BIOGENESIS OF ENDOPLASMIC RETICULUM MEMBRANES

I. Structural and Chemical Differentiation in Developing Rat Hepatocyte

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ABSTRACT

The development of the endoplasmic reticulum of rat hepatocytes was studied during a period of rapid cell differentiation, i.e., from 3 days before to 8 days after birth. Before birth, the ER increases in volume, remaining predominantly rough surfaced; after birth, the increase continues but affects mainly the smooth-surfaced part of the system. These changes are reflected in variations of the RNA/protein and PLP/protein ratios of microsomal fractions: the first decreases, while the second increases, with age. The analysis of microsomal membranes and of microsomal lipids indicates that the PLP/protein ratio, the distribution of phospholipids, and the rate of P^{32} incorporation into these phospholipids show little variation over the period examined and are comparable to values found in adult liver. Fatty acid composition of total phosphatides undergoes, however, drastic changes after birth. During the period of rapid ER development in vivo incorporation of leucine-C^{14} and glycerol-C^{14} into the proteins and lipids of microsomal membranes is higher in the rough- than in the smooth-surfaced microsomes, for the first hours after the injection of the label; later on ($\sim$10 hr) the situation is reversed. These results strongly suggest that new membrane is synthesized in the rough ER and subsequently transferred to the smooth ER.

INTRODUCTION

At present, the biosynthesis of lipid and protein molecules is intensively investigated and relatively well understood, but the next steps in biological organization—i.e. the biogenesis of specific cell structures, starting from lipids, proteins, and other macromolecules—are hardly explored and practically unknown. Biogenesis of membranes is a particularly interesting topic of investigation because membranes are relatively simple, but widely occurring, elementary structures. Although they are used in many different situations in the cell as diffusion barriers, partitions, or as frameworks to which enzyme units or multienzyme systems are attached, they seem to have a number of structural and chemical features in common, such as their layered structure and high content of lipid and protein. Hence, a certain extent of interconversion among various cellular membranes is conceivable.

The purpose of this paper is to investigate some intermediary steps in membrane biogenesis: namely, the order of assembly of various molecules (lipid) and macromolecules (protein) into a functional membrane, and the possible biogenetic relations of one type of membrane to other membrane types within the same cell.

A prerequisite for this kind of study is the choice

of a suitable system which exhibits rapid membrane synthesis at some period in its development, and which is well characterized morphologically and chemically, at least at some developmental stages.

The ER[1] of the developing rat hepatocyte fulfills these conditions. Its membranes can be isolated as a microsomal fraction and the latter can be used to study changes in membrane composition in situ as a function of time during the development of the ER system. Much information concerning the ER of the fetal and newborn hepatocytes has already been published; thus morphological (1–15) and cell fractionation (16–20) studies as well as chemical (21–24) and enzymic (*see* 25) analyses were carried out on the developing livers of rat, mouse, chicken, guinea pig, rabbit, pig, and man.

Our data show that in the fetal and the newborn hepatocytes large quantities of membranes are synthesized, and that the membranes of the rough ER (those with ribosomes attached) are probably the precursors of the membranes of the smooth ER. The new membranes are virtually indistinguishable from those of the fully differentiated cell as regards the amount and composition of their phospholipids. Data regarding their rather different enzymic pattern are published in a companion paper (25). A brief report of this work has already been published (26).

MATERIALS

Animals

All animals used were rats of the CFN strain (Carworth Farms Inc., New City, New York) fed ad libitum on a standard lab chow diet (Ralston Purina Co., St. Louis). Pregnant rats were received on the 7th day of pregnancy and maintained on the same diet in the laboratory until the end of the experiment.

Reagents

Carrier-free, isotonic, sterile sodium phosphate-P^{32} solution was obtained from the Volk Radiochemical Co., Burbank, California; DL-leucine-1-C^{14} (4 mc/mmole) and glycerol-1,3-C^{14} (20 mc/mmole) were purchased from the New England Nuclear Corp., Boston.

[1] Abbreviations used are: ER, endoplasmic reticulum; PLP, phospholipid; RNA, ribonucleic acid; RNP, ribonucleoprotein; TCA, trichloroacetic acid; DOC, deoxycholate.

METHODS

Electron Microscopy

Liver specimens were fixed either in 1% OsO_4 in 0.1 M phosphate buffer, pH 7.4 (3 hr at ~0°), or in 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4 (3 hr at ~0°), followed by 1% OsO_4 in the same buffer (15 hr at ~0°). Pellets were fixed in situ (at the bottom of the centrifuge tubes) in 1% OsO_4 in 0.25 M or 1 M sucrose (15 hr at 0°). All specimens were dehydrated in graded ethanols and embedded in Epon. The pellets were dehydrated in their tubes up to 100% ethanol, when they were hard enough to be removed and cut into orientable strips without loss or displacement of their components. The strips were embedded in grooved Epon plugs in such a way as to permit the sectioning of the pellet from top to bottom, parallel to the direction of sedimentation. Sections were cut with diamond knives on Porter-Blum or LKB microtomes, mounted on Formvar-coated grids, doubly stained with uranyl acetate followed by "alkaline lead" solutions (27), and finally examined in a Siemens Elmiskop I operated at 80 kv with a double condensor and 50-μ apertures in the objective lens. Survey micrographs were taken at a magnification of 3,000, and structural details were studied at direct magnifications ranging from 10,000 to 40,000.

Cell Fractionation

a) MICROSOMES: Liver microsomes were prepared from 0.25 M sucrose homogenates as described by Ernster et al. (28), except that adult animals (90-day-old females) were not starved, since we had no means of reducing the glycogen content of the liver in other age groups (fetus, newborn). The high glycogen content of the homogenates led, as expected, to substantial losses of microsomes to the nuclear-mitochondrial fraction during the preliminary centrifugation at 10,000 g.[2] The microsomes were centrifuged down at 105,000 g (60 min) and resuspended to a final tissue equivalent of 0.5 g/ml in 0.25 M sucrose.

b) SEPARATION OF ROUGH AND SMOOTH MICROSOMAL SUBFRACTIONS: Subfractionation of microsomes was performed with a two-layered sucrose gradient, containing CsCl (29). As the centrifuge used (Spinco, model L) had a lower capacity and produced a lower centrifugal field than the one previously employed (Christ, model Omega), the procedure was modified as follows. Livers were homogenized in 0.25 M sucrose at a tissue concentration of 15 to 18% (w/v) in the case of fetus and newborns, and 25% in the case of adults. The homogenate was centrifuged in a No. 40 rotor, at 10,000 g for 20 min. The supernate, called "10,000 g supernate" was decanted

[2] All g values given in this paper are calculated for the middle of the tube.

and mixed with 0.15 ml of 1 M CsCl (optical grade) for each 9.85 ml, to give a final CsCl concentration of 15 mM. This suspension was used to prepare *total microsomes* as well as *microsomal subfractions.*

For *total microsomes,* an appropriate sample of the CsCl-containing "10,000 *g* supernate" was diluted with 0.25 M sucrose-15 mM CsCl solution to a final volume of 11 ml and centrifuged in a No. 40 rotor at 105,000 *g* for 90 min. The pellet was rinsed 3 times and resuspended by homogenization in 0.25 M sucrose.

For the separation (Fig. 1) of rough and smooth microsomal subfractions (for convenience, these subfractions will be hereafter referred to as *smooth microsomes* and *rough microsomes,* respectively), 3 ml of the CsCl-containing "10,000 *g* supernate" were layered with care over 2 ml of 1.3 M sucrose-15 mM CsCl. After centrifugation in a SW 39 rotor for 210 min. at 39,000 RPM, a fluffy layer appeared at the interface (*smooth microsomes*)[3] and a brown pellet at the bottom (*rough microsomes*). The reddish, clear upper phase above the fluffy layer was removed with a pipette provided with a rubber aspirator and was discarded. The fluffy layer was completely collected together with about 0.6 ml of the 1.3 M clear sucrose solution, and transferred into a 5-ml tube (No. 40.3 rotor) or an 11-ml tube (No. 40 rotor). It was then diluted with 3 ml of distilled water brought up to tube volume with 0.25 M sucrose, mixed, and centrifuged at 105,000 *g* for 90 min. The resulting pellet was rinsed 3 times and resuspended by homogenization in 0.25 M sucrose to give the *smooth microsomes.*

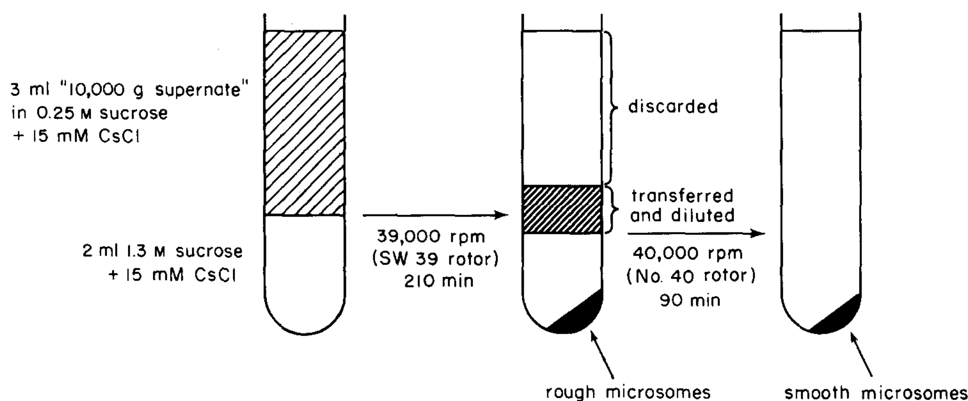


FIGURE 1 Schematic representation of the procedure for subfractionation of liver microsomes by density gradient centrifugation in the presence of CsCl.

Since the pellet of the *rough microsomes* obtained at the bottom of the centrifuge tube after the first centrifugation was not tightly packed, the overlaying clear 1.3 M sucrose layer was not completely removed. A few drops were left behind, diluted with distilled water to bring the sucrose concentration to 0.25 M and used for the resuspension of the pellet.

