CORRELATED MORPHOMETRIC

AND BIOCHEMICAL STUDIES

ON THE LIVER CELL

II. Effects of Phenobarbital
on Rat Hepatocytes

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ABSTRACT

The changes occurring in rat hepatocytes during a 5 day period of treatment with pheno-
barbital were determined by morphometric and biochemical methods, particular attention
being paid to the endoplasmic reticulum. The hepatocytic cytoplasm played an over-
whelming part in the liver hypertrophy, while the hepatocytic nuclei contributed to
only a moderate extent. The endoplasmic reticulum accounted for more than half of the
increase in cytoplasmic volume. The increase in the volume and number of hepatocytic
nuclei in the course of phenobarbital treatment was associated with changes in the ploidy
pattern. Until the 2nd day of treatment both the rough-surfaced endoplasmic reticulum
(RER) and the smooth-surfaced endoplasmic reticulum (SER) participated in the increase
in volume and surface of the whole endoplasmic reticulum (ER). Subsequently, the values
for RER fell again to control levels, whereas those for SER continued to increase, with the
result that by the 5th day of treatment the SER constituted the dominant cytoplasmic
element. The specific volume of mitochondria and microbodies (peroxisomes) remained
constant throughout the duration of the experiment, while that of the dense bodies increased.
The specific number of mitochondria and microbodies displayed a significant increase,
associated with a decrease in their mean volume. The phenobarbital-induced increase in the
phospholipid and cytochrome P-450 content of the microsomes, as well as in the activities of
microsomal reduced nicotinamide-adenine dinucleotide phosphate–cytochrome c reductase
and N-demethylase, was correlated with the morphometric data on the endoplasmic retic-
ulum.

INTRODUCTION

Phenobarbital belongs to the large and chemically
heterogeneous group of agents the metabolic con-
version of which induces in hepatocytes of verte-
brates (1, 2) a reversible adaptive response. The
metabolic situation of the "induced" hepatocyte is
mainly characterized by an increase in activity of
several microsomal enzymes which may handle
either foreign (or xenobiotic, [3]) lipid-soluble
compounds (for reviews see 4–8) or endogenous
substrates (9). Several observations indicate that
this stimulation is the result of *de novo* synthesis
(10–12). The oxidases (13) of these enzymes are,

functionally, closely linked to or integrated into an electron-transport chain which is also part of the microsomal membranes (14–16). Phenobarbital treatment of animals causes an increase in the activity of some constituent enzymes of that chain, such as NADPH-cytochrome c reductase[1] (11, 17, 18) and cytochrome P-450 (3, 11, 19, 20).

Furthermore, phenobarbital provokes an increase in liver weight and, at the subcellular level, a proliferation of smooth-surfaced membranes of the endoplasmic reticulum (21, 22), though apparently without drastically affecting the rough-surfaced variety (21, 23). Since the structural interdependences between rough-surfaced and smooth-surfaced elements of the endoplasmic reticulum are largely unknown, the disproportionate synthesis of one morphological variety under the stimulus of phenobarbital might represent a valuable model for the study of biosynthetic interrelationships between the two membrane types.

This paper presents a correlative morphometric and biochemical description of the changes in hepatocytes caused by acute treatment of rats with phenobarbital. The results of this study are based on those reported in the preceding communication dealing with the untreated liver (24).

MATERIAL AND METHODS

Animals

Male albino rats (Wistar-derived), weighing 176–227 g and fed *ad libitum* with standardized laboratory chow, were used.

Treatment

Sodium phenobarbital (100 mg/kg body weight), dissolved in distilled water, was injected intraperitoneally on 5 consecutive days. The animals were fasted for 24 hr prior to sacrifice in order to deplete glycogen.

Preparation of Microsomes

All experiments were started between 7 and 8 a. m. The rats were lightly anesthetized with ether and sacrificed by decapitation. Their livers were quickly removed, weighed, and chilled in ice. The livers were then homogenized in 5 volumes of 1.15% KCl with the aid of a glass homogenizer equipped with a

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[1] *The abbreviations used are:* NADPH, reduced nicotinamide-adenine dinucleotide phosphate; ER, endoplasmic reticulum; RER, rough-surfaced endoplasmic reticulum; SER, smooth-surfaced endoplasmic reticulum.

Teflon pestle. Both homogenization and fractionated centrifugation were performed at a temperature of $\sim 4°C$. Following centrifugation at 800 g for 10 min he resultant supernatant was centrifuged at 20,000 g for 20 min. Sedimentation of the microsomes was achieved by submitting this supernatant to centrifugation at 105,000 g for 60 min in a Spinco Model L ultracentrifuge (Spinco Div., Beckman Instruments, Inc., Palo Alto, Calif.) equipped with rotor No. 39.

Enzyme Assays

The results of biochemical assays represent averages of six animals per dose. Enzyme activity or content was expressed as indicated in the legend to Fig. 6.

NADPH-CYTOCHROME c REDUCTASE: The activity of this enzyme was determined essentially according to Williams and Kamin (25). NADPH and cytochrome c were obtained from C.F. Boehringer, Mannheim, Germany. The reduction of cytochrome c was followed at 550 mμ in a Zeiss PMQ II spectrophotometer. A millimolar extinction coefficient (mM^{-1} cm^{-1}) of 18.5 (26) was used to calculate the concentration.

