ISOLATION OF CENTROLOBULAR AND PERILOBULAR HEPATOCYTES AFTER PHENOBARBITAL TREATMENT

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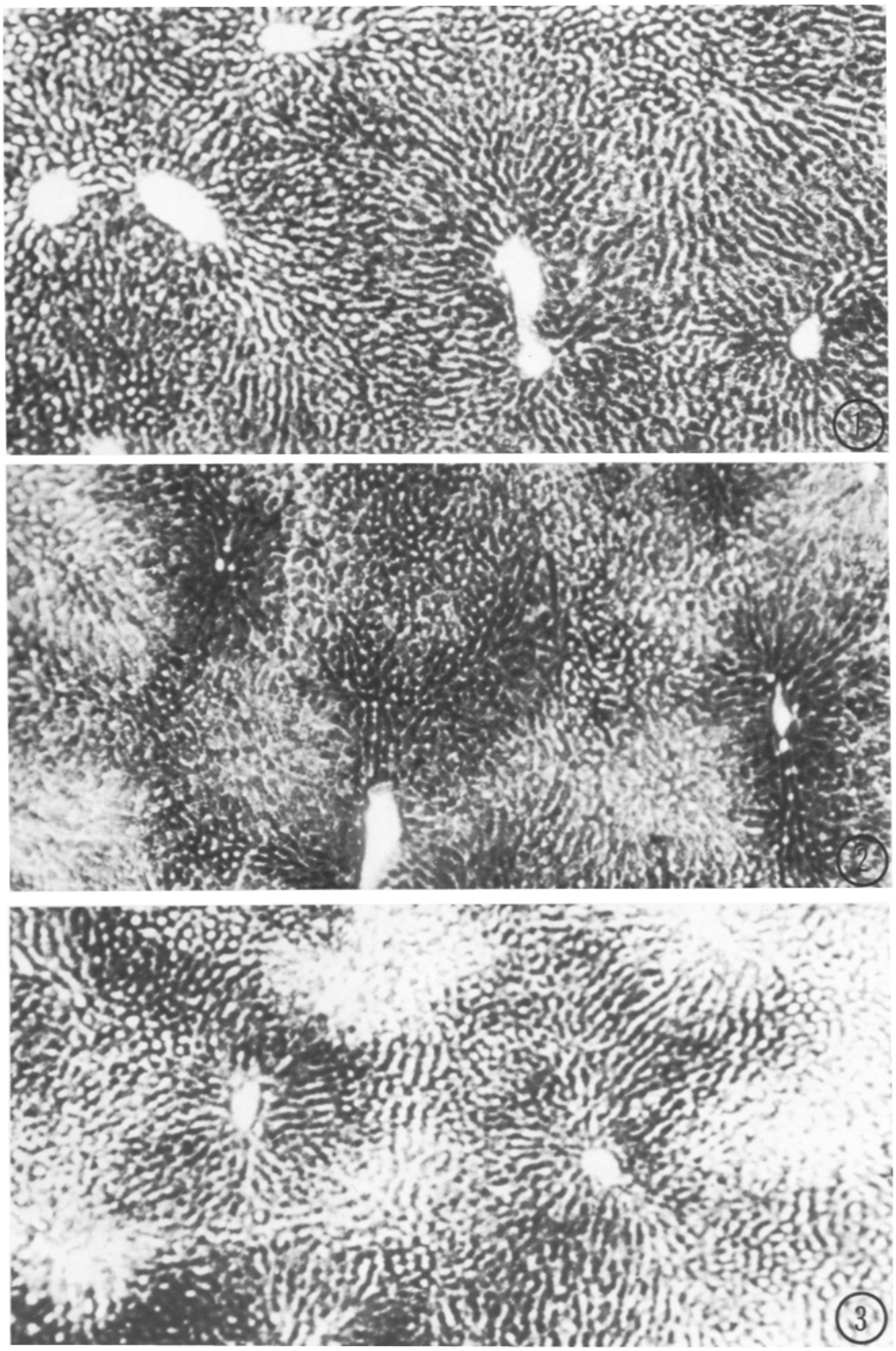
ABSTRACT

Daily phenobarbital (PB) injections, on 3–7 consecutive days, induce an intense proliferation of smooth endoplasmic reticulum (ER) associated with a decrease of the glucose-6-phosphatase activity. This situation first affects the centrolobular hepatocytes, enhancing the degree of liver lobule heterogeneity. This experimental model was used for isolation and further subfractionation of hepatocytes on Ficoll density gradients, as described in the preceding paper. Profiles of protein, DNA, RNA, glycogen, phosphorylase, and glucose-6-phosphatase were determined all along the gradient. Two liver cell populations were distinguished: (*a*) light hepatocytes (mean density 1.10) present the same morphological characteristics as centrolobular cells, i.e., an abundant smooth ER composed of tubular elements, numerous small mitochondria, and few glycogen particles; (*b*) heavy hepatocytes (mean density 1.14) are characterized by large and compact glycogen areas and prominent rough endoplasmic cisternae, as are the perilobular cells. After incubation in the Wachstein-Meisel medium, centrolobular hepatocytes exhibit dispersed reaction sites of glucose-6-phosphatase activity, whereas perilobular cells present a continuous and intense reaction. Morphometric determinations were carried out for both cell populations. Centrolobular PB hepatocytes are considerably enlarged (mean diameter: 23.7 μm); perilobular hepatocytes have a significantly smaller mean diameter of 19.2 μm, which is close to the values of control liver cells.

The efforts directed to separate hepatocytes of different functional significance originate in the observation of a morphological and functional heterogeneity of the liver lobules. Most of the authors attracted by this feature have insisted on the contrast between hepatocytes located in the center of the lobule and those of the periphery (2, 7, 14). In normal liver, the difference in cell structure and composition between centro- and perilobular hepatocytes is faint, and the proportion of the light hepatocytes, as shown in the accompanying paper (4), is relatively low, amounting to about 15% of the total hepatocyte population. The fact remains that if we succeed in enhancing the heterogeneity by influencing selectively some cells in the lobule, we may separate the modified hepatocytes from those which have not been affected with a better efficienty. Phenobarbital (PB) administration to rats induces changes in the central cells of the liver lobule which progressively involve more peripheral cells (1). Ménard et al. (6) analyzed the response of the hepatocytes to barbi-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turates and showed that the most striking heterogeneity was obtained in the lobule at the 3rd day of PB administration (100 mg/kg).

In the present work, we have induced, by intraperitoneal injections of PB in rats, a clear selective response in the centrolobular hepatocytes; this consisted of an important proliferation of the smooth endoplasmic reticulum (ER). A centrifugation of the isolated hepatocytes on a Ficoll gradient leads to the separation of light and heavy cells corresponding, respectively, to the centrolobular modified hepatocytes and the perilobular unaffected cells. The ultrastructural changes are correlated with biochemical determinations carried out on both cell populations. Morphometric studies show that the modified hepatocytes are of low density are increased in volume, and all behave in a similar way.

MATERIAL AND METHODS

Animals

Adult female Sprague-Dawley rats, weighing 190–220 g, were used. The rats were fed standardized laboratory diet and had free access to water.

PB Treatment

Animals received 100 mg per kg body weight sodium PB (E. Merck, Darmstadt, Germany) by daily intraperitoneal injection, on 3–7 consecutive days. Body weights were recorded daily. An average weight increase of 5–10 g per animal per day was observed. Animals appeared healthy throughout the drug administration and were sacrified on the 3rd and 7th days after medication had commenced.

Isolation Procedure and Centrifugation of Hepatocytes

Suspensions of isolated hepatocytes were obtained by the method described in the preceding paper. No significant differences in behavior were observed either during perfusion or during mechanical treatment of the cell aggregates. Liver weights of the PB-treated animals were determined after perfusion and compared to the controls. The separation of low and high density hepatocytes was obtained by applying the methods detailed in the accompanying paper (4).

Biochemical Assays

Protein, DNA, RNA, and glycogen content and glucose-6-phosphatase activity were determined on homogenates of the isolated cells and on homogenates of the different cell subfractions obtained after centrifugation on Ficoll gradients. These assays were performed according to the biochemical techniques reported in the preceding communication.

The profile of phosphorylase activity was determined along the Ficoll gradient. This enzyme activity was assayed as previously described (13).