C) SEPARATION OF SUBMICROSOMAL FRACTIONS BY DOC TREATMENT: To separate membrane components from ribosomes and microsomal contents, both rough and smooth microsomes were treated with low concentrations of DOC by a modification[4] of the procedure of Ernster et al (28). The treated suspensions were centrifuged for 4 hr at 105,000 *g* to yield tightly packed pellets consisting of ribosomes and membranes, and supernates which represent the microsomal contents together with an undetermined amount of "solubilized" or no longer sedimentable membrane components. The pellets were rinsed 3 times with 0.25 M sucrose, resuspended in the same solution, and treated with 0.5% DOC (final concentration). The clarified suspensions were recentrifuged at 105,000 *g* for 2 hr to yield ribosome pellets and supernates containing the remaining membrane components in "soluble" form. For convenience, the supernates thus prepared from rough and smooth microsomes will be referred to as "rough" and "smooth membranes," respectively.

Chromatography of Phospholipids and Fatty Acid Analyses

Lipids were extracted from TCA-precipitated, rather than from fresh, microsomal fractions. We

[3] This fluffy layer appears as a double band, of which the upper part is red and the lower is white; these two parts can be separated in the presence of $MgCl_2$ (29), but in the experiments here reported the double band was investigated as a whole.

[4] We could not obtain the type of separation described by Ernster et al. (28), presumably on account of the large amount of glycogen present in our preparations.

realize that this procedure can cause hydrolysis of certain phospholipids, especially plasmalogens, but we have used it because it allows the thorough washing needed for our ^{32}P-incorporation experiments, and because it is known that the plasmalogen content of liver microsomes is negligible (30). The low amounts of lysophosphoinositides and phosphatidic acid found in our extracts (*see* Table I) indicate that breakdown of phospholipids is limited, if present, and thus justify the use of our procedure.

Microsomes (330 mg tissue equivalent) resuspended in 0.25 M sucrose were precipitated with 5% cold TCA, washed twice with 5% TCA and once with distilled water (recentrifuging each time), and extracted with 4 ml of chloroform-ethanol (1:1) overnight in the cold room in a nitrogen atmosphere. The chloroform contained DL-α tocopherol (Sigma) ($\sim$10 mg/ml chloroform) as an antioxidant; under these conditions 90% recovery of phospholipids is possible by chromatography. In air and without an antioxidant, the heme compounds present in the microsomes initiate lipid peroxidation and thereby prevent satisfactory recovery of phospholipids. The protein in the chloroform-ethanol extract was removed by washing with 0.1 N HCl (5 ml per 4 ml extract) (31), and the protein-free extract was chromatographed on silicic acid–impregnated paper according to Marinetti (32, 33). A 0.1 ml extract sample was evaporated under nitrogen, redissolved in chloroform, and placed on the paper as one spot. Diisobutylketone-acetic acid–0.9% NaCl (40:25:5) was used as the solvent system. The development at room temperature required 30 hr. Detection of phosphatides was made by staining with Rhodamine 6G. The phosphatides were eluted with methanol-HCl (34, 35), and the amounts of phosphorus were measured according to Marinetti (32). The phospholipids were characterized by two-dimensional chromatography of the eluted samples, after deacylation, according to Dawson (36).

For fatty acid analysis, phospholipids were extracted as described above and the extracts passed through a silicic acid column according to Borgström (37) to remove the small amount of neutral lipids usually present in microsomes. After methylation (38) the fatty acids were determined by gas-liquid chromatography (39).

Incorporation Experiments

In experiments with P^{32}, the radioactive solution was injected intraperitoneally (0.5 mc/100 g), and the livers were removed and fractionated after 3 hr. After chromatography of microsomal extracts, radioautography of chromatograms was performed by using Kodak nonscreen X-ray film, exposed for about 2 days ($\sim$1 day per 10,000 CPM in the original spot). To detect less radioactive lipids, the chromatogram were also stained with Rhodamine. The radioactive and Rhodamine-stained spots were cut out, placed on planchettes, and counted in a Geiger-Muller gas flow counter. Radioactivity was corrected for decay. In control experiments, incorporation into individual phosphatides was found to be linear up to 6 hr.

DL-leucine-1-C^{14} in isotonic NaCl solution was injected intraperitoneally (15 μc/100 g). The livers were removed and fractionated at selected time points. The 0.5% DOC-soluble fractions (rough and smooth membranes) were precipitated with 12% TCA, and phospholipid and RNA were removed from the precipitate by the procedure of Siekevitz (40). The final pellets were dissolved in 88% formic acid, and the solution plated and counted in a Geiger-Muller gas flow counter.

Glycerol-1,3-C^{14} in isotonic NaCl solution was injected intraperitoneally (15 μc/100 g). Total lipids

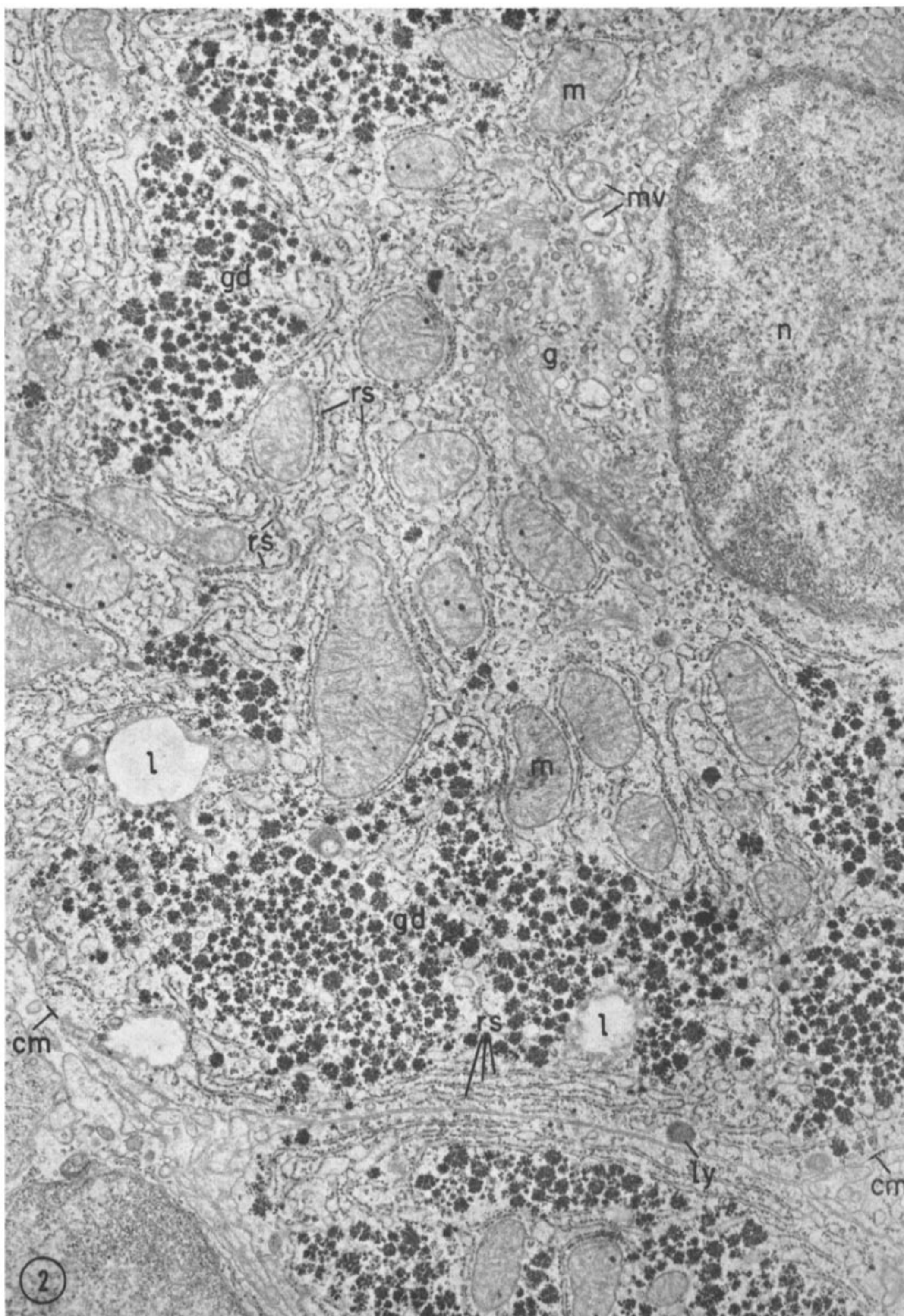
FIGURE 2 Hepatocyte at 3 days before birth (−3 days).

The cytoplasm contains a relatively well developed ER with numerous rough-surfaced cisternae (*rs*) (some of them in parallel arrays), few smooth-surfaced vesicular elements, and a voluminous Golgi complex (*g*).

The glycogen deposits (*gd*) consist primarily of aggregates of α particles (rosettes), penetrated by few ER elements both rough- and smooth-surfaced. Few glycogen particles appear individually scattered throughout the rest of the cytoplasm.

Numerous mitochondria (*m*), a few multivesicular bodies (*mv*) and lysosomelike bodies (*ly*), and a few (extracted) lipid droplets (*l*) complete the set of cytoplasmic components.

The cell membrane is marked *cm* and the nucleus *n*. Note the unusual, central, juxtanuclear position of the Golgi complex, and the absence of dense granules in the Golgi cisternae; together with the scarcity of microbodies, they are characteristic features of this early stage in hepatocyte differentiation. Another hepatocyte appears in the lower right corner and a reticuloendothelial cell in the lower left one. × 11,000.


m
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2

were extracted from rough and smooth microsomes and HCl-washed as described above. The lipids in chloroform were plated directly and counted as in the leucine-C^{14} experiments.

Chemical Analyses

The various microsomal fractions and subfractions were precipitated with 12% TCA and washed twice with 5% cold TCA. Separate aliquots were used for RNA, phospholipid, and protein N determinations. Protein N was determined by nesslerization of Kjeldahl digests of fractions from which RNA and phospholipids had previously been removed (41). The values were multiplied by 6.25 to obtain the amount of protein. RNA was obtained by extracting for 20 min with hot (90°) TCA pellets previously washed first with cold TCA and then with cold distilled water to remove beforehand glycogen and sucrose. The Mejbaum orcinol method was used to measure RNA (42), with a purified yeast RNA as a standard. Phospholipids were extracted as described above, and the phosphorus was measured according to Marinetti (32). The amount of phospholipid was obtained by multiplying the phosphorus values by 25.

RESULTS

A. Structural Changes during Hepatocyte Differentiation

The following description applies primarily to those subcellular components of hepatocytes directly related to the problem under study, i.e., endoplasmic reticulum and deposits of particulate glycogen. Details concerning other cytoplasmic structures can be found in the legends of Figs. 2 to 9.