CYTOCHROME P-450: The content of cytochrome P-450 in the microsomes reduced by dithionite (30 mM) was determined by the method of Klingenberg (27). The incubation system was gassed with carbon monoxide for 1 min. The difference in optical density between 450 and 490 mμ was taken as an estimate of cytochrome P-450. The relevant concentrations were calculated from the optical density values, by using a millimolar extinction coefficient of 91 (28).

N-DEMETHYLASE: The assay system used was that described by Orrenius et al. (29), except that no nicotinamide was added (30). The formaldehyde formed was determined by the method of Nash (31).

Other Assays

Protein was estimated by the method of Lowry et al. (32), with crystallized bovine plasma albumin (Armour Co. Chicago, Ill.) as a standard. For the determination of phospholipids the method of Morrison (33) was employed.

Electron Microscopy

The tissue subjected to electron microscopic examination was removed from the same livers as those used for the biochemical assays. The tissue was prepared by the methods described in the preceding paper (24).

Morphometry

As the general sampling and counting procedures employed, as well as the methods of stereological analysis, have already been described in detail in the preceding paper (24), we shall review briefly the different sampling steps. Five animals were used for each phenobarbital dose. From each rat five tissue blocks were randomly selected. From each block one large ($\sim$0.4 $\times$ 0.6 mm), thin section was cut, from which altogether 12 randomly chosen micrographs at two primary magnification levels (six micrographs at 2500 $\times$ and six at 10,000 $\times$) were made.

In contrast to our previous report (24), the sampling level I was omitted. Hence, the extralobular space of liver tissue was not estimated, and the specific volumes of different compartments refer strictly to lobular parenchyma. It was shown in the foregoing paper that the extralobular space accounts for roughly 4% of the total liver tissue. Thus, in the present investigations, the extrahepatocytic space has been underestimated by 4% in favor of the hepatocytes. The volume of the hepatocytic nuclei was determined on $\sim$1 μ thick Araldite sections stained with toluidine blue by employing two independent methods, described in detail in the preceding paper (24): (*a*) morphometric volumetry and (*b*) determination of the diameters of the hepatocytic nuclear profiles using a Zeiss Particle Size Analyzer. The profile diameters were converted into nuclear diameters with the aid of the Wicksell transformation (34). Some 350 nuclear profiles were measured per animal.

Computation and Statistical Treatment of Results

The biochemical and morphometic values were preferentially expressed per 100 g of body weight "specific dimensions," but also in terms of the average "mononuclear hepatocyte." Details of computation are to be found in our preceding paper (24). Individual biochemical and morphometric data for each phenobarbital dose were averaged, and the standard deviation of the mean (standard error, $\pm$SE) was calculated. In order to compare the means of different doses, Student's two-sided t test was used. Two means were considered to differ significantly if the probability of error P was smaller than 0.05.

RESULTS

Liver Growth

After 2 and 5 days of phenobarbital administration the relative liver weight of treated animals was significantly higher than that of the controls (Table I). As the mean specific weight of the liver (determined by pyknometry) did not change during treatment, the observed increase in wet weight reflects a proportionate increase in volume.

As shown in Fig. 1, in which the morphometric data are reproduced in the form of a histogram, the cytoplasmic compartment provided the largest contribution to the liver hypertrophy. In comparison with the controls, the cytoplasm was already

TABLE I
Changes in Relative Liver Weight

Duration of treatment	Mean body weight	Relative liver weight		
		in g/100g body weight	Increase in %	P*
	g			
Control	176.2	3.6 $\pm$ 0.040‡	—	—
16 Hr	227.3	3.7 $\pm$ 0.050	2.9	<0.1
2 Days	186.2	4.1 $\pm$ 0.064	14.2	<0.001
5 Days	212.7	4.6 $\pm$ 0.201	28.5	<0.001

* Level of significance.
‡ Standard error.

significantly enlarged by the 2nd day of treatment, and became further enlarged until the 5th day. The total volume of the cytoplasmic compartment increased by approximally 1 ml/100 g body weight during the entire period of treatment. As will be shown later, the endoplasmic reticulum (ER) played the largest part in this increase. This preponderance of the cytoplasm was also found at the level of the individual hepatocyte: as can be seen from Table II, the hepatocytic volume rose by 1032 μ^3, or 21% (Fig. 14), during the 5 day period of phenobarbital treatment; during the same interval of time the cytoplasmic volume increased by 993 μ^3 (Table II), which is likewise equivalent to a rise of 21% (Fig. 14).