Electron Microscopy, Cytochemistry, and Size Measurements

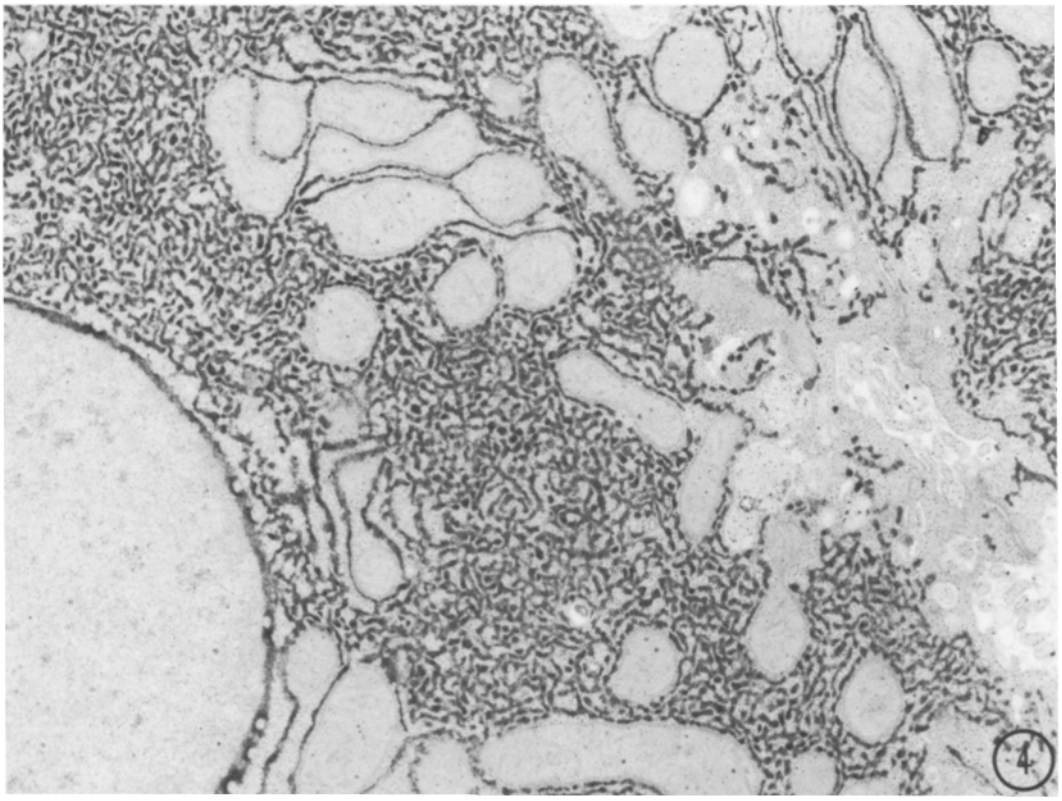
The light and heavy hepatocyte fractions were subjected to electron microscope examination under the same conditions as described in the first paper. The cytochemical demonstration of glucose-6-phosphatase was carried out on 40-μm thick frozen sections of livers fixed by perfusion for 1 min with 2.5% glutaraldehyde buffered with 0.1 M cacodylate, pH 7.4 (9). Short incubation time (5 min) in the Wachstein-Meisel medium was used so that the importance of the lead deposits would be more or less proportional to the enzyme activity. Incubations for longer periods of 30 min were also used when a clear, dense precipitate was needed. Diameters were measured on thick sections of Epon-embedded material by means of the Zeiss TGZ3 particle size analyzer (Carl Zeiss, Oberkochen, Wuerttenberg, West Germany). Mean diameters, surfaces, and volumes were calculated from the measured values.

RESULTS

Morphology and Glucose-6-Phosphatase Activity of Livers After PB Administration

The characteristic and classical response of the parenchymal liver cells to the administration of PB was reproduced in our assays. We have chosen the

FIGURES 1–3 Light micrographs of 20-μm thick frozen sections of liver tissue, fixed for 1 min with 2.5% distilled glutaraldehyde and incubated in the Wachstein-Meisel medium in order to reveal the glucose-6-phosphatase activity. A homogeneous distribution occurred in liver sections of control animals (Fig. 1), whereas an important heterogeneity of the distribution of glucose-6-phosphatase activity was detected in the liver lobules after 3 days (Fig. 2) and 7 days (Fig. 3) of PB treatment. In this last condition, a more extended reduction of enzymatic activity was observed involving a larger portion of the lobule. The light regions correspond to centrolobular areas and the heavily stained regions to periportal areas. × 70.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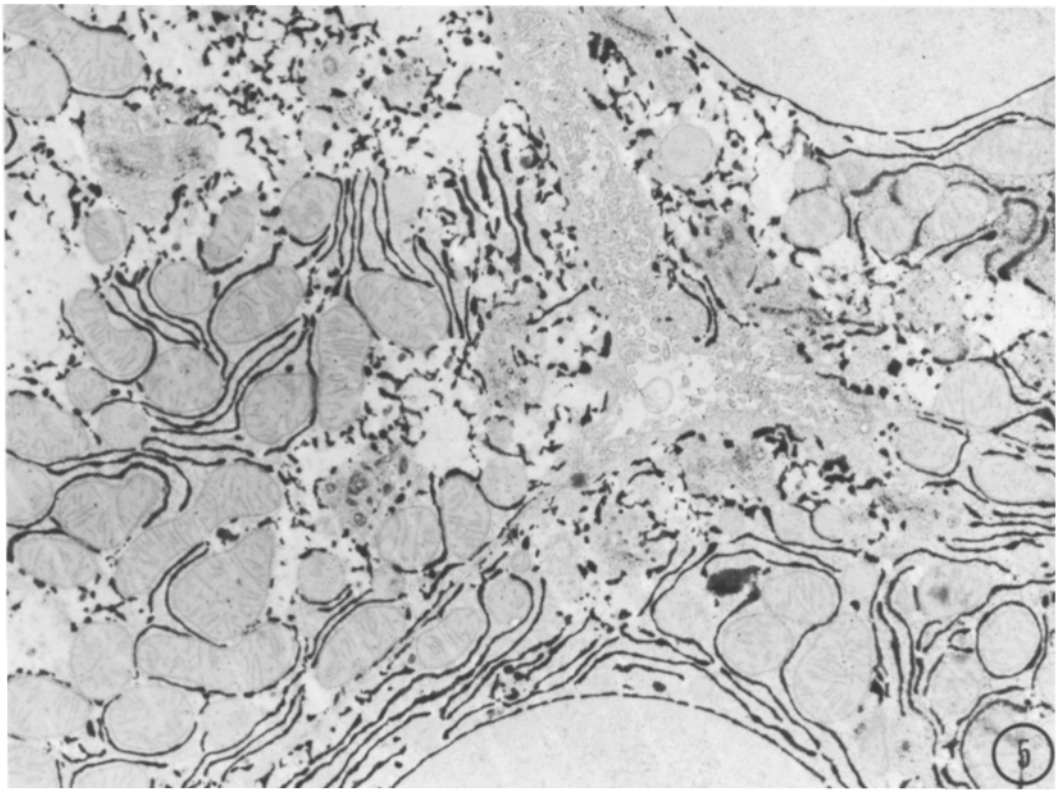
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TABLE I

Isolated Rat Liver Cells Obtained from Control and PB-Treated Rats

Assays	Control	7-days PB
Protein		
*mg/g**	99.2 ± 8.0	134.8 ± 11.5
%	100	135.8
DNA		
*mg/g**	2.0 ± 0.1	1.5 ± 0.1
%	100	75
RNA		
*mg/g**	4.2 ± 0.8	4.8 ± 0.5
%	100	116
Glycogen		
*mg/g**	22.0 ± 4.5	11.0 ± 3.0
%	100	50
Glucose-6-phosphatase		
*μmol/min/g**	5.6 ± 1.0	2.0 ± 0.5
%	100	36
Wet weight of liver		
g	8.2 ± 0.4	10.2 ± 0.2
%	100	125

Mean values and standard deviations calculated for five experiments.

* The data are expressed per gram wet weight of perfused liver.

cytochemical demonstration of glucose-6-phosphatase to follow the evolution and the topography of the cell modification in the liver tissue.

Livers of untreated control animals are characterized by an evenly distributed enzymatic reaction throughout the lobule (Fig. 1). In our experiments, with the rat species used, we found a gradual decrease of the glucose-6-phosphatase activity starting from the centrolobular regions and extending progressively into the lobule. After 3 days' PB treatment, the reduction of the glucose-6-phosphatase activity was confined to the inner third portion of the lobule (Fig. 2); after 7 days treatment, a more extended reduction of enzymatic activity was observed involving a larger portion of the lobule (Fig. 3).

The centrolobular regions of the lobules, affected by the drug administration, were examined in the electron microscope. An intense proliferation of the smooth ER was noticed. The glucose-6-phosphatase activity, revealed by a lead phosphate precipitate, demonstrates clearly in these centrolobular hepatocytes the extent of the hyperplasia of the smooth-walled cisternae and a slight reduction of the intensity of the reaction (Fig. 4) compared to that found in the periportal hepatocytes (Fig. 5).

Biochemical Determinations on Hepatocytes Isolated from Livers of PB-Treated Animals and Controls

The hepatocytes were isolated from livers of animals treated for 7 days with PB and from control animals in order to carry out basic biochemical determinations on the global population of isolated cells and on the two cell fractions which we were able to separate after centrifugation on a Ficoll density gradient.

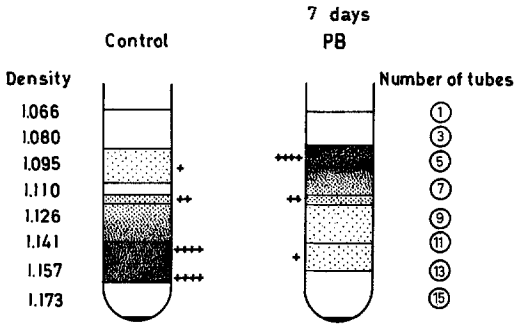
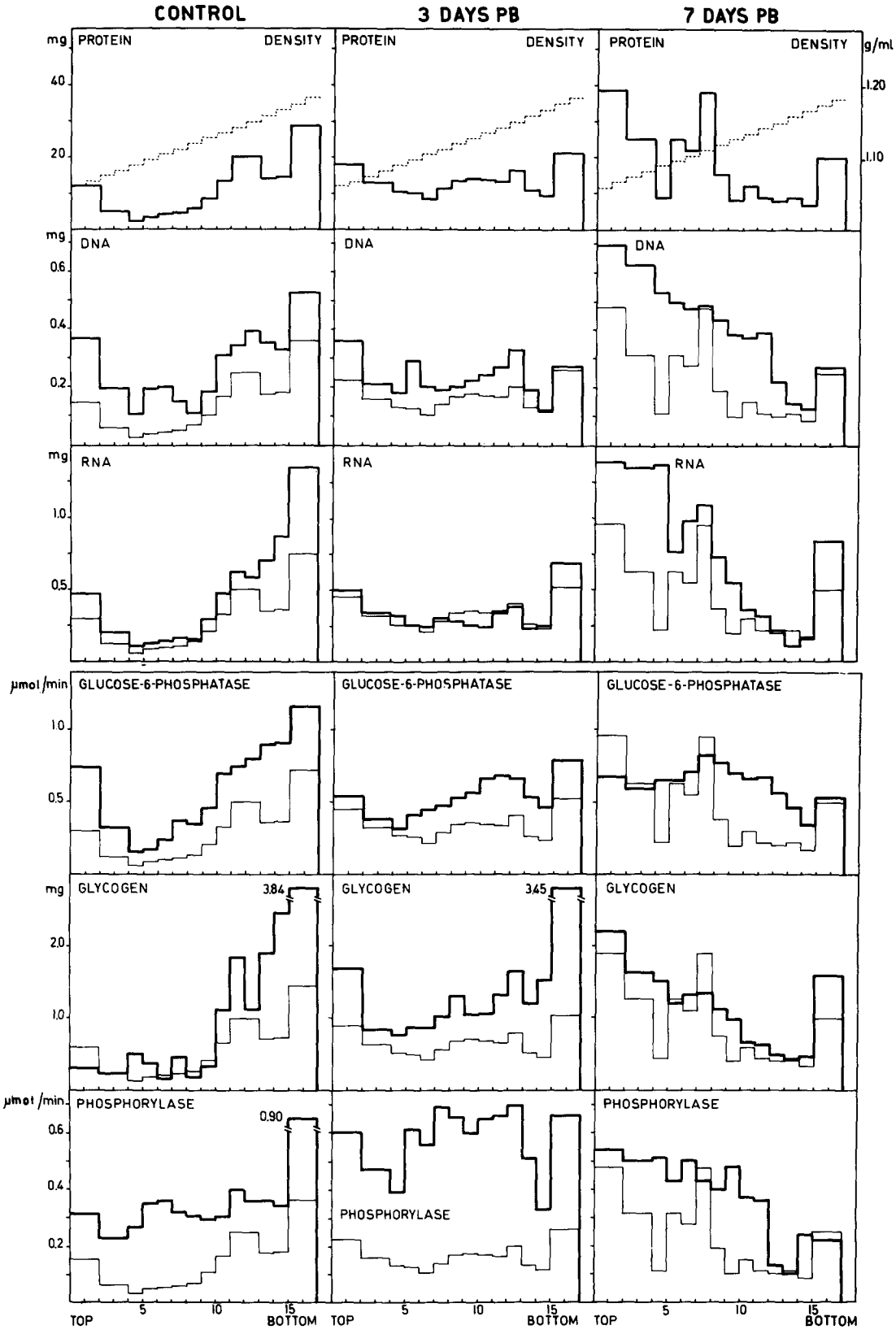