At 3 days before birth, the hepatocytes represent only one-third to one-half of the total cell population of the liver, the balance being comprised mainly of cells of the erythropoietic series which range from erythroblasts to reticulocytes. The hepatocytes are small (diameter $\simeq$15 μ) and are characterized by a still incompletely developed ER and by relatively large glycogen deposits (Fig. 2). The ER consists primarily of randomly disposed, rough surfaced, tubular, and cisternal elements whose cavities appear moderately distended and filled with an amorphous or finely fibrillar material of low density. The attached ribosomes are disposed in usual patterns (i.e., rosettes, spirals, double rows) tightly packed at small intervals (up to $\sim$500 A) from one group to another.[5] The surrounding cytoplasmic matrix contains a large population of free ribosomes, most of them disposed in clusters. Smooth-surfaced elements of the ER occur only in small numbers in the form of randomly scattered vesicles, but the Golgi complex is well developed and appears comparable in size and complexity to that seen in fully differentiated hepatocytes. Part of the smooth-surfaced vesicles, and most of the vacuoles and cisternae of the Golgi complex contain a material similar in texture and density to that found in the rough-surfaced cisternae of the ER. Most glycogen particles are of α type (diameter $\simeq$200 mμ) and occur in large, irregular aggregates within which elements of the ER, both rough- and smooth-surfaced, are only occasionally encountered. In addition, a few glycogen particles appear individually scattered throughout the cytoplasmic matrix.

[5] The spacing of ribosomes, center to center, in a group or pattern (polysomes?) is generally constant at $\sim$250 A. The spacing among groups is variable.

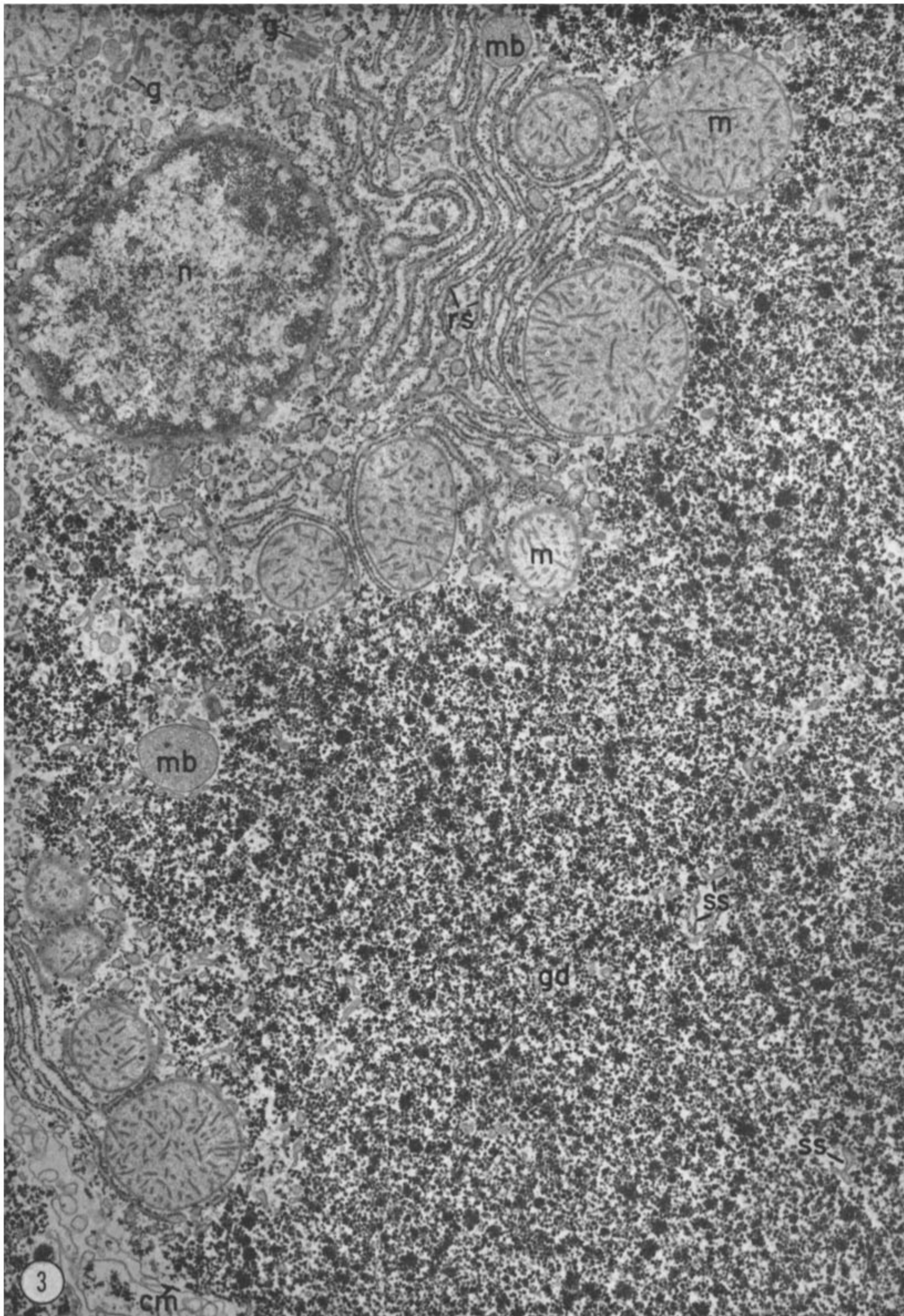
FIGURE 3 Hepatocyte at birth.

The ER consists predominantly of rough-surfaced cisternae (*rs*), disposed in parallel arrays and partially distended by a content of moderate density. Smooth-surfaced elements (*ss*) appear in small numbers at the periphery of, and scattered throughout, glycogen deposits (*gd*). Part of a Golgi complex is seen at *g*.

A large continuous glycogen deposit (*gd*), which consists of numerous β particles and a few α particles, occupies practically the entire lower half of the figure.

The relatively vast size, the displacement of all other cell components, the predominance of β particles, and the scarcity of associated ER elements characterize glycogen deposits at this age.

In addition, the cytoplasm contains numerous, rounded, apparently swollen mitochondria (*m*) and a few microbodies (*mb*). The number of microbodies increases sharply around this age. The cell membrane is marked *cm*, and a grazing section through the nucleus, *n*. $\times$ 10,000.


g
g
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gd
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3
cm

At birth, the situation is greatly changed. The cell population becomes more homogeneous because of a sudden decrease in the number of erythroblasts and cells derived therefrom, and of a rapid increase in the number and size of hepatocytes (diameter $\simeq 25\ \mu$). The ER is greatly enlarged (Fig. 3). Its elements are still predominantly rough surfaced, and frequently appear disposed parallel to one another in more or less elaborate arrays. There is little change in the frequency and appearance of the smooth-surfaced elements of the ER, Golgi complex included, but the cavities of the entire system are markedly distended and generally filled with a material similar in texture to, but slightly denser than, that seen in the same location at -3 days.[6] The attached ribosomes[7] are increased in number and, as a result, the spacing among ribosome groups (patterns) is greatly reduced ($\simeq 350$ A) (Figs. 3 and 5). In many places, the membrane of the ER appears covered by a continuous layer of tightly packed ribosomes. The glycogen deposits are strikingly increased in size and number, consist of mixtures of α and β (diameter $\simeq 300$ A) particles, and occupy large regions in the cytoplasm from which most other components seem to be crowded away (Figs. 3 and 9). The number of smooth- and rough-surfaced elements of the ER included in the glycogen masses is even smaller than at -3 days.

At 3 days after birth, the cell population of the liver is comparable to that seen in the adult and the hepatocytes have almost reached the appearance of fully differentiated cells. Their size has decreased, presumably as a result of a drastic reduction in the size and number of their glycogen deposits (Fig. 4). The rough-surfaced elements of the ER are disposed with less order than at birth and their attached ribosomes, still arranged in characteristic patterns, appear much more loosely packed (Figs. 4 and 6). Ribosome groups are now separated by relatively large stretches (up to ~ 1500 A) entirely free of attached particles. The smooth-surfaced elements of the ER appear greatly increased in number, are predominantly tubular, and form tightly meshed tridimensional networks. As in the hepatocytes of the adult, the meshes of these networks are occupied by glycogen particles mostly of the α type (Fig. 7). Thus the typical relationship found in the fully differentiated hepatocyte between glycogen deposits and smooth surfaced ER (44) is already established at $+3$ days. Images which suggest that between $+1$ day and $+3$ days the glycogen deposits are surrounded and penetrated (Fig. 8) by smooth-surfaced ER are frequently encountered. The new situation is in contrast with that prevailing in the compact glycogen deposits present at birth (Fig. 9). The Golgi complexes show little change (not illustrated), but their amorphous content, like that of the rest of the ER, has disappeared to be replaced only in the vacuoles and dilated cisternae of the complex by the aggregates of dense granules (diameter $\simeq 500$ A) that constitute the characteristic content of the Golgi complex in the hepatocytes of the adult. In addition to a still large population of free ribosomes, the cytoplasmic matrix now contains numerous, large lipid droplets.

[6] The days before birth are designated from -3 to -1, and after birth from $+1$ to $+8$.

[7] The ribosomes are attached to the ER membrane by their large subunit, with their groove parallel to the membrane, as already shown in adult liver (43).