In comparison with the controls, the specific volume of the hepatocytic nuclei was significantly elevated on the 5th day of phenobarbital treatment (Fig. 2); its contribution to the increase in liver volume was only moderate (approximately 0.05 ml/100 g body weight) after 5 days of pheno-
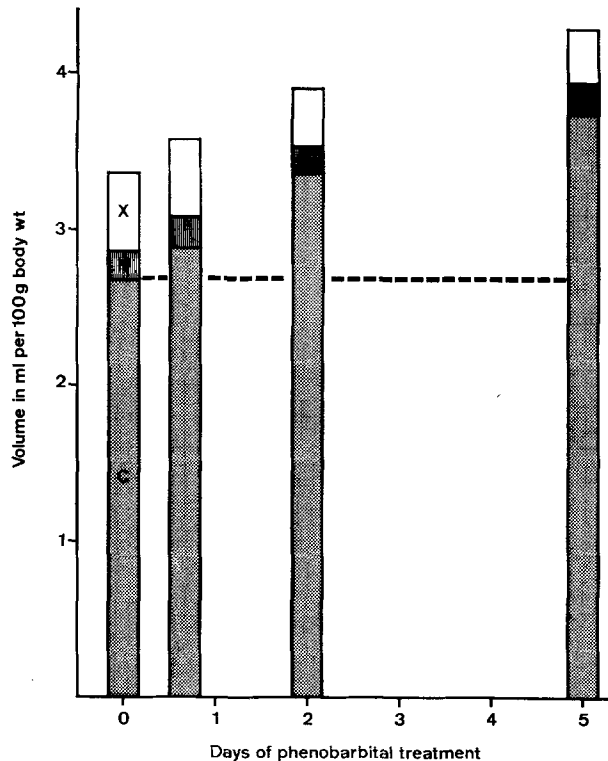
FIGURE 1 The extent to which cytoplasmic (C), nuclear (N), and extrahepatocytic (X) space are involved in liver growth induced by phenobarbital. The values for the cytoplasmic space at 2 and 5 days are significantly (P < 0.001) higher than those of the control.

Days of phenobarbital treatment

barbital treatment (Fig. 1). The cell nucleus accounted for some 40 μ^3 of the increase of 1032 μ^3 in hepatocytic volume measured at the end of the period (Table II, Fig. 14).

The specific number of parenchymal cell nuclei was already significantly higher than in the controls after the second phenobarbital injection and remained at this elevated level throughout the duration of the experiment (Fig. 2).

If the profile diameters of the cell nuclei measured in 1 μ thick Araldite sections were converted into mean nuclear diameters with the aid of the Wicksell transformation (34), the histograms reproduced in Fig. 3 are obtained for the various phenobarbital doses. In accordance with the arbitrary procedure explained in the legend to Fig. 3, these histograms were classified into three sizes, the diploid cell nuclei (2n) being assigned to Class I, the tetraploid (4n) to Class II, and the octaploid (8n) to Class III. It can be seen from Fig. 3 that the classification we have selected increases the percentage share of the 8n nuclei at the expense of the 2n nuclei, in comparison with the findings reported by various authors (35, 36) in experimentally dissociated cells from rat livers. This discrepancy might be due to the arbitrary nature

of our classification, to differences in the ages of the rats (35), or to the fact that, when cell suspensions are used, the yield of 2n cells is increased (possibly because nonhepatocytic 2n cells are also counted) and that the yield of the 8n cells is decreased for unknown reasons. In this connection, it should be pointed out that on the basis of microphotometric DNA determinations in rat liver sections Alfert and Geschwind (37) arrived at a ploidy pattern which is rather similar to ours (Fig. 3). Whatever may be the reasons for these discrepancies, the classification illustrated in Fig. 3 allows one to follow the relative shifts in nuclear size as a function of the number of phenobarbital doses. Fig. 4 shows, in the form of a histogram, the changes in the incidence of nuclei belonging to the three classes (I–III) at various times during phenobarbital treatment. After 16 hr the 2n nuclei (Class I) had appreciably increased and the 8n nuclei (Class III) had decreased, whereas the percentage share of the 4n nuclei (Class II) had undergone only a slight change. Along with this shift in ploidy the specific number of nuclei rose (Fig. 2). In the course of further phenobarbital treatment, the percentage share of 8n nuclei increased progressively, whereas that of the 2n and

TABLE II

Morphometric Parameters Expressed per Average Mononuclear Hepatocyte

Component	Control	Duration of phenobarbital treatment		
		16 hr	2 days	5 days
Number of nuclei per 100 g body weight	568×10^6	620×10^6	645×10^6	640×10^6
Hepatocyte				
Volume (μ^3)	4,938	4,806	5,225	5,970
Volume of nuclei (μ^3)	298	276	294	337
Volume of cytoplasm (μ^3)	4,640	4,530	4,931	5,633
Endoplasmic reticulum				
ER Volume (μ^3)	756	1,160	1,186	1,734
Surface (μ^2)	63,000	81,600	88,840	95,160
Rough ER Volume (μ^3)	467	666	591	516
Surface (μ^2)	37,900	41,000	41,860	37,500
Smooth ER Volume (μ^3)	289	494	595	818
Surface (μ^2)	25,100	40,600	46,980	57,660
Number of bound ribosomes	12.7×10^6	17.7×10^6	17.0×10^6	14.7×10^6
Mitochondria				
Volume (μ^3)	1,070	1,016	1,163	1,094
Number	1,665	1,693	1,628	2,203
Microbodies				
Volume (μ^3)	67	53	57	59
Number	370	439	713	708
Dense bodies				
Volume (μ^3)	41	31	40	59

The values are not corrected for systematic errors (cf. reference 24).

4n nuclei diminished (Figs. 3 and 4); at the same time, the specific nuclear volume now also displayed a significant increase (Fig. 2). Finally, it should also be pointed out that the use of tissue sections made it impossible to count binucleated and multinucleated cells.

Protein and Phospholipid Content of Microsomes

As illustrated in Fig. 5 the microsomal protein did not increase significantly until the 5th day, whereas the approximately 40% increase in phospholipid was already highly significant by the 2nd day of treatment; after five doses of phenobarbital, the phospholipid content of the microsomes per 100 g body weight had doubled.