FIGURE 6 Schematic representation of cell distributions along Ficoll density gradients (15–40% Ficoll, buffered with calcium-free Hanks' solution, containing 5 mM EDTA and 2% albumin, pH 7.4) after centrifugation of isolated hepatocytes at 25,000 rpm for 3 h. The cell opalescence along the gradient was compared in both situations, control and 7-day PB-treated rats, and revealed a reverse distribution of hepatocytes after PB treatment.

FIGURES 4 and 5 Electron micrographs of portions of hepatocytes incubated for glucose-6-phosphatase, 30 min at 37°C, in the Wachstein-Meisel medium.

Figure 4 Centrolobular hepatocytes characterized by an important proliferation of smooth ER, where the reaction product is found slightly reduced in intensity. × 18,000.

Figure 5 Perilobular hepatocytes presenting heavy deposits of reaction product throughout the rough and smooth ER, including the perinuclear cisterna. × 18,000.


CONTROL
3 DAYS PB
7 DAYS PB
mg
PROTEIN
DENSITY
g/ml
40
20
1.20
1.10
mg
DNA
0.6
0.4
0.2
mg
RNA
1.0
0.5
µmol/min
GLUCOSE-6-PHOSPHATASE
1.0
0.5
mg
GLYCOGEN
3.84
3.45
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0.90
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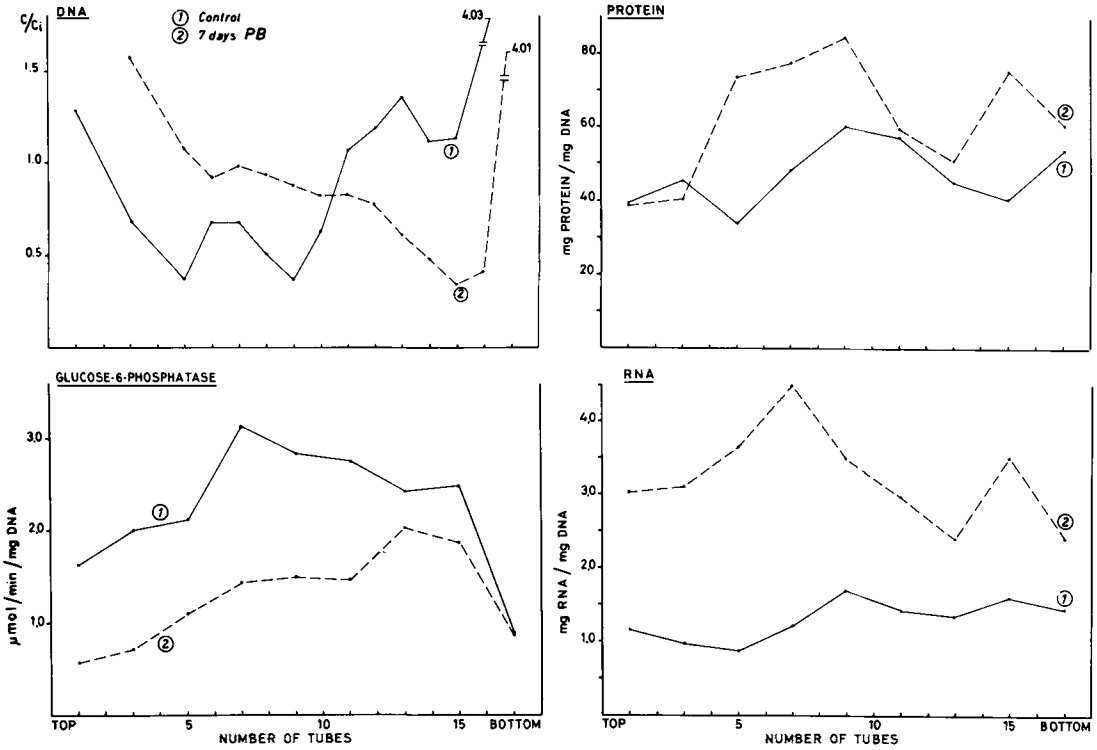


FIGURE 8 Protein:DNA, glucose-6-phosphatase:DNA, and RNA:DNA ratios along Ficoll gradients after centrifugation of the hepatocytes under the same conditions as described for Figs. 6 and 7. Curve 1: control rats; curve 2: 7-day PB-treated rats. The protein:DNA ratio and the RNA:DNA ratio are increased in light hepatocytes whereas the glucose-6-phosphatase:DNA ratio appears decreased. The upper left graph illustrates the reversed distribution of DNA along the gradient in both situations. C/C_i is the relative concentration, i.e. the ratio of the concentration C in the fraction to the concentration C_i that the constituent would have if it was uniformly distributed in the whole gradient.

In Table I, we collected the determinations of protein, DNA, RNA, and glycogen content, and glucose-6-phosphatase activity of hepatocytes obtained from rats treated for 7 days with PB and from controls. The drug administration resulted in protein and RNA increase, which is even more important than reported in Table I when the wet weight increase of the liver is taken into account. The DNA content per gram of hepatocytes is decreased, but the total DNA per liver remains about the same. The glycogen content and glucose-6-phosphatase activity are decreased when expressed in both relative and absolute values.

Properties of Low and High Density Cell Fractions Prepared from PB-Treated Rats and Controls

SEDIMENTATION PROPERTIES AND BIOCHEMICAL DETERMINATIONS: Fig. 6 represents schematically the cell distribution evaluated by indicating the opalescence of the gradient in the

FIGURE 7 Profiles of protein, DNA, RNA, glucose-6-phosphatase, glycogen, phosphorylase, and density along Ficoll gradients (15–40%) after a 25,000-rpm centrifugation, at 4°C for 3 h, of hepatocytes from control and from 3-day and 7-day PB-treated rats: 17 fractions of 2.5 ml were collected. In the control situation, most cells sedimented in tubes 10–15 (heavy hepatocytes), fewer in tubes 4–9 (light hepatocytes). An equal distribution on the gradient appeared after 3-day PB treatment. After 7-day PB treatment, the situation was reversed: numerous light hepatocytes (tubes 4–9) were isopycnically banded at the level of mean density of 1.10. The density of each fraction was measured at 20°C.

TABLE II

Cell Fractions Separated on Ficoll Density Gradients after Centrifugation of Isolated Hepatocytes Obtained from Control and 7-Day PB-Treated Rats

Gradient Subfractions	Protein		DNA		RNA		Glucose-6-phosphatase		Glycogen	
	Control	PB	Control	PB	Control	PB	Control	PB	Control	PB
		%	%			%	%			%
Non sedimented material	5.9	9.8	8.5	19.2	5.0	14.9	6.9	9.0	2.2	7.2
Low density fraction	12.3 ±1.8	19.2 ±3.7	15.0 ±2.6	28.0 ±5.0	7.5 ±2.0	22.1 ±0.3	13.2 ±0.5	27.3 ±7.2	6.6 ±2.9	15.9 ±3.9
High density fraction	21.7 ±5.4	10.3 ±0.5	23.3 ±5.2	17.2 ±1.7	14.5 ±2.8	10.3 ±4.6	22.5 ±5.5	21.9 ±1.9	15.3 ±7.2	8.6 ±0.4
Bottom	21.3	29.8	30.7	19.9	20.9	14.3	12.5	11.2	14.9	24.0
Recovery	61.1	70.5	76.7	84.3	47.8	61.6	55.0	66.9	38.2	55.7

TABLE III

DNA, RNA, Glycogen, and Glucose-6-Phosphatase Activity per Milligram of Protein from Control and 7-day-PB Hepatocytes