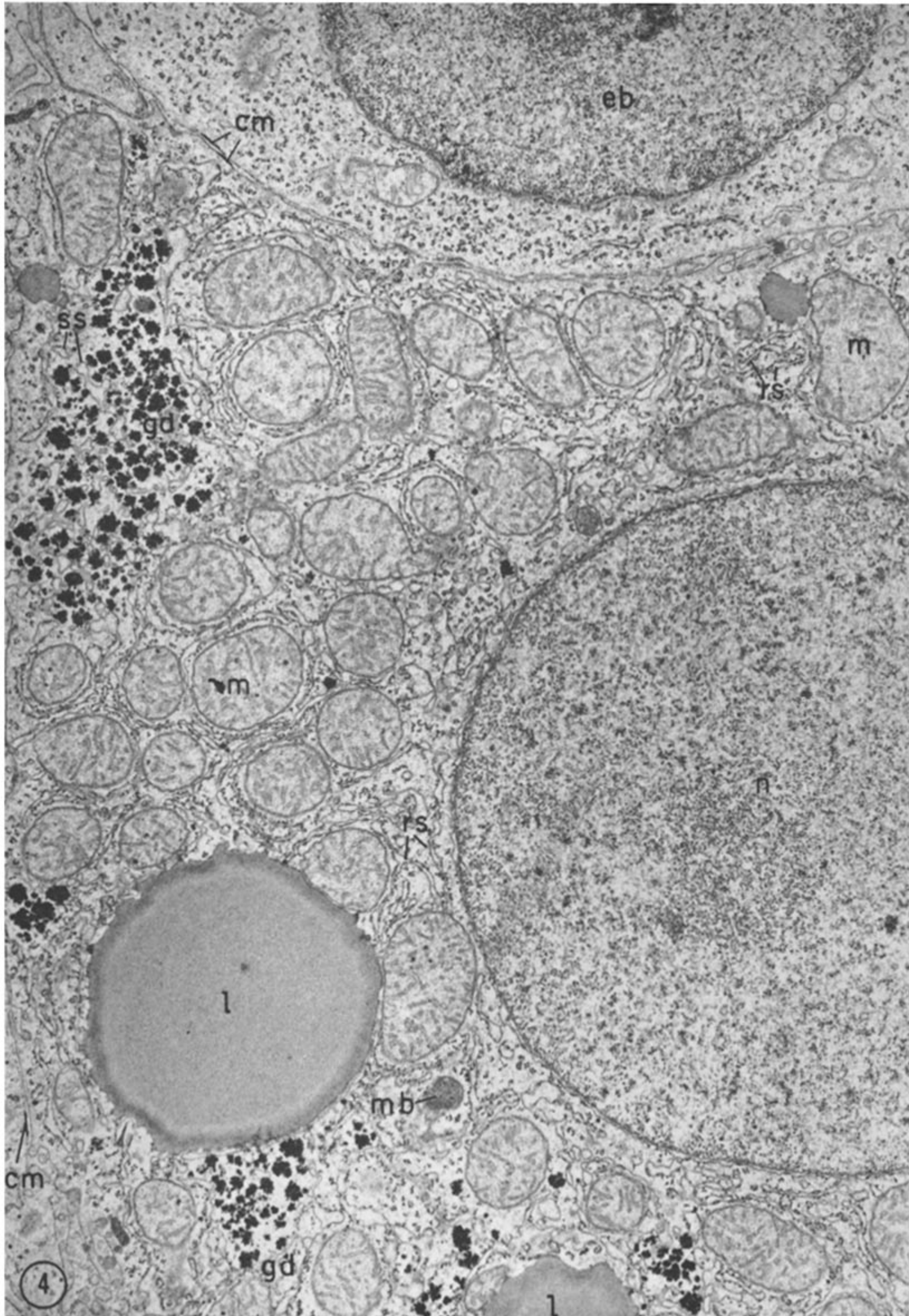
FIGURE 4 Hepatocyte 3 days after birth.

The rough-surfaced cisternae (*rs*) of the ER are slightly distended and have lost their moderately dense content and their arrangement in parallel arrays. Their attached ribosomes are less numerous than at birth (*See* Fig. 3). Smooth-surfaced elements (*ss*) are concentrated around and within the glycogen deposits (*gd*) which are greatly reduced in size and again consist mainly of α particles.

Mitochondrial profiles (*m*) have decreased in size but increased in numbers. Numerous, usually large, lipid droplets (*l*) occur in the cytoplasmic matrix.

At this age, the population of microbodies decreases, that of the lysosomes increases, and the cisternae of the Golgi complex acquire their content of dense granules which characterizes the fully differentiated hepatocyte.

The nucleus is marked *n*, the cell membrane *cm*, and an adjacent erythroblast *eb*. $\times$ 11,000.


eb
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B. Morphology of Cell Fractions

Since the cell population of the liver is both heterogeneous and rapidly changing during the period examined, it was of interest to find out to what extent these conditions are reflected in the cell fractions used in the present investigation. To this intent, pellets of the 10,000 *g* fraction and of the microsomal fraction were systematically examined.

10,000-G PELLETS: At −3 days, this fraction consisted mainly of intact or slightly damaged cells of the erythropoietic series and contained, in addition, a few hepatocyte debris, many hepatocytic nuclei (mostly damaged), many mitochondria, and a certain amount of microsomes.

At +1 day, intact erythroblasts and reticulocytes were still present but in greatly reduced numbers, the main constituents of the pellets being damaged hepatocytic nuclei, mitochondria, and a sizable amount of microsomes, primarily rough surfaced.

At +3 days, the composition of the pellets was comparable to that seen at +1 day, except that the erythroblasts and reticulocytes were only occasionally encountered.

MICROSOMAL PELLETS (TOTAL MICROSOMES): At −3 days, the fraction was comprised of rough-surfaced vesicles, few smooth-surfaced elements, and a large amount of free ribosomes.

At +1 and +3 days, there was a progressive decrease in the free ribosome content of the fraction, and a concomitant increase in microsomal vesicles of both rough- and smooth-surfaced varieties. Contamination by mitochondria and lysosomes was negligible, and contamination by reticulocytic or nuclear fragments was absent at all time points investigated.

Since cells of the erythrocytic series appear intact or only slightly damaged in the 10,000 *g* pellets, and since nuclei and cytoplasmic fragments derived from such cells are not found in any of the pellets examined, it can be concluded, in agreement with Oliver et al. (45), that these cells are not disrupted during homogenization. Hence, the microsomal fractions studied represent mainly the hepatocytic contingent of the liver cell population. In addition, it should be noted that all our microsomal fractions are incomplete, since a sizable proportion of microsomes, primarily of the rough-surfaced variety, is centrifuged down at the preliminary centrifugation in the 10,000 *g* pellet.

ROUGH MICROSOMES: The corresponding pellets consist primarily of microsomal vesicles with attached ribsomes. Golgi cisternae and vacuoles filled with a dense homogeneous material (up to +1 day) represent the only contaminant that occurs with some frequency. At the top of the pellets there is a thin layer of free ribosomes.

SMOOTH MICROSOMES: The pellets are comprised mainly of smooth vesicles of various sizes and of smooth membrane fragments. They contain also, especially in their lower half, a noticeable amount of free ribosomes.

C. Chemistry

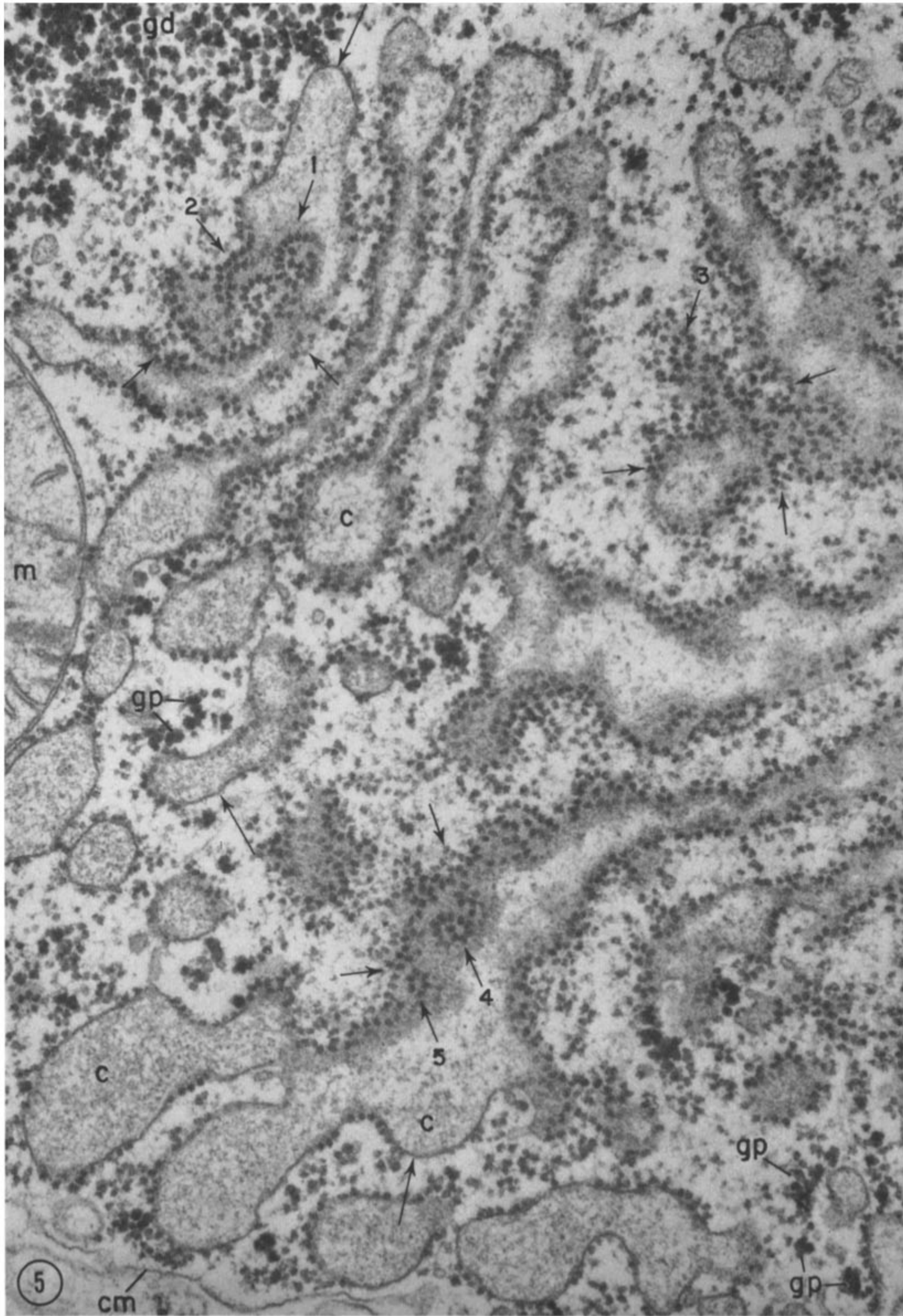
1. TOTAL LIVER RNA, PROTEIN, AND PHOSPHOLIPIDS: The protein content of rat liver is 80 to 90 mg/g wet weight liver before birth, and 110 to 140 mg/g in the newborn and adult. The RNA content is highest at −3 days (about 13 to 14 mg/g liver), which is the earliest time point in our series, and gradually decreases to about 8 mg/g liver in the adult. The phospholipid content of the liver is low before birth (12 to 13 mg/g) and rises rapidly after birth to 20 to 25 mg/g.

2. TOTAL MICROSOMES: Our data concerning the gross chemistry of the total microsomal

FIGURE 5 Hepatocyte at birth.