Enzyme Activity or Content of Microsomes

Fig. 6 illustrates the activation of some constituent microsomal enzymes as a function of the duration of phenobarbital treatment. All the enzyme activities investigated underwent a highly significant increase by the 2nd day of treatment at the latest, irrespective of the terms of reference selected (per milligrams of protein, per total liver, or per 100 g body weight). In agreement with other authors (11, 29), we found that the activity of NADPH-cytochrome c reductase and of N-demethylase, as well as the P-450 content, increased in roughly linear fashion along with the number of phenobarbital doses. It should be mentioned, however, that, in contrast to the findings of Ernster and Orrenius (11, 29) and in agreement with other reports (8, 19), we found an asynchronous increase in activity[2] of the different micro-

[2] These differences in the rate at which the activity of individual microsomal enzymes increased could result, however, from the fact that we have not established optimal assay conditions for each enzyme tested. For example, we observed, in agreement with Gram
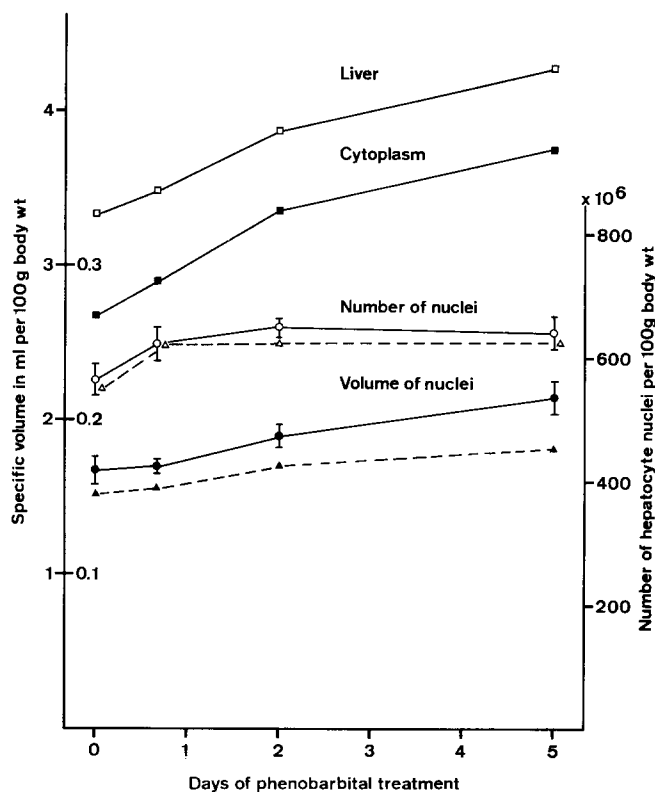
FIGURE 2   Changes in volume and number of hepatocytic nuclei: (The figures on the right of the ordinate (0.1–0.3) refer to the nuclear volume, and those on the left (1–4) to the specific volume of the liver and the cytoplasm). The nuclear volume was determined by morphometric volumetry (solid line) or by measuring the nuclear profiles and then applying the Wicksell transformation (hatched line) on $1\,\mu$ thick toluidine blue–stained Araldite sections. The value at 5 days was significantly ($P < 0.01$) higher than the control value. Why the volumes arrived at by using the Wicksell transformation should be 10–15% below those ascertained by morphometry is not known; the discrepancy might perhaps be connected with the fact that the geometrical simplifications introduced in the course of the Wicksell transformation (34) give rise to a systematic error. As regards the specific number of hepatocytic nuclei, the morphometric values (solid line) tally well with those obtained from the Wicksell transformation (hatched line). The value on the 2nd day of phenobarbital treatment differed to a weakly significant extent ($P < 0.05$) from the control value. For a detailed description of the procedures and computations used, see the preceding paper (24). For comparison, the specific volume of whole liver and of hepatocytic cytoplasm is shown. Standard errors are indicated.

somal enzymes (Fig. 6). The emergence of the individual microsomal enzyme activities during postnatal differentiation of rat hepatocytes also follows differential kinetics (38).

Cytoplasmic Components

VOLUME OF THE ER: The specific volume of the entire ER increased significantly throughout

and Fouts (39), a marked stimulation of aminopyrine demethylase by using HEPES buffer instead of Tris buffer.

the duration of treatment, whereas the corresponding values for the RER were significantly higher at 5 days (Fig. 7). The values for the volume of ER, RER, and SER per mononuclear hepatocyte are summarized in Table II, whereas Fig. 13 shows the percentage change in these values during the course of phenobarbital treatment.