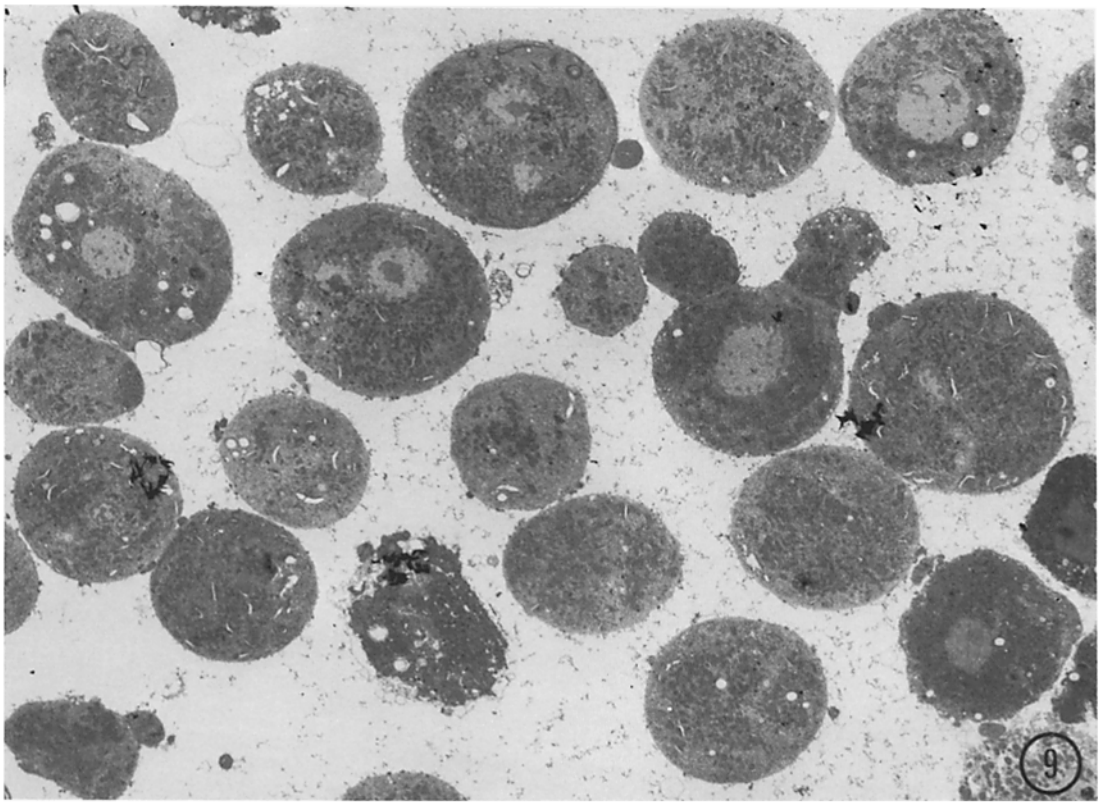
	Low density fraction (*d*: 1.10)	High density fraction (*d*: 1.14)
DNA		
Control	0.031 ± 0.005	0.025 ± 0.001
PB	0.015 ± 0.002	0.020 ± 0.001
RNA		
Control	0.029 ± 0.005	0.027 ± 0.006
PB	0.042 ± 0.006	0.035 ± 0.006
Glycogen		
Control	0.075 ± 0.009	0.106 ± 0.007
PB	0.068 ± 0.011	0.069 ± 0.010
Glucose-6-phosphatase		
Control	0.045 ± 0.007	0.042 ± 0.003
PB	0.021 ± 0.003	0.031 ± 0.004

Mean values for three experiments.

centrifuge tube after centrifugation of hepatocytes from PB-treated rats and controls. The sedimentation behavior of the PB hepatocytes differs from that of the control: an increased number of cells sediment in the upper layers of the gradient corresponding to densities of the control light hepatocytes (mean density: 1.10); inversely, there are fewer PB cells which reach the high density regions (mean density: 1.14) than there are in the control. This reversed distribution is typical of the rats treated for 7 days with PB, and an intermediate situation occurs for the animals treated for 3 days. Protein, DNA, RNA, glycogen, phosphorylase, and glucose-6-phosphatase determinations were carried out on the subfractions collected from gradients after centrifugation of PB and control hepatocytes. The results are represented in the graph of Fig. 7: in the control hepatocytes most cells, as mentioned before, sediment in tubes 10–15, fewer in tubes 4–9; in the 3-day-treated animals the curve is flattened, which results in an equal distribution of the hepatocytes in both groups of light and heavy cells; in the 7-day-treated animals, the situation is reversed showing a maximum accumulation of cells in the low density region. One may notice the parallelism of distribution between RNA and glucose-6-phosphatase and between glycogen and phosphorylase activity. Little attention was paid to fraction 1–3, which were characterized by the presence of cell debris, nuclei, and altered cells. One may notice that more material is present on top of the Ficoll gradient

FIGURE 9 Low power electron micrograph illustrating the low density subfraction (tubes 4–9) of the Ficoll gradient: light hepatocytes from 7-day PB-treated rats appear well preserved and form a homogeneous population presenting a mean diameter of 23.7 μm. Little contamination is detected in this representative fraction. × 1,000.

FIGURE 10 Low power electron micrograph illustrating the high density subfraction (tubes 10–15) of the Ficoll gradient: heavy PB hepatocytes are spherical cells presenting a mean diameter of 20.0 μm. 80–90% of the hepatocytes recovered after centrifugation appear intact. × 1,000.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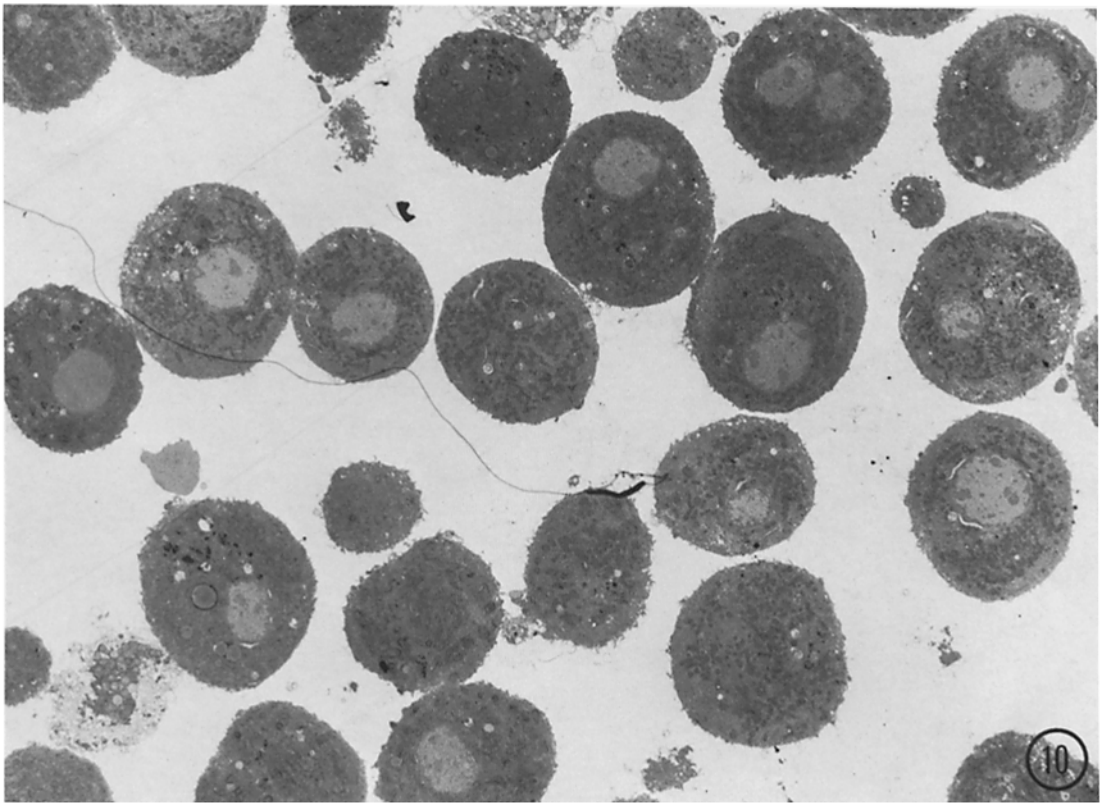
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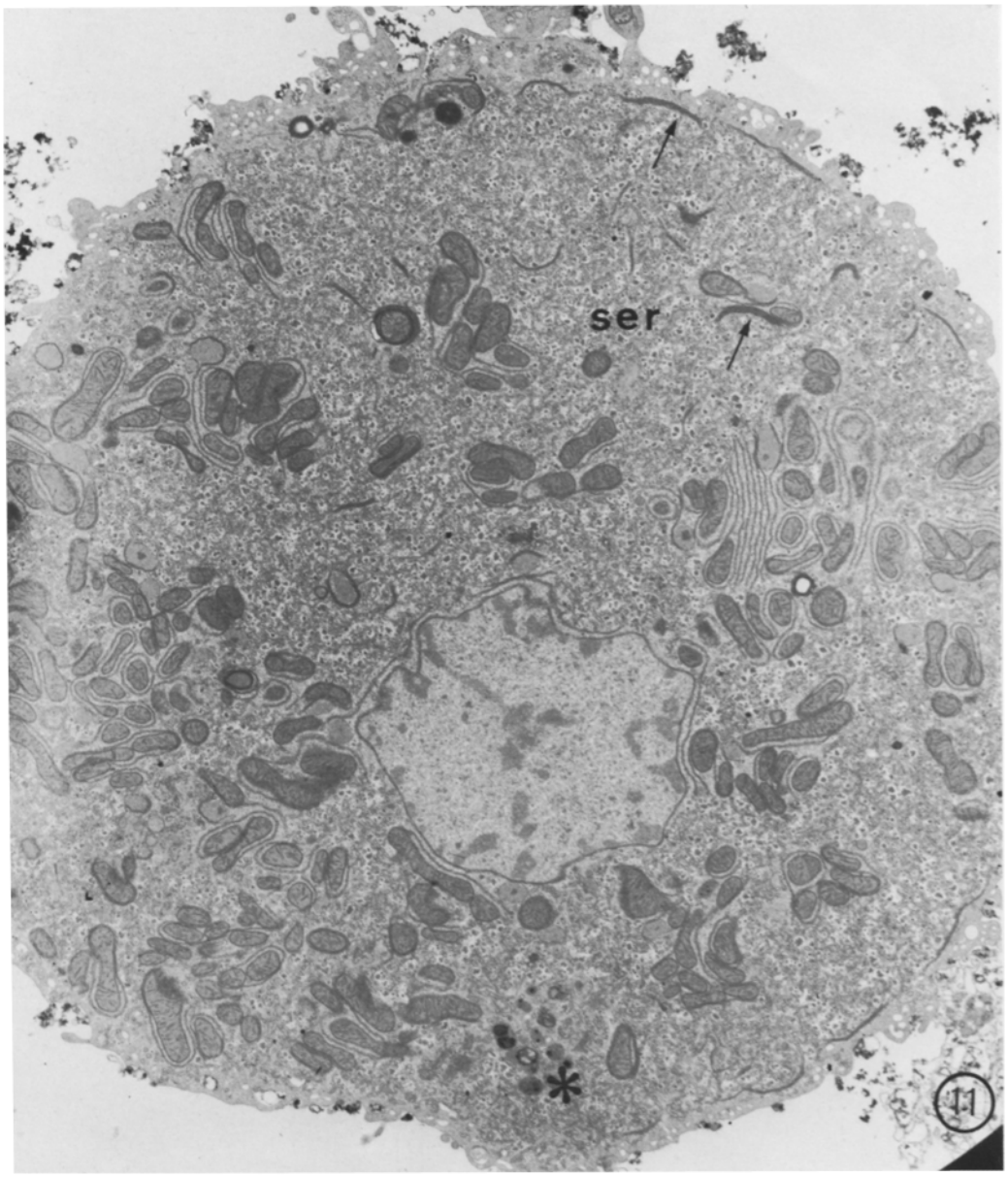