Group of rough-surfaced ER cisternae whose limiting membranes are cut normally in a few places (long arrows) and obliquely in the rest of the field. Where the section grazes the surface of the cisternae (short arrows), it reveals the high frequency of attached ribosomes, the characteristic patterns they form—e.g. rosettes (*1*), double rows (*2*), loops (*3*), spirals (*4*), and circles (*5*)—as well as the short distances separating the patterns from one another. Ribosome-free membrane stretches appear only at the margins of some cisternae. The cisternal space is generally distended and shows a content of moderate density (*c*).

The cell membrane can be seen at *cm*, a mitochondrion at *m*, the periphery of a glycogen deposit at *gd*, and scattered glycogen particles (mostly β particles or small clusters thereof) at *gp*. × 46,000.


gd
1
2
3
c
m
gp
4
5
c
c
gp
gp
5
cm

fractions must be interpreted with the following reservations in mind: (*a*) The fractions are incomplete, i.e., they do not represent the whole ER of the liver cells. We lose a large number of ER elements, primarily rough- surfaced (~50%), especially in the case of +1-day-old animals because we have increased the centrifugal field of the first separation to avoid the contamination of microsomes by other membrane-containing subcellular components (mitochondria, microbodies, lysosomes). (*b*) The microsome losses to the 10,000 *g* pellet[8] are greater in the presence of glycogen, and the liver of the fetus and the newborn contains large and variable amounts of particulate glycogen which, for obvious reasons, cannot be eliminated or reduced.

Hence, we are obliged to disregard the absolute amounts found in our fractions and to rely primarily on relative values. The latter show that the RNA/protein ratio decreases as a function of age, while the PLP/protein ratio increases concomitantly in both homogenates (Fig. 10 *A*) and microsomes (Fig. 10 *B*).

3. MICROSOMAL PHOSPHOLIPIDS: Since our morphological observations show a rapid production of rough and especially smooth membranes in the hepatocyte of the newborn, experiments were carried out to find out whether the newly produced membranes have a phospholipid composition different from that of those already present, and whether the rates of labeling of various phosphatides change as a function of age during the period of rapid development of the ER. Since we cannot separate "new" from "old" membranes, we must rely on data obtained on mixtures of the two. We assume, however, that differences between the two kinds of membranes would be detected, if present, by comparing "old" membranes to mixtures of "old" and "new" ones, because of the rapid substantial increase of ER membrane already mentioned that occurs during this period.

The distribution of individual phosphatides in the total hepatic microsomes of the newborn, 2-day-old and adult rats is shown in Table I. The data show an increase in phospholipid amount with age, but the significance of this finding is difficult to assess, because of the already mentioned incomplete and variable recovery of microsomes. The results show, however, that no major differences occur among the microsomal membranes of 2-hr, 2-day, and 90-day-old rats, as far as types and relative amounts of phosphatides are concerned. The small variations present are within the accuracy of the method used, since the experiments were performed four times with variations of from 2 to 6% in the various phospholipids, and since the recoveries of the separated phospholipids were satisfactory.

Incorporation of P^{32} into total and individual phosphatides extracted from total microsomal fractions prepared 3 hr after the intraperitoneal injection of the tracer is shown in Table II. Fig. 11 shows a radioautogram of P^{32}- labeled microsomal phospholipids; the chromatogram was also stained with Rhodamine 6G to detect weakly labeled components. Again, while the specific activity of the total microsomal phospholipids in newborn and 2-day-old rats is higher than in the adult, the per cent recovery of radioactivity in individual phosphatides is similar in all three instances. Again, experiments were performed three times, with but little variations. Hence, the relative rates of incorporation into various phopholipids do not vary with age, except perhaps for a decrease in sphingomyelin labeling and an increase in phosphatidylethanolamine labeling. The data indicate that, during this period of rapid membrane synthesis

[8] Similar losses of liver microsomes of fetal and newborn rats to the low speed pellet have also been observed by Chatterjee et al. (60).

FIGURE 6 Hepatocyte 3 days after birth.

The rough-surfaced cisternae of the ER have fewer attached ribosomes than at birth, as indicated by the long spacings in between ribosome rows in normal or oblique sections (long arrows), and by the relative scarcity of ribosome patterns in grazing sections (short arrows) of the cisternal membranes.

The cisternal space contains a few intracisternal granules (*cg*), similar in appearance to those seen at this age in the smooth-surfaced ER and in the vascular spaces.

The field comprises, in addition, a microbody (*mb*) and a number of mitochondrial profiles (*m*), one of which represents a bending and branching mitochondrion (m_1). The cell membrane can be seen at *cm*. × 46,000.

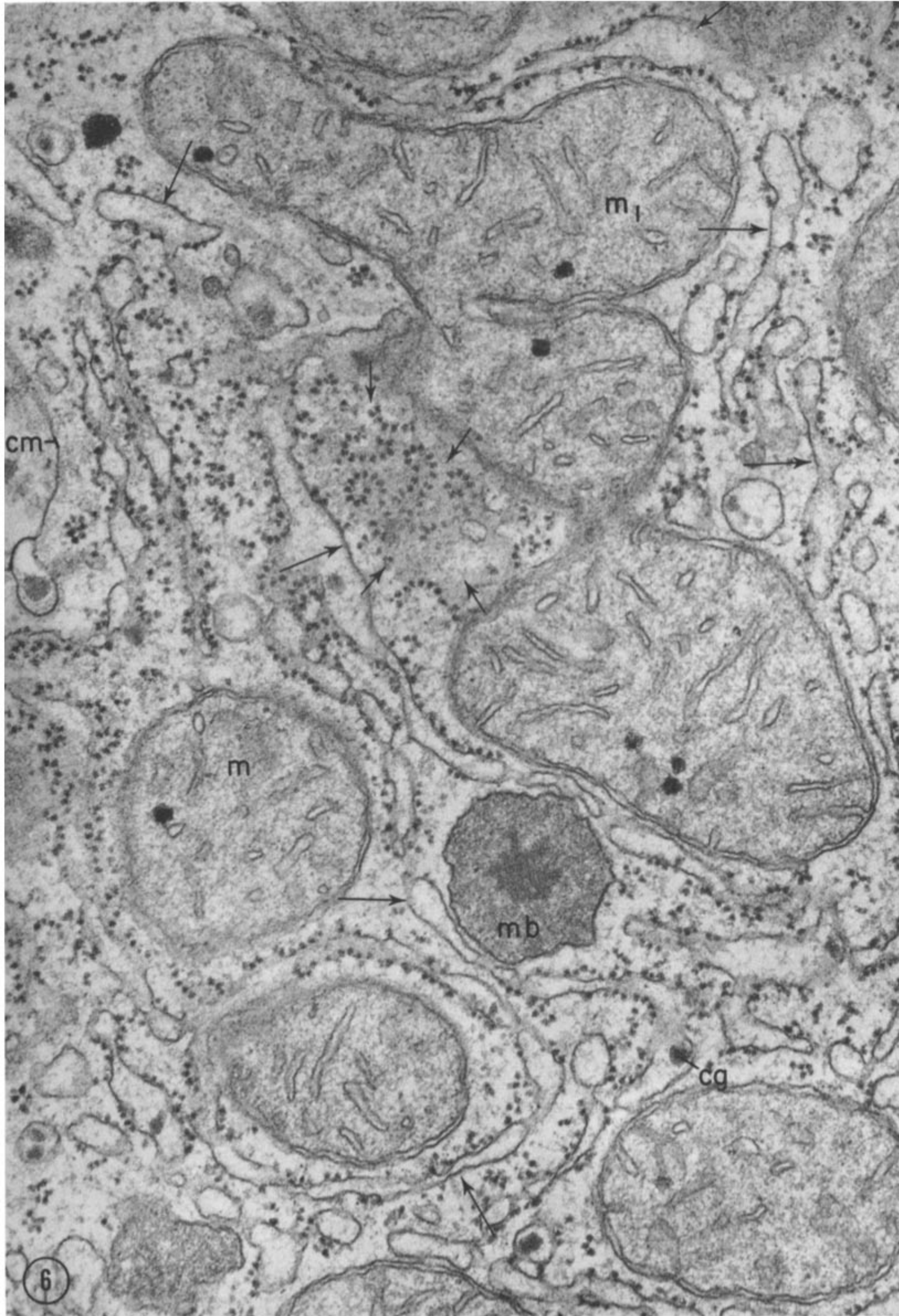

m1
cm
m
mb
cg
6

TABLE I

Phosphatide Composition of Hepatic Microsomal Membranes as a Function of Age in Rats

Abbreviations as in Fig. 11. Representative experiment from a series of four. Variations from one experiment to another were within 6%.

Age of rats:	2 hr		2 days		Adult	
	μg PLP-P / g liver	% of total	μg PLP-P / g liver	% of total	μg PLP-P / g liver	% of total
Total	97.5	100	155.0	100	196.0	100
PE	17.5	17.9	23.7	15.3	29.8	15.2
PS	8.7	8.9	19.7	12.7	15.3	7.8
PC	46.6	47.8	61.6	39.8	87.3	44.6
SPM	8.5	8.7	18.6	12.0	19.2	9.8
PI*	5.9	6.1	13.3	8.6	15.7	8.0
LPI	2.5	2.6	5.9	3.8	7.4	3.8
PA	1.8	1.8	1.9	1.2	3.1	1.6
Recovery	91.5	93.8	144.7	93.4	177.8	90.8

* The content of the PI area of the chromatogram is heterogeneous: it comprises mono-, di-, and triphosphoinositides.

there is no great preferential labeling of any one phospholipid. A comparison of Tables I and II shows that the percentage of radioactivity found in the individual phosphatides is very close to the percentage amounts of these phosphatides in the lipid fraction, except for phosphatidylethanolamine which seems to be synthesized or turned over more rapidly than the others.

4. FATTY ACIDS OF TOTAL PHOSPHOLIPIDS: The fatty acid composition of the extracted, washed, neutral fat-free phospholipid fractions from total hepatic microsomes of 0-, 5- and 90-day-old rats was determined (Table III), taking particular care to avoid peroxidation by using a nitrogen atmosphere throughout the whole procedure. The distribution pattern varies during development for almost every fatty acid examined. Some fatty acids increase, others decrease, while still others show transient drops or peaks, in their per cent distribution in 5-day-old rats.