SURFACE OF THE ER: The results of morphometric surface determinations are summarized in Fig. 8. The specific surface of the entire ER showed the most pronounced increase (+38%)
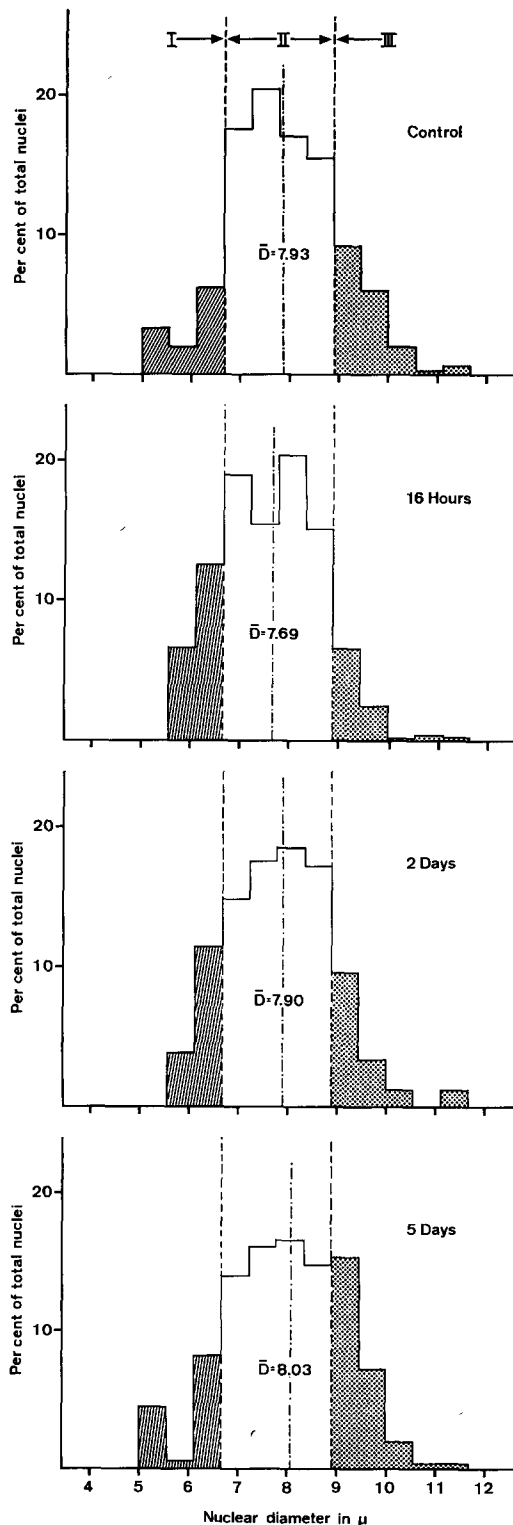
during the first 16 hr. Both the SER (+58%) and the RER (+23%) were involved in this increase. After 16 hr of treatment the surface ratio of SER to RER was raised from 0.75 in the controls to approximately 1.

During the period between 16 hr and 2 days the specific surface of the RER remained constant, whereas that of the SER increased further (+24%), though at a slower rate (Fig. 8). Between the 2nd and the fifth day of treatment the surface of the SER increased by approximately 8 m² per 100 g body weight, while that of the RER decreased by about 5.1 m² to the control levels. During this period the surface of the entire ER showed no more than a slight increase.

Although the specific volume of the SER on the fifth day of treatment was 4 times greater than the control value and the specific surface was only 2.5 times greater, the surface-to-volume ratio of the SER did not differ to a significant extent from the corresponding ratio of controls at any point during the experiment.

Table II reveals that in the course of the 5-day period of phenobarbital treatment the surface of the SER of the hepatocyte increased from 25,100 μ^2 to 57,660 μ^2, i.e. by 130% (Fig. 13).

RIBOSOMAL LOADING OF THE RER: As Fig. 9 shows, the packing density of the ribosomes on the surface of the RER membranes increased much more markedly than the latter's specific surface. In the first 16 hr of phenobarbital treatment the number of membrane-bound ribosomes per 100 g body weight showed a significant rise from 73 × 10¹⁴ to 115 × 10¹⁴, and remained at this level until the 2nd day of treatment. Between the 2nd and the 5th day of treatment the number of ribosomes decreased to the control values, as did the specific surface of the RER.

FIGURE 3 Distribution of nuclear diameters. The classification is based on the following assumptions: (a) The mean diameter $\bar{D}$ of the nuclear population in the controls was taken as being equal to the mean diameter of the tetraploid (4n) nuclei occurring most commonly in the adult liver (35); (b) The volume of diploid (2n) nuclei was half and the volume of octaploid (8n nuclei) twice that of the 4n nuclei. Hence, Class I contains the 2n nuclei with a diameter $d < 6.6\ \mu$, Class II the 4n nuclei with a diameter of 6.6 $\mu \leqslant d \geqslant 8.9\ \mu$, and Class III the 8n nuclei with a diameter $d > 8.9\ \mu$. Each histogram is based on about 1,700 measurements.
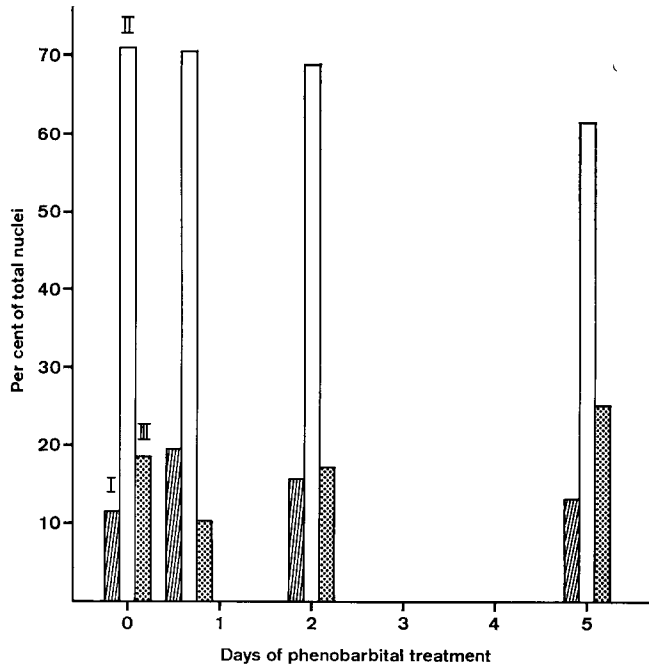
FIGURE 4 Histogram showing the frequency distribution of nuclear diameters in the three classes (I–III) as defined in the legend to Fig. 3.
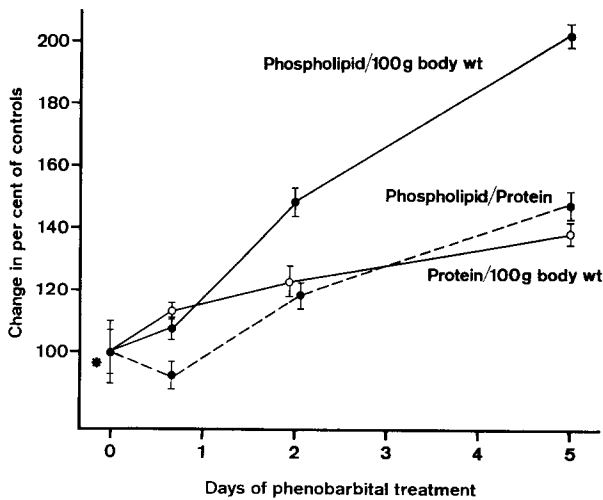