FIGURE 11 Light hepatocyte or centrolobular cell from 7-day PB-treated rats, isolated on Ficoll gradient: an important proliferation of smooth ER is noticed (*ser*). Glycogen particles are dispersed in the meshes of the extended network of smooth tubules. Compact lamellar bodies (arrows) present at the periphery of the cells seem to correspond to alterations of the endoplasmic reticulum. Accumulation of lysosomes at the lower part of the cell (asterisk) suggest that this portion of the cytoplasm corresponds to a peribiliary space. × 8,400.

after centrifugation of the 7-day PB cells (Fig. 7). This situation may explain why, in some experiments, contaminations of the first tubes of the low density subfraction may occur. Results obtained for the 7-day PB-treated animals may be further analyzed by expressing the protein and RNA contents and the glucose-6-phosphatase activity per milligram of DNA contained in each subfrac-

tion: these values are plotted in the graph of Fig. 8. In the upper left graph, we reproduce the DNA profiles of the different gradient subfractions for treated and control animals; in the upper right graph, the protein:DNA ratio shows an increase in the light hepatocytes (tubes 4–9) and practically no change in the heavy cells (tubes 10–15); a similar situation exists for the RNA:DNA ratio; the glucose-6-phosphatase specific activity, on the contrary (lower left graph), is more reduced in the low density cells than in the heavy cells. The glycogen: DNA ration in the PB cells is about constant throughout the gradient, whereas in the controls this ratio is often low in the light cells. A relative inconstancy in the glycogen content of the different control rats used and a possible degradation of the polysaccharide during cell preparation can lead only to general conclusions.

The subfractions, collected from the three centrifuged gradients (control, 3 day PB, and 7 day PB), were pooled in 4 different fractions: the nonsedimented material (tubes 1–3), the low density fraction (tubes 4–9), the high density fraction (tubes 10–15), and the bottom fraction (tubes 16 and pellet). The biochemical determinations carried out on these four fractions are reported in Table II. These results confirm the previous observations according to which the PB hepatocytes of the low density fraction are increased in number with a parallel increase in the different compounds that were determined.

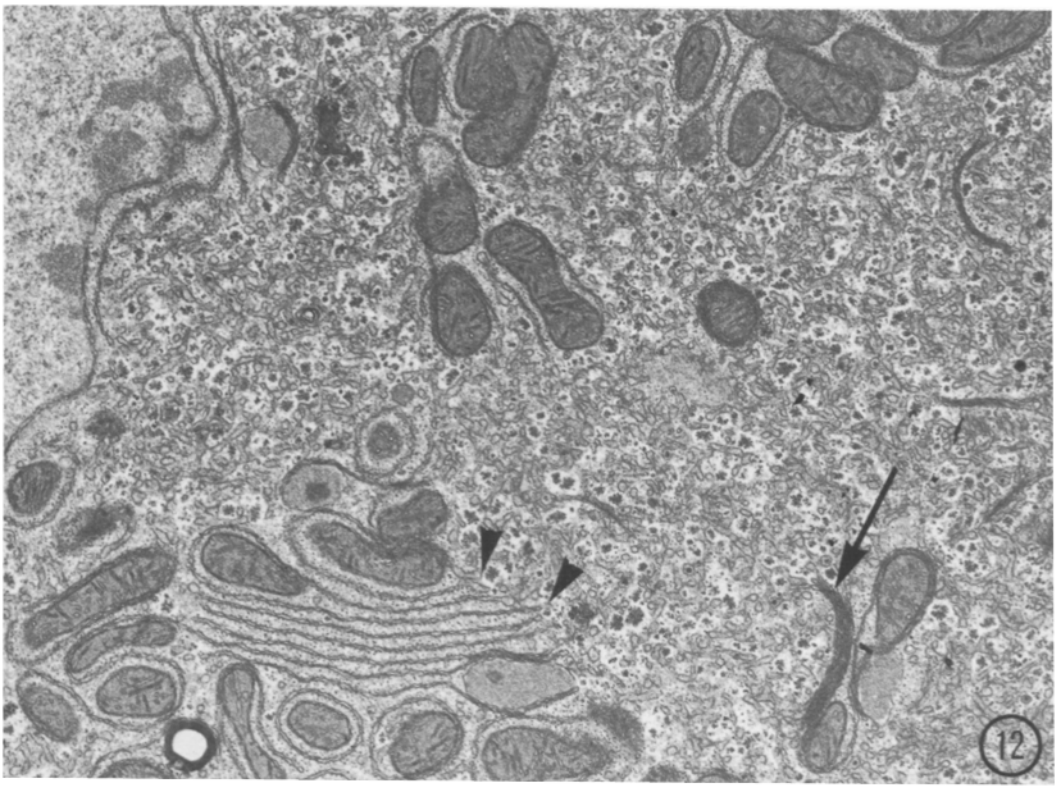
The distribution on Ficoll gradients of the same cell components expressed per milligram of protein is given in Table III. If we compare low and high density hepatocytes in the controls, as emphasized in the accompanying paper, both fractions contain similar concentrations of DNA, RNA, and glucose-6-phosphatase, but glycogen concentration in the light hepatocytes is lower than in the heavy cells. In the figures obtained for PB-treated rats, the values for both cell fractions are equivalent except that the glucose-6-phosphatase activity is significantly lower in the low density fraction than in the high density fraction, the glycogen concentration being leveled in these conditions.

MORPHOLOGY AND GLUCOSE-6-PHOSPHATASE ACTIVITY: Figs. 9 and 10 are low magnification electron micrographs of 7-day PB cells isolated on Ficoll gradients: both light PB hepatocytes (Fig. 9) isolated from tubes 4–9 and heavy PB hepatocytes (Fig. 10) from tubes 10–15 demonstrate an excellent morphological preservation. 80–90% of the cells recovered in these fractions appear intact. Little cell debris is detected in between the cells, partly due to the isolation procedure and partly due to the high speed centrifugation technique. However, the cell debris does not interfere with biochemical determinations carried out on the different cell fractions because these fractions were washed as previously described.

The light hepatocytes isolated in the low density fraction present, under the electron microscope (Fig. 11), the same characteristics as the centrolobular hepatocytes of the liver tissue: an abundant smooth ER forms a network of tubular elements, the α-glycogen particles are dispersed in the mesh of the reticulum, the rough-surfaced ER is arranged in parallel arrays and presents the characteristic relationships with other cytoplasmic organelles, such as mitochondria and peroxisomes (Fig. 12). At the periphery of the cells, some alterations of the ER may occur. They consist of compact lamellar bodies (Figs. 11 and 12, arrows) probably derived by condensation of smooth-surfaced membranes.

The glucose-6-phosphatase activity was demonstrated in the entire ER. The enzymatic reaction in the light hepatocytes is relatively low in intensity and consists of small accumulations of electron-dense lead deposits (Figs. 13 and 14, arrows) in the smooth and rough cisternae of the ER. In control liver sections prepared under the same conditions of fixation and incubation, the centrolobular cells present a more intense reaction: the lead deposits practically fill the cisternal lumen. We may mention that rough and smooth ER in many regions are easily distinguished at high magnification (Fig. 13, *rer* and *ser*) because the ribosomes are exceptionally well preserved when short fixation times are used. These observations are in agreement with the glucose-6-phosphatase specific activities determined in the low density fraction of hepatocytes (Fig. 8). The presence of numerous glycogen particles dispersed between the meshwork of smooth tubules (Figs. 11–14) can be correlated with quantitative determinations which reveal similar concentrations of glycogen in both light and heavy hepatocytes (Table III).