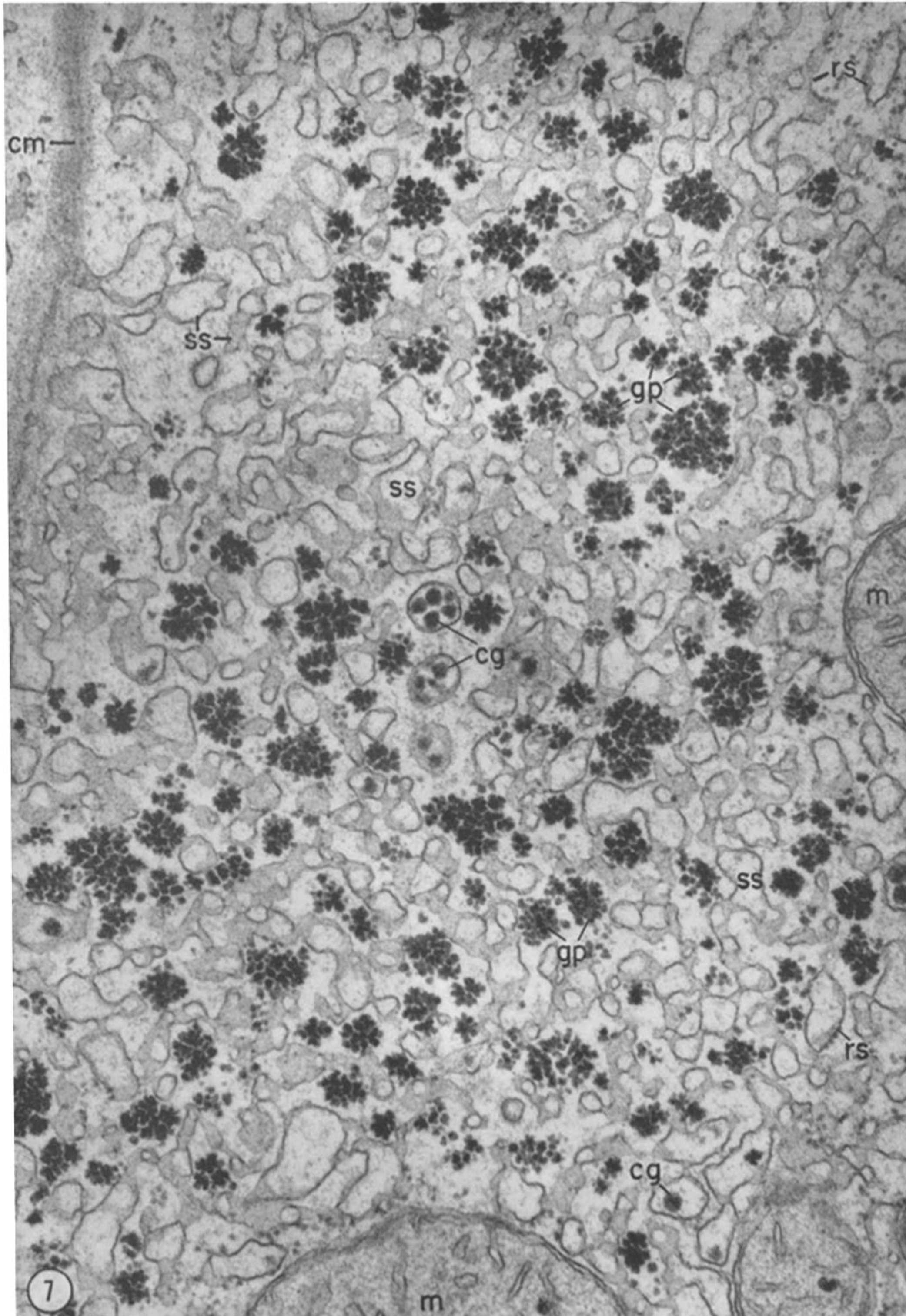
5. INCORPORATION OF LEUCINE-C^{14} INTO ROUGH AND SMOOTH MICROSOMAL MEMBRANES: The rapid production of ER membranes during the period investigated raises such questions as whether there are specific sites of membrane synthesis in the system and whether protein and lipid syntheses are coordinated or not. To get information on these questions, the incorporation of leucine-C^{14} and glycerol-C^{14} into protein and lipid components of membranes derived from rough and smooth microsomes was comparatively studied. Prepared as indicated under Methods, these membranes are free of intravesicular and absorbed proteins, as well as of ribosomal proteins.

When leucine-C^{14} is injected into 2-hr-old rats and rough and smooth membranes are prepared

FIGURE 7 Hepatocyte 3 days after birth.

The field shows a glycogen deposit which consists mainly of α particles (*gp*) and is already extensively penetrated by elements of the smooth-surfaced ER (*ss*). The latter form throughout the glycogen mass a randomly disposed, tridimensional network, so finely meshed that practically every glycogen particle is surrounded by ER elements. This is the type of relationship usually found in fully differentiated hepatocytes.

Some ER elements contain intracisternal granules (*cg*) of a type already mentioned. Rough-surfaced ER elements (*rs*) and mitochondria (*m*) appear at the periphery of the field. The obliquely cut cell membrane is seen at *cm*. × 48,000.



cm
rs
ss
gp
ss
m
cg
cg
ss
gp
rs
cg
7
m

TABLE II

Incorporation of P^{32} into the Individual Phosphatides of Developing Hepatic Microsomal Membranes

Abbreviations as in Fig. 11. Representative experiment from a series of three. Livers were removed 3 hr after intraperitoneal injection of $^{32}PO_4$.

Age of rats*:	2 hr		2 days		Adult	
	CPM / μg PLP-P	% of total radioactivity	CPM / μg PLP-P	% of total radioactivity	CPM / μg PLP-P	% of total radioactivity
Total	5,260	100	3,790	100	1,910	100
PE	7,890	25.7	6,440	23.5	5,070	35.0
PS	3,500	8.3	2,790	8.4	1,820	7.8
PC	6,320	38.5	4,470	42.5	1,570	31.8
SPH	3,380	8.0	2,570	7.3	920	4.1
PI	2,820	4.4	2,370	4.8	1,510	5.5
LPI	1,620	1.2	1,620	1.5	800	1.4
PA	1,060	0.5	870	0.3	980	0.7
Recovery		86.6		88.3		86.3

* At the time of injection.

TABLE III

Fatty Acid Composition of Microsomal Phospholipids as a Function of Age of Rat

Phospholipids were extracted as described in Methods; the extracts from 10 to 14 livers were pooled in each case and fatty acids determined as described in Methods.

Type of fatty acid	% of total fatty acids		
	0 day	5 days	90 days
16:0	23.9	24.8	10.6
16:1	4.8	1.0	0.3
18:0	17.7	18.9	22.1
18:1	17.1	5.0	13.9
18:2	7.1	6.4	23.5
20:4	15.6	22.9	17.4
22:6	13.7	21.1	12.1

after 30 min in vivo incorporation, both types of membrane are found to contain high radioactivity in their proteins, but the specific radioactivity of the rough membranes is about twice that of the smooth ones (Fig. 12 *A*). At 4 hr after the injection of the tracer, when peak incorporation of total counts occurs, this ratio has declined to ~1.3. After 4 hr, the radioactivity decreases more rapidly in the rough than in the smooth membranes, and after 8 to 10 hr the situation is reversed: the specific radioactivity of the smooth membranes is higher than that of the rough ones. Thereafter, the radioactivity decreases slowly and in parallel in both types of membranes.

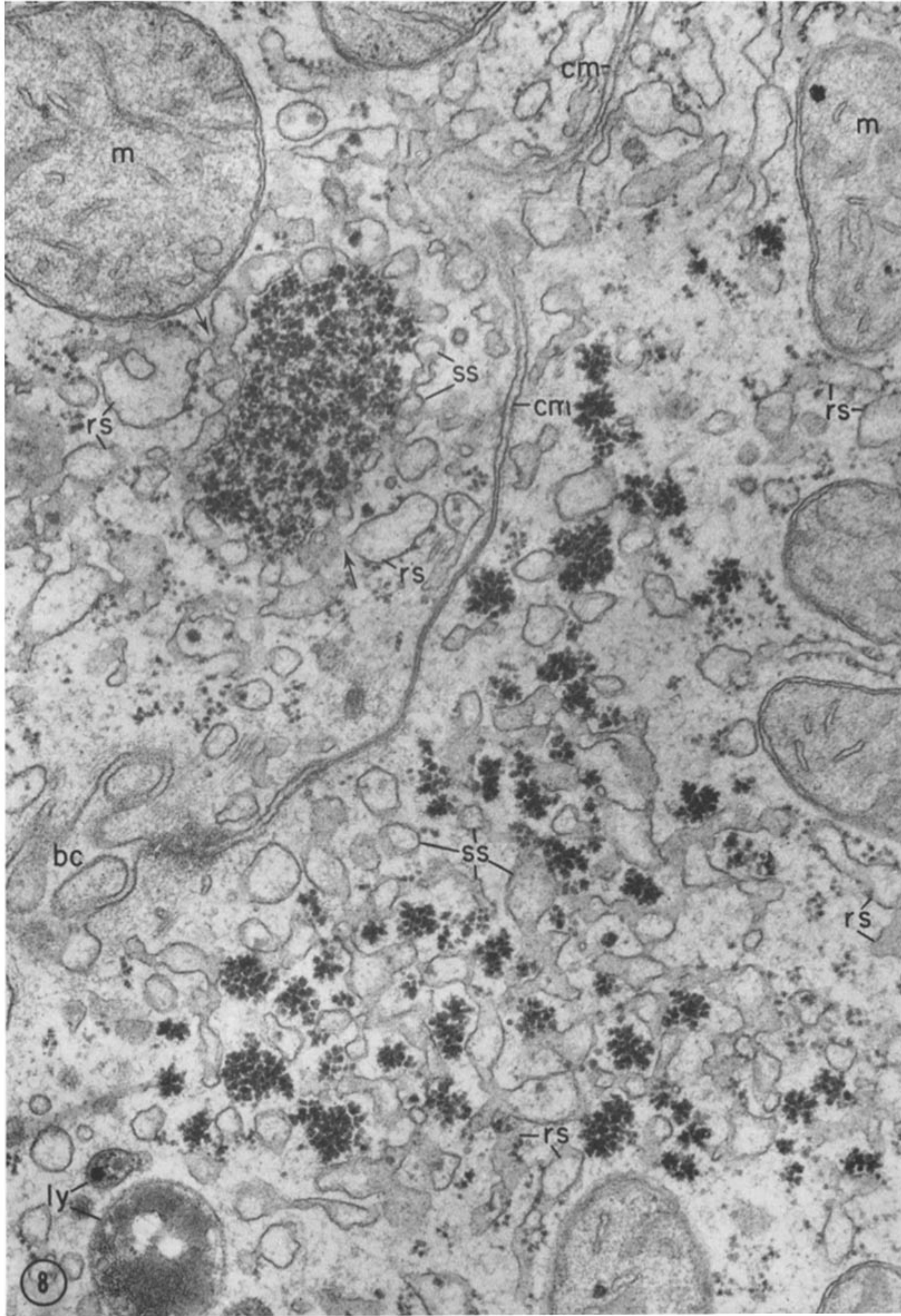
When the leucine-C^{14} incorporation experiments are repeated at the age of 18 hr instead of 2 hr (2 experiments with identical results), a similar picture emerges (Fig. 12 *B*) except that peak incor-

FIGURE 8 Glycogen deposits in two adjacent hepatocytes 3 days after birth.