FIGURE 5 Changes in protein and phospholipid content of microsomes. By the 2nd day of treatment, the increase in phospholipid is already highly significant ($P < 0.001$), whereas the increase in protein is only weakly significant ($P < 0.05$) after 5 days. *The control values, in mg per 100 g body weight, are 89.9 ± 8.92 (± SE) for the protein and 43.4 ± 2.86 for the phospholipid. The standard error is ±7% for the phospholipid and phospholipid/protein ratio, and ±10% for the protein.

A mononuclear hepatocyte contains approximately 13 million membrane-bound ribosomes (Table II); the highest value was attained 16 hr after the commencement of phenobarbital treatment and amounted to some 18 million, which is equivalent to an increase of 40% (Fig. 13).

VOLUME OF CYTOPLASMIC PARTICLES: The specific volume of mitochondria and microbodies did not change throughout the period of phenobarbital treatment, whereas that of the

dense bodies increased significantly in comparison with the control values on the 5th day of treatment (Fig. 10). The relative constancy in the volume of the mitochondrial and peroxisomal compartment in the hepatocytes is also apparent from Table II and Fig. 14.

NUMBER AND MEAN VOLUME OF CYTOPLASMIC PARTICLE: On the 2nd and 5th day of treatment the number of mitochondria per 100 g body weight showed a significant increase
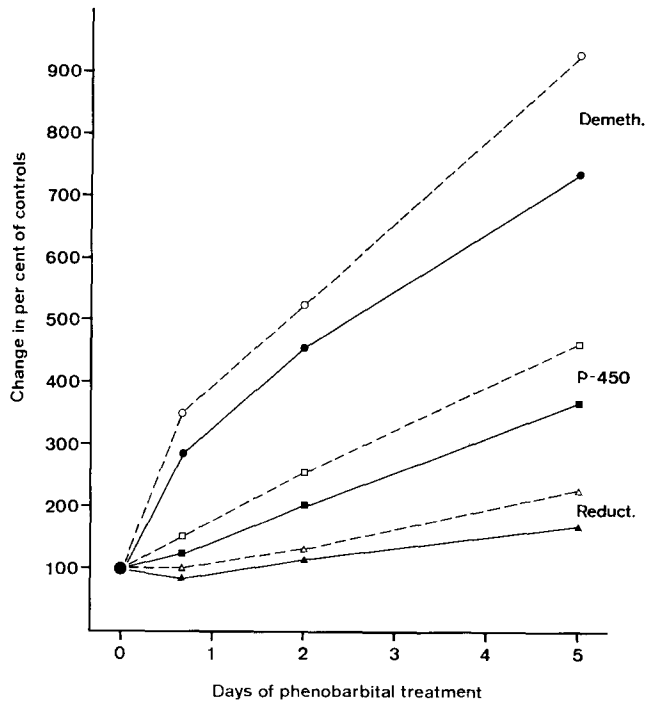
FIGURE 6 Changes in enzyme activities or content of microsomes. With the exception of NADPH-cytochrome c reductase, all the values differed highly significantly ($P < 0.001$) from the controls 16 hr after the first dose of phenobarbital. After 2 days the NADPH-cytochrome c reductase activity was likewise significantly higher ($P < 0.001$). The control values, per 100 g body weight, are 0.21 ± 0.023 ($\pm$ SE) μmoles/min for NADPH-cytochrome c reductase (Reduct.), 19.69 ± 3.37 μmoles for cytochrome P-450 (P-450), and 3.70 ± 1.05 μg formaldehyde/30 min for N-demethylase (Demeth.). Hatched lines: values expressed per 100 g body weight; solid lines: values expressed per milligram microsomal protein.

in comparison with the control values, whereas their mean volume decreased between the 2nd and the 5th day (Fig. 11). The same trend can be observed when the values are related to the individual parenchymal cell (Table II, Fig. 14).

