthesized G6Pase, while light areas represent old membrane lacking G6Pase molecules. In each model the figure at the left illustrates the composition of the rough ER membrane, the diagram in the upper right, the pattern of lead phosphate deposit seen after in situ cytochemical incubation, and the diagram in the lower right, the population of rough microsomes after in vitro incubation with G6P and lead ions. In model A the reacted microsomal vesicles are either completely positive or completely negative for the enzyme, in model B vesicles partly reactive can be found, while in model C only reacting vesicles are obtained.
small number of growing points where new membrane is formed. If enzyme is inserted only into these regions, cytochemical reaction product would be limited to well-defined areas in most cisternae. Cell fractionation would yield a heterogeneous population of microsomes comprised of both nonreacting and reacting vesicles.

In the third model (Fig. 7, C) new membrane components are introduced at closely spaced sites throughout the pre-existing framework. In this situation, membrane growth and differentiation are diffuse rather than regional processes. With this model the cytochemical reaction should produce a continuous or quasi-continuous deposit throughout the ER. Cell fractionation, followed by in vitro incubation should yield a homogeneous population of vesicles all containing deposit, provided that the microsomes formed be larger than the spaces between neighboring G6Pase sites, and provided that the hepatocyte population be homogeneous for the enzyme.

The results obtained in situ and in vitro do not support either of the first two models. The only way to reconcile the findings with these models of assembly is to assume that membrane components are turning over rapidly enough for old membrane, lacking G6Pase, to be replaced by new membrane containing the enzyme. There are no experimental data on the turnover of ER membrane constituents in the fetal liver. In the adult rat liver, however, ER membrane proteins turn over with a half-life of 2–4 days (19, 20). If the turnover rate in the fetal liver were comparable to that in the adult, and assembly took place by mechanism A or B, then cytochemical tests should show both reacting and nonreacting areas of the ER within a single cell, since the G6Pase development takes approximately 3–4 days. Such cells are not found, and so unless the turnover rate in the fetus is much more rapid than in the adult, these models of membrane assembly cannot explain the findings. In fact, in the fetus as well as in the newborn rat, hepatocytes and their components are in a state of rapid growth, and under such conditions the turnover rate of membrane components is expected to be even slower than normal.4

The results predicted by the third model are exactly those seen both in situ throughout the development process, and in vitro at birth (and thereafter) when the cell population is homogeneous for the enzyme. It appears, therefore, that only the third model, based on diffuse expansion of the pre-existing framework, can adequately explain the results. This type of expansion or change could occur, however, by introducing either individual molecules or molecular aggregates of increasing complexity into a pre-existing structure. Large molecular aggregates could, in the extreme, contain all membrane components and would thus form minute scattered “growth regions” of the type considered in model B. Although either mechanism for the diffuse growth model is consistent with our findings, we favor the introduction of new components as individual molecules (or possibly small aggregates) for the following reasons:

(a) Available information concerning the turnover of membrane components suggests that these components turn over independently of each other. The phospholipid and protein constituents of the ER membrane apparently have different half-lives, and protein fractions and specific ER enzymes appear to have different turnover rates (18, 23, 24).

(b) During hepatocyte development, the activities of different ER enzymes appear at different times and increase at different rates, even for proteins involved in a common biochemical pathway (25).

(c) During the period of rapid smooth ER membrane formation after treatment with phenobarbital, the induced enzymes are added to the membrane at different rates even though they are part of the same electron transport chain (22).

We have interpreted the results of our previous studies on membrane development as evidence for a multistep assembly of membrane components into new membrane. But since these studies were carried out on isolated cell fractions, they could not determine whether membrane growth and change take place via a regional or a diffuse process; hence, they would not be able to distinguish between models B and C. With the new responding sudden growth of new membrane, the phenobarbital findings suggest that during times of rapid growth the turnover of membrane components is slowed down.

4 During development of the smooth ER induced by phenobarbital treatment, membrane components turn over more slowly than usual (20–22). Although in the developing fetal hepatocytes there is no cor-
evidence presented in these two papers we no longer have to consider restricted "growing points." The results indicate that during development new components are introduced throughout the entire pre-existing membrane; at any time ER membranes are mixtures of old and new molecules. A distinction between "old" and "new" membrane appears, then, artificial. The new results strongly suggest that the ER membrane, and possibly other membranes, grow, differentiate, and turn over by the introduction of new molecules throughout their structural matrix. Since this concept could have important implications for our understanding of how membrane specificity is generated and maintained, its applicability for other membrane components should be checked using similar localization procedures.

Our interpretation of the results obtained by localization procedures depends on the assumption that, upon its insertion into the membrane, a given molecule retains its position because diffusion within the membrane either does not occur or it is too slow to affect the observations. There is, however, evidence that most of the lipids of the membrane are liquid at the temperature of the body (26, 27), which will favor diffusion within the lipid phase, but the evidence on proteins is limited and apparently conflicting. Observations on the production of virus envelopes indicate that newly assembled membrane proteins do not diffuse in the plane of the membrane but retain their position relative to one another (28-31). Yet, observations on heterokaryons suggest that surface antigens (i.e., glycoproteins) spread relatively rapidly over the fused cell membranes (32). Our observations could be explained by (a) regional insertion followed by rapid two-dimensional diffusion in the plane of the membrane, or (b) generalized insertion irrespective of subsequent two-dimensional diffusion. We favor the second alternative since we have observed no gradient of G6Pase activity within the ER at any time during differentiation.

The model discussed concerns only membrane assembly and not membrane function. It does not suggest that molecules are inserted at random. Rather, we feel that functionally distinct units exist in membranes (e.g., electron transport chains). The model postulates, however, that even within such specialized regions, molecules are introduced and turn over individually.

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