The deposit in the upper left cell consists of β particles and is completely surrounded, but not penetrated, by smooth-surfaced elements (*ss*) of the ER. Points of continuity between this smooth-surfaced corona and adjacent rough-surfaced elements (*rs*) are marked by arrows.

The glycogen deposit in the lower right cell is comprised mainly of α particles and is extensively penetrated by a network of smooth-surfaced ER elements. Rough-surfaced elements (*rs*) bearing few attached ribosomes appear at the periphery of the field.

Mitochondria are marked *m*, lysosomes *ly*, the cell membrane *cm*, and a bile capillary *bc*. × 42,000.


cm
m
m
ss
rs
cm
rs
rs
bc
ss
rs
rs
ly
8

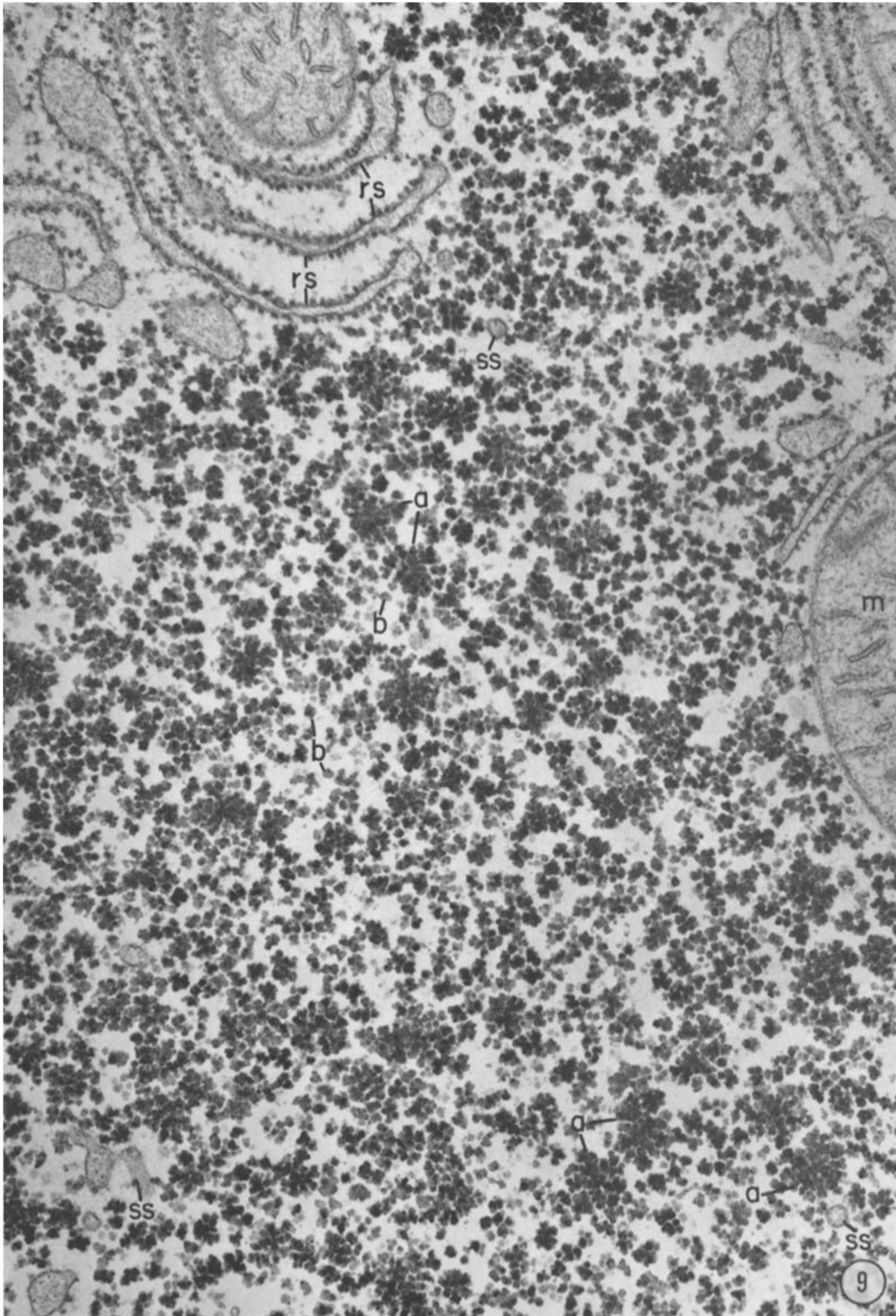


FIGURE 9 Hepatocyte at birth.

The field shows the periphery of a large glycogen deposit which consists of a mixture of α and β particles (*a* and *b*, respectively). There are very few smooth-surfaced ER elements (*ss*) within the deposit. Around it the cytoplasm contains mitochondria (*m*) and rough-surfaced cisternae (*rs*) of the ER. × 40,000.

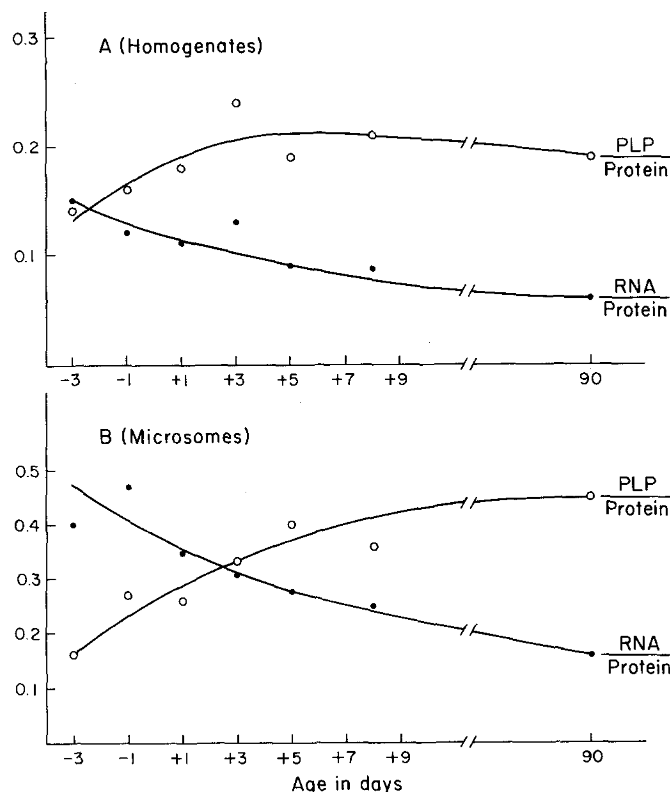


FIGURE 10 Phospholipid/protein (mg/mg) and RNA/protein (mg/mg) ratios of homogenates (*A*) and of microsomes (*B*) as a function of age.

poration and peak difference are reached sooner and that 4 to 10 hr after the administration of the isotope the differences in specific radioactivity between the proteins of rough and smooth membranes are no longer so evident as in the 2-hr-old rats. It should be noted that only the specific radioactivity ratios of the rough to smooth membranes are significant. The absolute incorporation values are meaningless, because the parent fractions of rough and smooth microsomes are incomplete, and because the recovery of membrane proteins after low DOC-treatment is only partial (see page 75).

6. INCORPORATION OF GLYCEROL-C^{14} INTO ROUGH AND SMOOTH MICROSOMES: It is generally believed that polar lipids play an important role as constituents in the formation of unit membranes (46). For this reason glycerol-C^{14} incorporation into the total extractable lipids of rough and smooth microsomes was followed. Since glycerol is found only in lipids and since the amount of neutral lipids in microsomes is small (47), it was assumed that glycerol can be used as a satisfactory label for membrane phospholipids. In addition, since the amount of lipid in the ribosomes and microsomal contents is negligible, it was thought unnecessary to isolate from rough and smooth microsomes the corresponding membrane fractions. Hence the experiments describe glycerol-C^{14} incorporation into the lipid portion of the membranes of total rough and total smooth microsomes.

At 14 and 45 min after injection, the specific radioactivity of the total lipids extracted from rough microsomes was considerably higher than the corresponding value for smooth microsomes (Fig. 13). At later time points, however, this difference disappeared and the radioactivity declined rapidly, suggesting a high rate of turnover.

The experiments with leucine-C^{14} and glycerol-C^{14} thus demonstrate a preferential early incorporation into the proteins and lipids of the membranes of rough microsomes, the in situ equivalents of the membranes of the rough ER.