The heavy hepatocytes present a prominent rough ER in close relation with mitochondria; the smooth ER is relatively poorly developed and reduced to a few tubules which meander in the glycogen accumulations; these glycogen areas are generally large and compact. In these heavy cells, alterations of the ER consisting of lamellar bodies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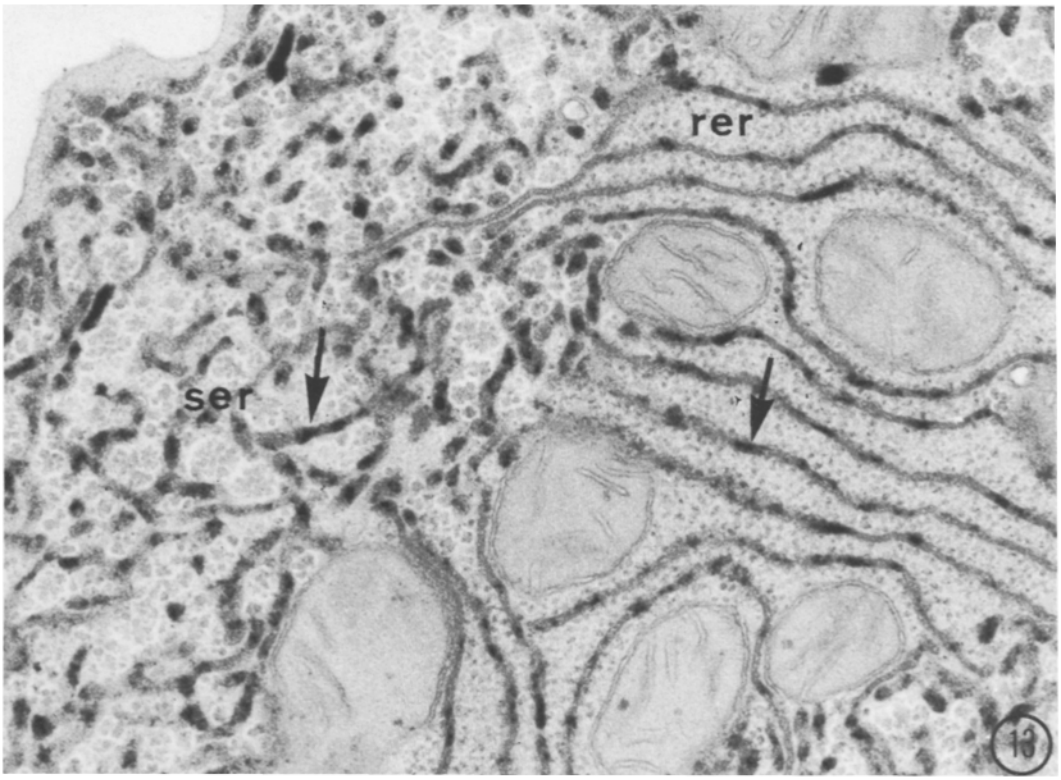
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(Fig. 15, arrows) similar to those described in the light hepatocytes were observed.

Fig. 16 illustrates the glucose-6-phosphatase activity demonstrated in the heavy hepatocytes. The lead phosphate deposits nearly completely fill the ER cisternae of these cells. A few smooth ER tubules marked with the reaction product infiltrate the glycogen areas (*gl*). This picture is typical also of a perilobular cell examined in tissue sections submitted to the same enzyme reaction. Some cisternae at the periphery of the cells may lack lead deposits. Nevertheless, considering the centrifuge forces imposed on the cells, the preservation remains excellent.

MORPHOMETRIC DATA: These data were obtained from measurements of diameters carried out on 1-μm thick sections of pellets of isolated PB-treated cells. Histograms of these diameter measurements were established for the original total cell population, and for the light and the heavy hepatocytes (Fig. 17, the three graphs of the first column). The corresponding histograms of cell diameters were calculated by means of the mathematical Wicksell transformation (Fig. 17, second column). The distribution curve of the diameters obtained for the total PB cell population presents a mode of 23.4 μm and a mean diameter (Table IV) of 20.1 μm, a value which is the same as that obtained for normal isolated hepatocytes. The distribution curve of the diameters obtained for the light PB hepatocytes also shows a mode at 23.4 μm, but the mean diameter is significantly higher, 23.7 μm, due to the fact that these cells are considerably enlarged. One may also notice that the light PB cells appear increased in diameter when compared to the light hepatocytes of the normal untreated liver. The heavy hepatocytes of the high density cell fraction, on the other hand, have a slightly lower mode and a significantly smaller mean diameter of 19.2 μm which is close to the values obtained for the equivalent cells of control livers. These measurements converted into volumes reveal that the light hepatocytes have gained in size, passing from a mean volume of 4,835–7,289 μm^3. The values of diameters plotted on a log-normal probability grid lie on a straight line except for the three smallest diameter classes measured.

DISCUSSION

The liver lobule represents an anatomical unit in which the hepatocytes differ in their morphology and enzyme content, depending on their position. The periportal hepatocytes are generally provided with higher enzyme activities than the centrolobular cells (7). For some functions, namely those related to drug-metabolizing enzymes, the centrolobular hepatocytes exhibit the greatest capacity of increasing their activity. This was demonstrated by Burger and Herdson (1) for PB-induced fine structural changes in the rat liver: the central cells are first affected by the drug, but the characteristic cellular changes consisting of a proliferation of the smooth ER, progressively involve more peripheral cells. At the third day of PB administration (6), the inner half of the lobule is profoundly transformed whereas the outer half remains unchanged. This situation was particularly suitable for increasing the degree of heterogeneity of the hepatocytes, dividing the lobules quite abruptly in about two equal parts. The induction of accumulations of smooth membranes in the centrolobular hepatocytes is also a good way to improve the clear separation of these cells by isopycnic centrifugation. Another technique designed to change the density of selected cells consists of loading them with glycogen, provided the hepatocytes are under physiological conditions which allow storage of the polysaccharide. Such an experimental model was obtained by using a concomitant administration of

FIGURE 12 Centrolobular hepatocyte from 7-day PB-treated rat. This high magnification allows one to visualize the extended network of anastomosing smooth tubules meandering in all directions. Furthermore, the process of condensation of smooth membranes in lamellar bodies is clearly demonstrated (arrow). The continuity between smooth and rough ER, classically described in untreated liver cells *in situ* (arrowheads), appears nicely preserved in these isolated cells. × 15,000.

FIGURE 13 Glucose-6-phosphatase preparation. Isolated centrolobular (light) hepatocytes from 7-day PB-treated rats were incubated for 30 min at 37°C in the Wachstein-Meisel medium. Small lead phosphate precipitates are dispersed in the smooth and rough cisternae of the ER (*ser*, *rer*). Reaction products appear scanty and isolated from one another (arrows). Notice the good preservation of mitochondria and ribosomes and the presence of numerous glycogen particles. × 60,000.

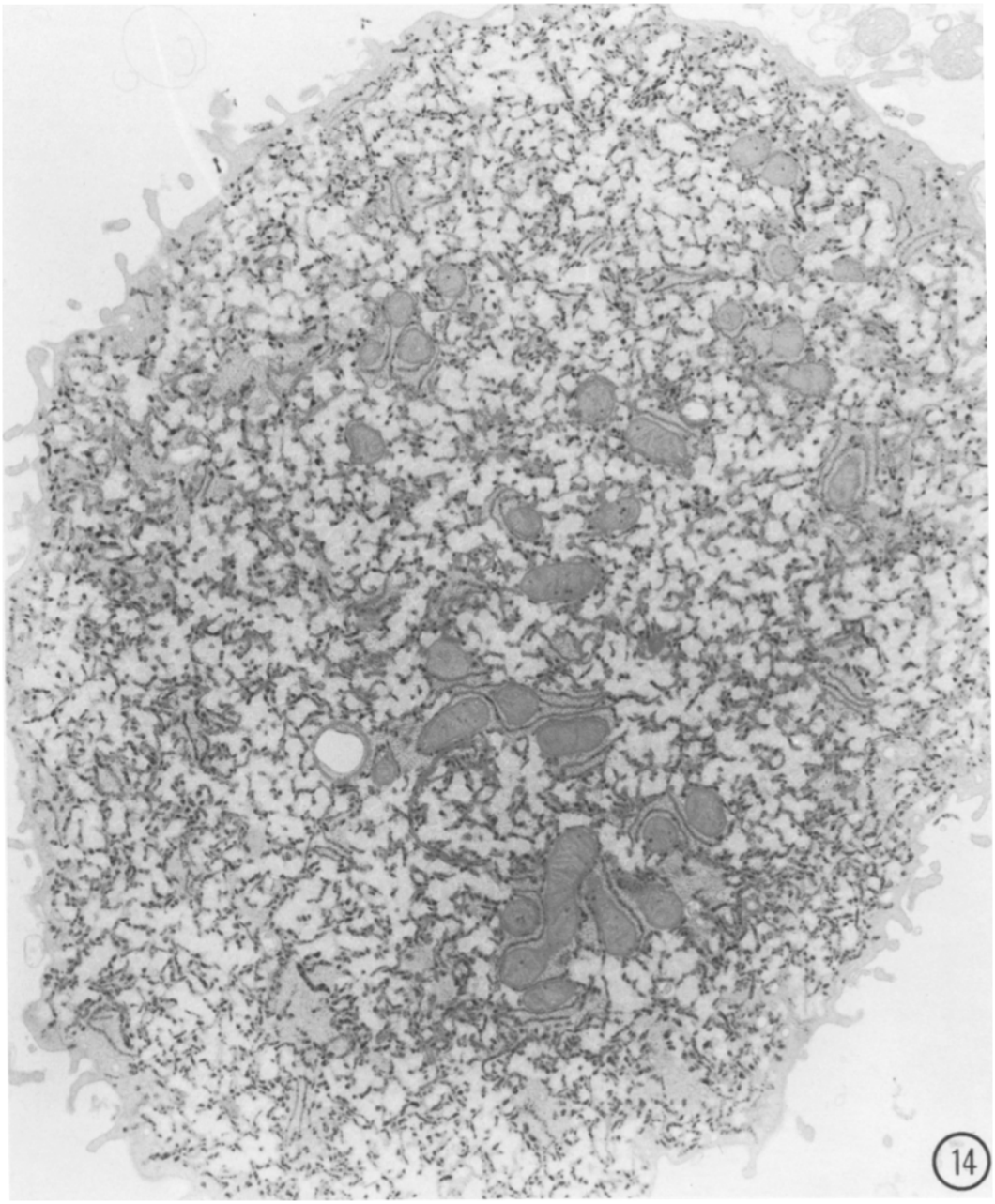

FIGURE 14 Glucose-6-phosphatase preparation. Low magnification of centrolobular (light) hepatocyte from 7-day PB-treated rat. This micrograph illustrates the dispersion of isolated glucose-6-phosphatase sites in the entire smooth and rough ER. The reaction product is irregularly distributed as small dots. × 12,000.