We also determined the following parameters of mitochondrial structure: surface of the internal membranes/surface of the external membrane, (the inner layer of the external membrane being included among the internal membranes), surface/volume ratio of the internal membranes, and surface/volume ratio of the external membrane. In none of these parameters did phenobarbital treatment cause any significant changes. The corresponding values for the controls have already been given in the preceding paper (24).

Fig. 12 shows that the specific number of microbodies doubled in the first 2 days of treatment, this increase being highly significant; the number then remained at this level from the 2nd to the 5th day of treatment. As the specific volume of the microbodies did not change throughout the duration of the experiment (Fig. 10), the increase in their number is connected with a significant reduction in their mean volume occurring by the 2nd day of treatment (Fig. 12). Table II and Fig. 14 show the behavior of these

two microbody parameters in terms of the average hepatocyte.

DISCUSSION

The morphometrically determined, quantitative data on surfaces and volumes of subcellular structures in the hepatocytes of untreated rats have already been extensively discussed in the preceding paper (24).

We must also consider briefly the question of the extent to which the lobular structure of the liver might have affected our results. According to the observations of Burger and Herdson (40), the centrolobular cells of rat liver react, to begin with, in a characteristic manner to phenobarbital stimulation; in the further course of phenobarbital treatment (for up to 10 days), the subcellular changes spread to the entire lobule. On the other hand, Remmer and Merker (21) report that the peripheral hepatocytes react most sharply to phenobarbital. Orrenius and Ericsson (41) point out that the response of individual hepatocytes varies following phenobarbital administration. Our morphometric studies were based on random sampling of the liver tissue, no account being taken of the localization of the hepatocytes within the lobule. In the light of the above-mentioned,
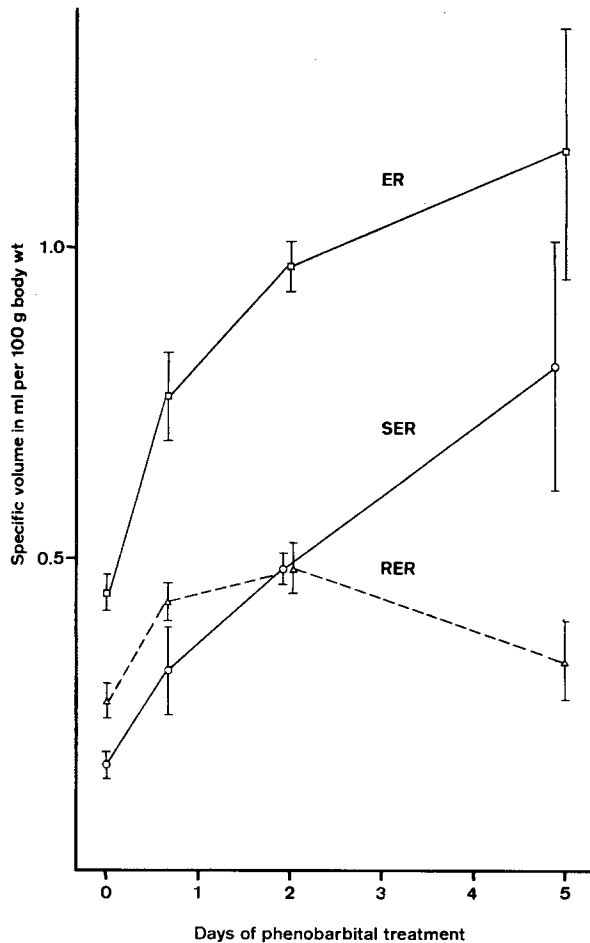
FIGURE 7 Changes in the specific volume of total (ER), rough-surfaced (RER), and smooth-surfaced (SER) endoplasmic reticulum. The levels of significance, as compared with controls, are for ER at 16 hr $P < 0.01$, at 2 days $P < 0.001$, and at 5 days $P < 0.01$; for RER at 16 hr and 2 days $P < 0.01$; for SER at 2 days $P < 0.001$ and at 5 days $P < 0.01$. Standard errors are indicated.

to some extent contradictory observations, it might be supposed that the results recorded by us tend to be minimum values; this limitation would apply particularly to the early stages of phenobarbital treatment.

Liver Growth

After five phenobarbital doses the relative weight of the liver, and thus its volume, had increased by about 30% (Table I); in absolute values, the increase in volume amounted to approximately 1 ml (per 100 g body weight). As Fig. 1 shows, the cytoplasm of the hepatocytes contributed the overwhelming part, whereas the extrahepatocytic space displayed, if anything, a tendency to decrease, although this decrease was not statistically significant. Although the specific volume of the hepatocytic nuclei underwent a significant increase (Fig. 2), its contribution to

the liver hypertrophy was to be regarded as slight (Fig. 1).

In agreement with Schulte-Hermann et al. (42), we found an increase in hepatocytic nuclei in the livers of phenobarbital-treated animals (Fig. 2). As the incidence of binucleated and multinucleated cells was not determined, it cannot be decided whether this increase resulted from an increase in multinucleated hepatocytes or from cellular proliferation.