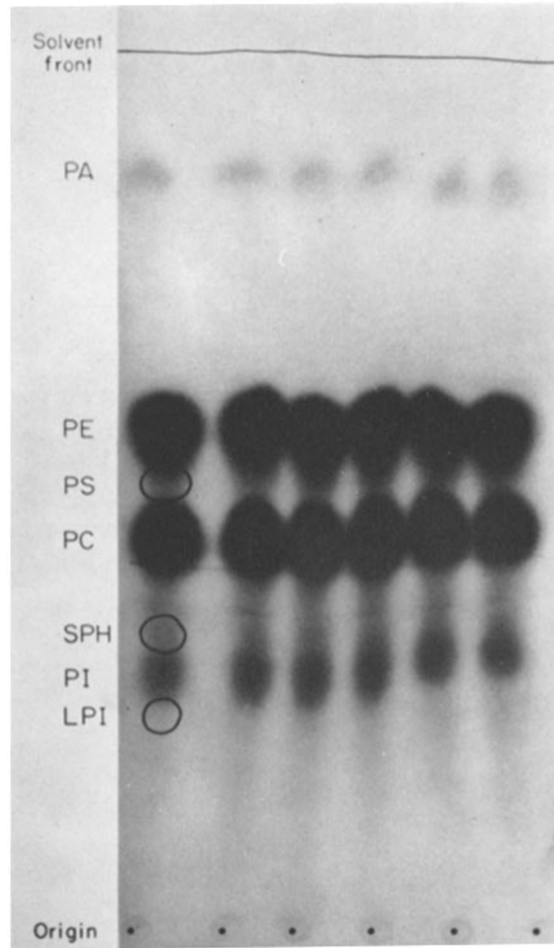


FIGURE 11 Radioautography of chromatograms of microsomal phospholipids (adult rat). Chromatography of P^{32}-labeled phospholipids was performed on silicic acid–impregnated paper as described under Methods. For radioautography, Kodak nonscreen X-ray film was exposed for 2 days to the chromatogram (each sample applied contained about 6,000 CPM, and 6 replicate samples were used in this experiment). The circles represent spots which exhibited low radioactivity but appeared in ultraviolet light after Rhodamine 6G staining. The following abbreviations are used: *PA,* phosphatidic acid; *PE,* phosphatidyl ethanolamine; *PS,* phosphatidyl serine; *PC,* phosphatidyl choline; *SPH,* sphingomyelin; *PI,* phosphatidyl inositol; *LPI,* lysophosphatidyl inositol.

Furthermore, experiments with leucine-C^{14} show that the ratio, specific radioactivity of the proteins in the smooth/rough membranes, increases continuously with time.

DISCUSSION

During the developmental stages we have studied, certain characteristic changes occur in the cytoplasm of the hepatocytes:

(*a*) The ER, which in the cells of the fetal liver consists mainly of rough-surfaced elements, increases in volume with time. The most pronounced proliferation occurs immediately after birth, and it is largely due to an increase in smooth-surfaced elements.

(*b*) Particulate glycogen increases rapidly in amount at the end of the fetal life, so that just before birth it occupies a large part of the cell volume. Fetal glycogen deposits differ from those seen in the hepatocytes of the adult; they are much larger and

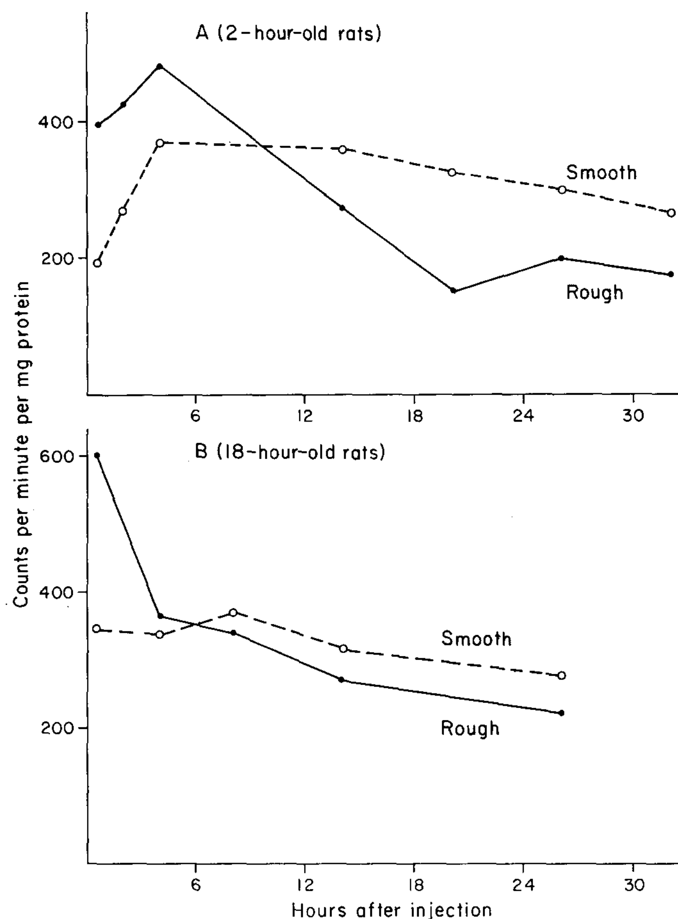


FIGURE 12 Incorporation of leucine-C^{14} into proteins of liver microsomal membranes of 2-hr-old (*A*) and 18-hr-old (*B*) rats.

contain only a few scattered smooth ER elements. After birth, most of the glycogen disappears and what remains becomes rapidly associated, as in the adult, with smooth ER, in this case newly formed. (*c*) The free ribosomes, which occur in large numbers in the cytoplasm of the fetal hepatocyte, decrease in frequency with time but, even at 3 days after birth, they remain more numerous than in the cytoplasm of the hepatocyte of the adult.

These changes are probably connected with the emergence of new hepatocyte functions, primarily control of glycemia, detoxication, and mass synthesis of proteins for export (plasma proteins). Evidence bearing on some of these aspects is presented and further discussed in the companion paper (25).

The results obtained in our cell fractionation experiments show that the microsomal fractions, isolated from fetal and newborn livers, represent mainly the hepatocytic contingent of the liver cell population. In agreement with previous findings (45), the fractions were not noticeably contaminated by contents of erythropoietic cells. Within the limitations already mentioned (see page 74), the properties of these microsomal fractions adequately reflect the morphogenetic events described above. The decreasing RNA/protein ratio and the increasing phospholipid/protein ratio can be correlated in time with the relative decrease of the rough-surfaced part of the ER, and the increase of the smooth-surfaced part of the system.

If we assume that all microsomal RNA is in ribosomes and that the latter have the same protein content as in adult liver, the amount of ribosomal proteins present in fetal microsomal fractions

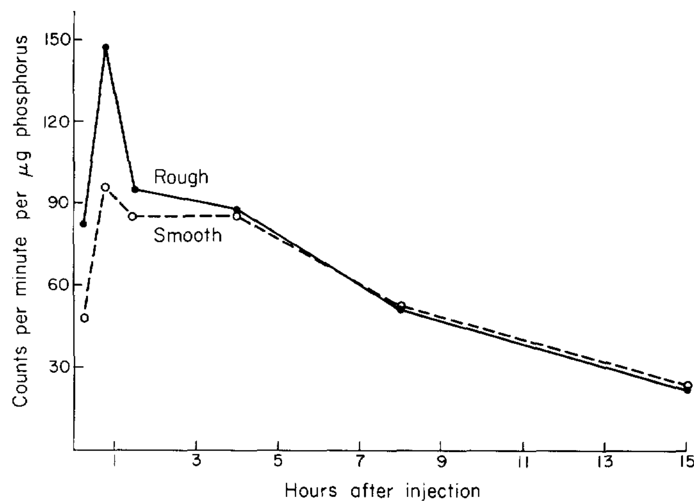


FIGURE 13 Incorporation of glycerol-C^{14} into total lipids of rough and smooth liver microsomes of 2-hr-old rats.

can be readily calculated. After correcting for ribosomal protein, the phospholipid/protein ratio of the microsomes is similar in fetal and adult livers. Hence, it follows that during the developmental period examined there are no quantitative differences in the amount of lipid in the ER (microsomal) membrane.

Our results further show that the phosphatide composition of the membrane and the relative rates at which new phosphatides are synthesized remain the same throughout the period of rapid ER development and are similar to the corresponding values for adult liver microsomes (*See* Tables I and II). Hence, as a first approximation, it appears unlikely that general or specific lipid deficiency is responsible for the low activity displayed by certain microsomal enzymes during ER development (25). Further work on this problem is presented and discussed in the companion paper (25). In other systems, it has been repeatedly shown that membrane-bound enzymes depend on phospholipids for full activity (48–50) and that certain phosphatides are required to restore activity after lipid extraction.

The stability of the phospholipid composition during ER development does not extend to the fatty acids of the phosphatides which vary greatly with age. These variations could be caused, however, by dietary changes inherent in the events studied (51–53); hence they may not be directly related to the differentiation process. Experiments reported in the companion paper (25) show, in fact, that the fatty acid composition of microsomal phospholipids can be modified by appropriate dietary changes, without significantly affecting the activity of the microsomal membrane-bound enzymes.

The results of our incorporation experiments lead to the conclusion that new membrane is formed from lipid and protein in the rough-surfaced ER and subsequently transferred to the smooth-surfaced part of the system. The protein components are probably synthesized by the attached ribosomes; the lipids may be synthesized elsewhere, but their interaction with proteins to produce membrane appears to take place in the rough ER.

Synthesis of membrane lipids and at least some membrane proteins seems to be linked probably through the assembly step. It has been shown in vitro that phospholipid synthesis is regulated by the amount of protein available for the binding of newly synthesized lipid; without protein in the system, phospholipid synthesis stops after a short initial period (54). The capacity of fetal liver to synthesize lipids is very high (55) and the products appear to be used exclusively for biogenesis of various membranes since they are not further metabolized (56). These data, together with our results, suggest that, during the formation of new ER membranes, the rate-limiting factor is not phosphatide synthesis, but the amount of membrane

protein available. Such a mechanism could explain the finding that newly synthesized ER (microsomal) membranes appear as finished structures as far as their quantitative and qualitative lipid composition is concerned.

The means by which newly formed membrane is transferred or converted from rough to smooth ER remain unknown, but we can envisage three different processes to account for the final results:

(*a*) Attached ribosomes detach from the ER membranes when the synthesis of the latter is completed. (Upon cell fractionation, the same membrane is recovered in the rough microsomes before ribosomal detachment and in the smooth microsomes after this event).

(*b*) New smooth membrane is formed between groups of attached ribosomes (polysomes) engaged in membrane protein synthesis, so that eventually large patches of smooth membrane appear between ribosomal clusters.

(*c*) Newly formed membrane "flows" from rough to smooth ER past the junction points between the two parts of the system.

The first two processes are compatible with the finding that the spacing among ribosomal groups (polysomes) increases coincidentally with rapid smooth membrane synthesis, but a choice between the 3 possibilities mentioned is not possible with the data now available. If hypothesis (*a*) or (*b*) applies, there must be subsequent redistribution of membrane within the system since, in the hepatocyte of the +3-day-old and especially in that of the adult animal, the rough and smooth parts of the ER, although continuous, are segregated in distinct masses in different regions of the cytoplasm (57–60). In the companion paper, these hypotheses will be further discussed in relation with the synthesis and transfer of membrane-bound microsomal enzymes during the same developmental period.

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