insulin and glucose (11). Recently, Ehrenreich et al. (5) used a similar artifice to separate the Golgi vesicles from rat liver homogenates. Overloading of some specific Golgi vesicles with lipoprotein particles of low density, 90 min after administration of ethanol to the rats, greatly facilitates the separation of these selected fractions. It might be that this treatment modifies the density of the

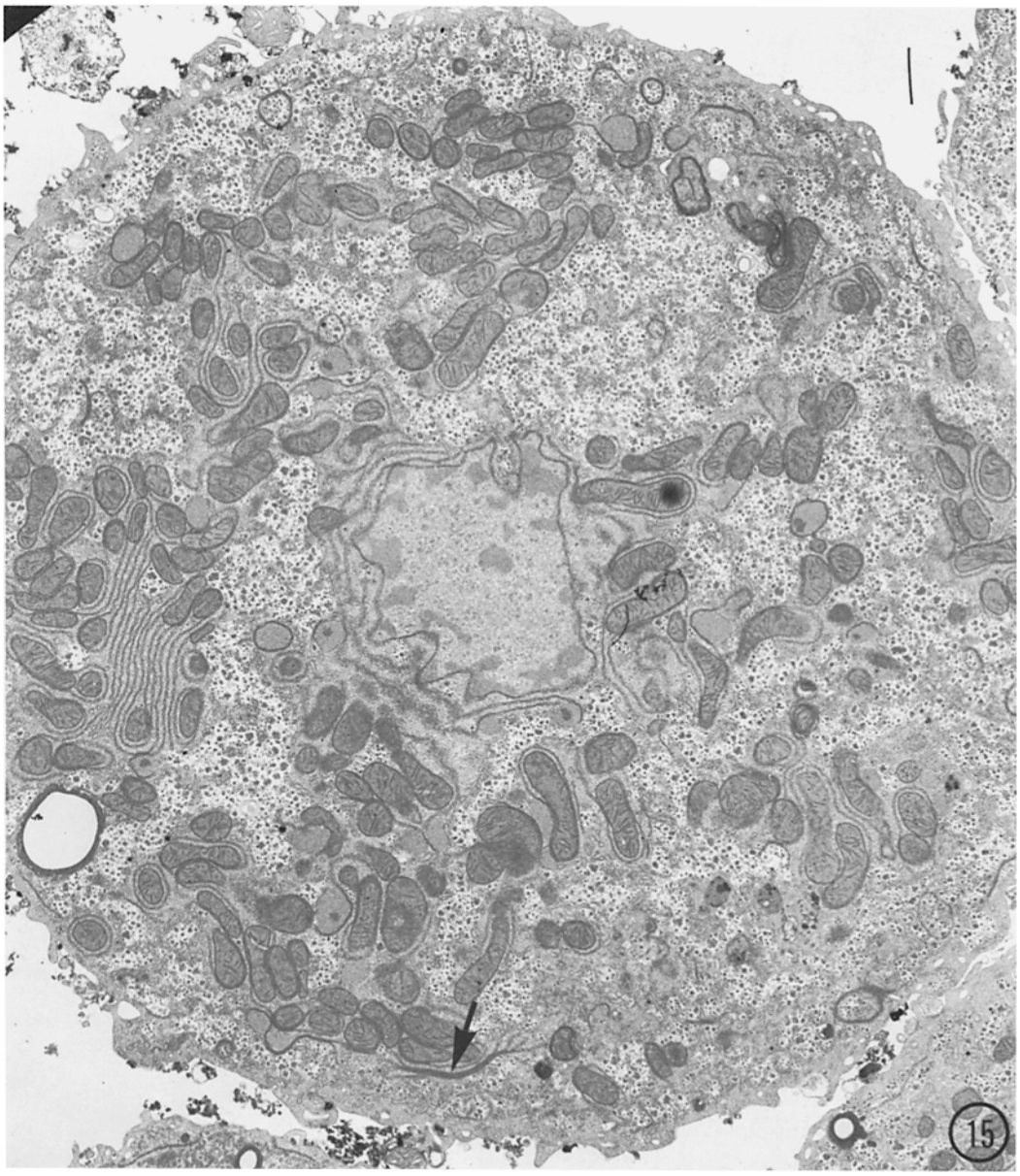

FIGURE 15 Perilobular (heavy) hepatocyte from 7-day PB-treated rat. Numerous large glycogen areas are present but smooth membranes of ER appear scarce and poorly developed. Notice the close topographic relationships between mitochondria and rough ER. Sometimes, lamellar bodies (arrow) composed of apposing smooth membranes are detected. × 8,400.

whole cell and could be used for separating those cells which respond to alcohol administration.

Castagna and Chauveau, using fasted Wistar rats, succeeded in separating two hepatocytic cell populations on Ficoll gradients (3). The light hepatocytes differ from the heavy hepatocytes by a higher lipid and glucose-6-phosphatase content and also by a more active incorporation of orotic acid in RNA and of acetate into cholesterol. After injecting the rats daily with 100 mg PB per kilogram of body weight these authors observed that the light cell fraction increased its nicotina-

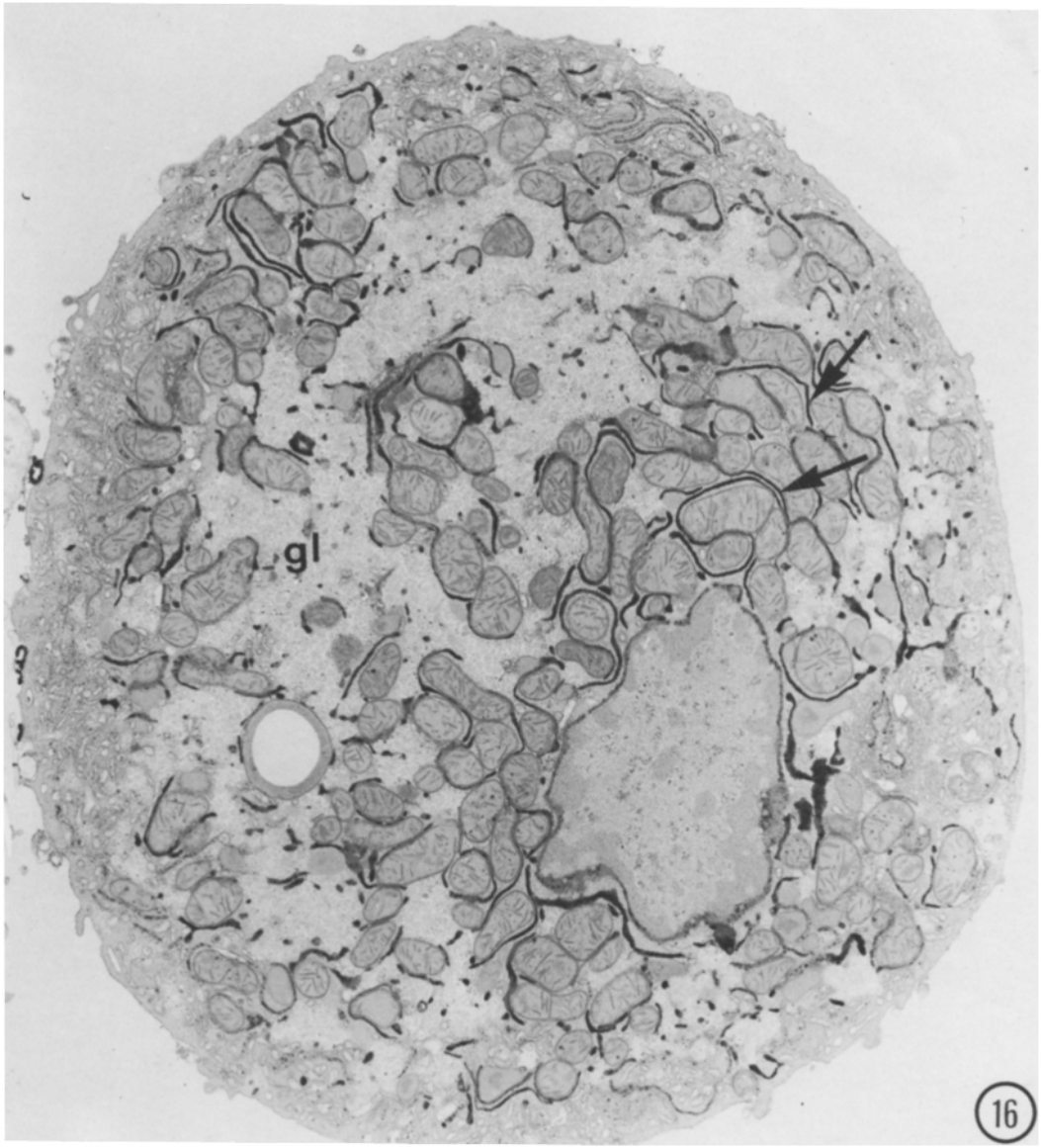

FIGURE 16 Glucose-6-phosphatase preparation. Perilobular (heavy) hepatocyte isolated from 7-day PB-treated rat. The lead phosphate precipitate fills nearly all the cisternae of the ER (arrows), as in tissue sections (cf. Fig. 4). The reaction appears homogeneous except for some cisternae disposed at the periphery of the cell. Glycogen areas (*gl*). × 9,400.

mide adenine dinucleotide phosphate (NADPH) cytochrome *c* reductase activity, the microsomal enzyme which is induced by barbiturate treatment. All these findings were confirmed by the results we obtained by applying correlative biochemical, cytochemical, and ultrastructural methods to a similar experimental model. As we mentioned in the Discussion section of the accompanying paper (4), our results differ from those of Castagna and Chauveau in localizing the light cells within the liver lobule. It is evident from the literature and from our own experience that different strains of rats do not respond identically to PB administration. This was the reason for defining the conditions to be realized in order to obtain a reproducible localization of the cell alteration (6). But, here also, some contradictions remain unclarified.

One also has to point out that the hepatocytes of

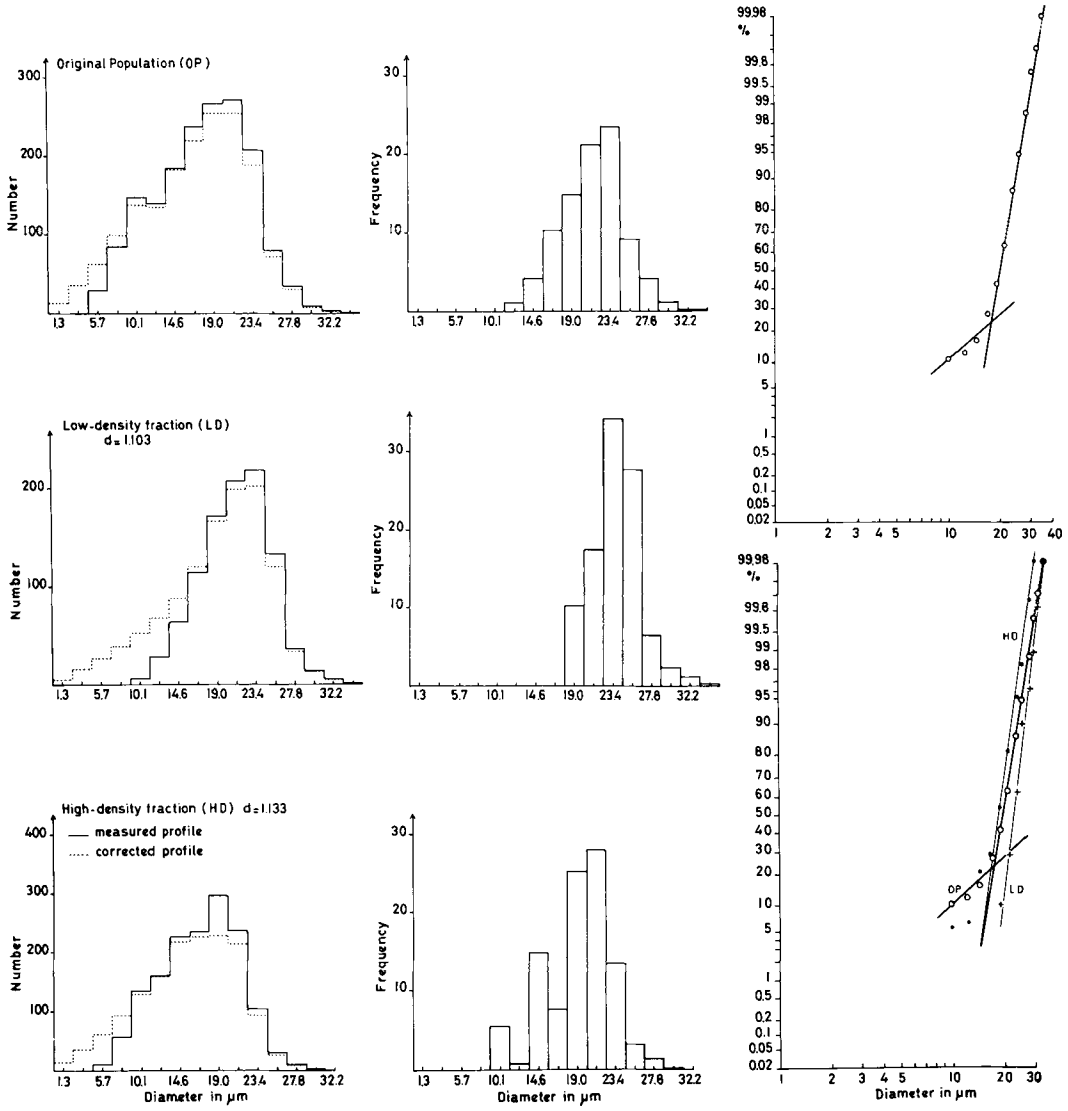

FIGURE 17 Size distribution histograms of fixed and embedded isolated hepatocytes from 7-day PB-treated rats. Size measurements of cell profiles determined on 1-μm thick sections, for the original cell population (*OP*) and for the light (low density fraction, *LD*) and heavy (high density fraction, *HD*) hepatocytes were recorded in the first column. These data obtained for the three cell populations were converted by means of the mathematical Wicksell transformation into cell diameters of hepatocytes, considered as spherical bodies (second column). Finally, the values of diameters were plotted on a log-normal probability grid (third column): in the upper part of the graph, distribution of diameters of the original population (*OP*); in the lower part, comparison between diameter distributions of light (centrolobular) hepatocytes (*LD*) and heavy (perilobular) hepatocytes (*HD*).

the low density fraction in the PB experiments differ from the light hepatocytes of untreated rats: the distribution in the gradient (Fig. 5) is quite different compared to the control, the mean diameter of the cells is larger, 23.7 μm compared to 20.5 μm, and finally the smooth ER is extensively hypertrophied.

PB induces a marked increase in the liver weight which reaches its maximum at the sixth day of drug administration (1). This liver hypertrophy is caused by a considerable enlargement of hepatocytes, the cytoplasm of which is filled with smooth-walled ER (12). Our morphometric data, determined for both light and heavy PB-isolated hepa-

TABLE IV

Morphometric Data of Isolated Hepatocytes Obtained after 7-day PB Treatment, Compared to Control Values

Fractions	Type	Mean cell diameter	Standard deviation of diameter	Mean cell area	Mean cell volume
		μm	μm	μm^2	μm^3
Isolated hepatocytes	Normal	20.0	3.2	1,296	4,547
	7 days PB	20.1	4.8	1,344	4,961
Light hepatocytes	Normal	20.5	3.3	1,349	4,835
	7 days PB	23.7	2.8	1,794	7,289
Heavy hepatocytes	Normal	19.0	3.0	1,160	3,839
	7 days PB	19.2	3.8	1,206	4,144

tocytes, reveal a striking difference in response: the light hepatocytes, corresponding mainly to centrolobular cells, measure 23.7 μm in diameter and have a mean volume of 7,289 μm^3; and the heavy hepatocytes, enriched in perilobular cells, exhibit a mean diameter of 19.2 μm and a mean volume of 4,144 μm^3. These values are not influenced by differences in the osmotic pressure of the medium because both hepatocyte fractions were washed, resuspended in an isosmolar medium and finally fixed in 2.5% glutaraldehyde buffered with 0.1 M cacodylate (440 mosmol). Despite these precautions, it may be that some hepatocytes are more susceptible to slight variations in the osmolality to which they were subjected during the density gradient centrifugation.

The importance of the cytoplasmic changes induced in the centrolobular cells after several days of PB administration was analyzed with morphometric methods by Stäubli et al. (12). The cytoplasmic compartment that contributes to the largest extent to cell hypertrophy is the smooth ER. Its volume after 5 days of PB treatment was 2.8 times greater than the control value. The dominant enlargement of this membranous organelle may explain the reduction in density of the modified hepatocytes, if we take into consideration the density of the smooth-walled cisternae. Measured after subfractionation of the microsomes obtained from normal liver, the median equilibrium density of the smooth ER vesicles varies from 1.05 to 1.18 (10). In PB-treated animals, the phospholipid content of the microsomes increases (12) and the ratio of the phospholipid P to the proteins increases 1.32-fold (15). As a consequence, the smooth ER influences significantly the final density of the modified cells, acting as a lightening substance.

The glucose-6-phosphatase activity revealed with histo- and cytochemical methods appears reduced in the centrolobular regions of the liver tissue from rats treated with PB and in the corresponding isolated light hepatocytes. The reactive enzymic sites located on the membranes which bound the proliferated ER are dispersed and form small spots of precipitate whereas in the unaffected perilobular cells the precipitate is more abundant and fills the cisternae (6). A decrease in the glucose-6-phosphatase specific activity (glucose-6-phosphatase:protein) noticed earlier by Orrenius et al. (8) in the total liver homogenate was clearly evidenced in the homogenate of the isolated light cell fraction. The glucose-6-phosphatase:DNA ratio of this fraction appeared diminished also, which suggests that there is, in addition to the dilution factor due to the proliferation of the smooth ER, a reduction in the absolute enzyme activity.

Glycogen concentrations, calculated on the basis of the DNA and protein content, are similar in both light and heavy hepatocytes. This is confirmed in the electron micrographs of centrolobular cells in which glycogen particles are present but widely spread in between the tubules of the smooth ER, whereas in the perilobular cells the particles form aggregates. It can be concluded that the separation of both cell types on Ficoll gradients is more subject to the degree of development of the smooth membranous component than to the amount of polysaccharide.

The total DNA content of the isolated hepatocytes expressed per liver is similar in 7-day PB-treated rats and in control rats. DNA:protein ratios were calculated for both light and heavy cell populations. These values appear lower than the corresponding control values reported in the ac-

companying paper, especially for the light centrolobular cells. A higher protein content of the centrolobular cells, which is related to the intense proliferation of the smooth ER, would account for these results.

The existence of a heterogeneous distribution of hepatocytic functions within a lobule is in general explained by two peculiarities of the liver: the blood supply, which is directed from the periphery to the center of the lobule, and the growth, which is restricted to the perilobular cells and supplies new cells to the center. The differential response of the centro- and perilobular cells to humoral stimuli may be partly based upon these topographical differences. Other factors that induce differentiations in hepatocytes may be even more important than the topography, namely the genetically controlled differentiations which may vary from one hepatocyte to another. All hepatocytes in a lobule are not necessarily equivalent and have not acquired the same differentiation. One may hope that further studies on isolated hepatocytes lead to the separation of different classes endowed with particular functions.

The authors are grateful to D. Ménard for stimulating discussions and help in the use of the experimental model of liver lobule heterogeneity, induced by phenobarbital. We are greatly indebted to Mr. J. Rummens for valuable technical help and to J. Verheyden for expert assistance in the preparation of the electron micrographs.

This work was supported in part by research grants of the Fonds de la Caisse Générale d'Epargne et de Retraite (C.G.E.R.). Portions of this manuscript were presented in abstract form at the meeting of the Belgian Society of Biochemistry, Louvain, February 1972 and at the Congrès de la Société Française de Microscopie électronique, Dijon, June 1973.

Received for publication 1 July 1974, and in revised form 18 February 1975.

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