At all events, in view of the results shown in Figs. 1 and 2, it must be assumed that the hepatomegaly induced by phenobarbital was due largely—but not exclusively—to an increase in hepatocytic cytoplasm (cf. also Table II and Fig. 14). This finding is to some extent in contrast to the data reported by Kunz et al. (43) and Barka and Popper (44), who consider phenobarbital-induced liver growth to be exclusively

the result of a hepatocytic enlargement in which the nuclear compartment is not involved at all.

However, it could be inferred from the significant increase in the number and volume of hepatocytic nuclei (Fig. 2) and the changes in nuclear size distribution (Figs. 3 and 4) that mitoses occur during phenobarbital-stimulated liver growth. In fact, several authors (10, 40, 45) have reported an increase in mitoses in the liver of phenobarbital-treated rats. More specifically, the pattern of nuclear size distribution represented in Fig. 4 is reminiscent of the findings of Nadal and Zajdela (35), according to whom the appearance of nuclei of higher ploidy in developing and regenerating rat livers is preceded by an increase in the umber of binucleated parenchymal cells. Although we did not determine the incidence of binucleated cells, it might be assumed, in line with the observations of Nadal and Zajdela (35), that the increase in 2n nuclei (possibly contained in binucleated diploid cells) occurring

16 hr after the commencement of phenobarbital treatment is causally connected with the increased proportion of 8n nuclei seen after 5 days of treatment (Fig. 4). This postulated selective increase in the incidence of binucleated diploid cells could also explain why the number of nuclei was already elevated 16 hr after the commencement of phenobarbital treatment, while the nuclear volume remained initially unchanged (Fig. 2). Whatever might be the functional meaning of polyploidization (cf. references 46, 47), it should be mentioned that ploidy shifts occur not only in hepatic differentiation and regeneration (35, 36, 48, 49), but also, for example, following carbon tetrachloride poisoning (50) and in the course of experimental inflammatory processes (51).

Attempts to Correlate Morphometry and Biochemistry

As regards drug metabolism, the surface of the ER—particularly the surface of the smooth-
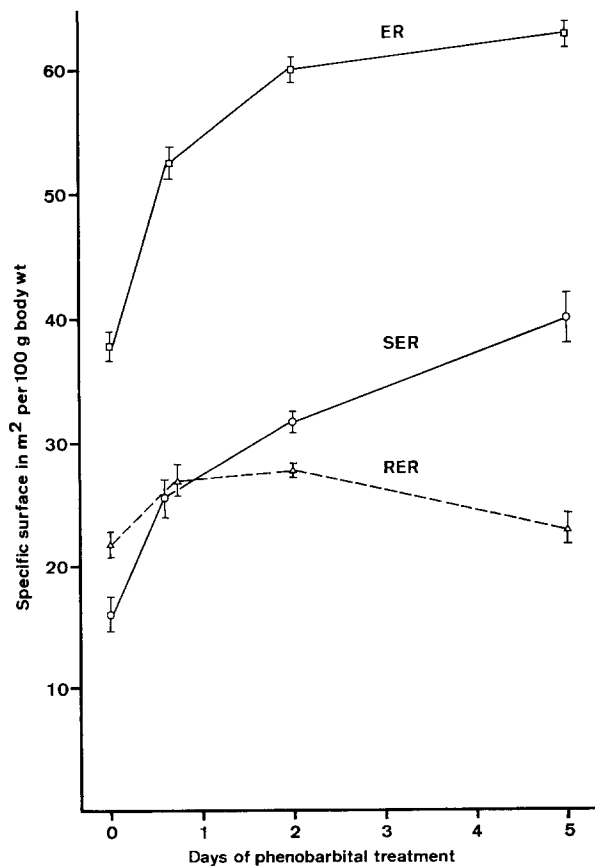

FIGURE 8 Changes in the specific surface of total (ER), rough-surfaced (RER) and smooth-surfaced (SER) endoplasmic reticulum. The levels of significance, as compared with controls, are for ER at 16 hr $P < 0.01$; at 2 and 5 days $P < 0.001$; for RER at 2 days $P < 0.01$; for SER at 16 hr $P < 0.01$ and at 2 and 5 days $P < 0.001$. Standard errors are indicated.
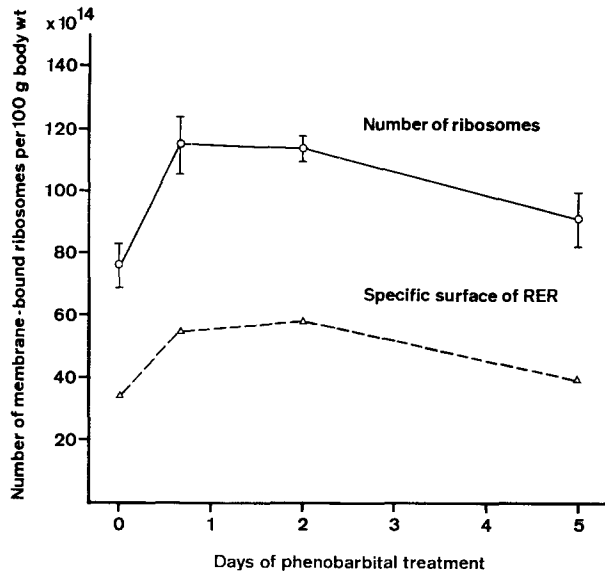
FIGURE 9 Changes in ribosomal loading of rough-surfaced membranes. The values at 16 hr and 2 days differ significantly ($P < 0.01$) from the controls. For comparison, the specific surface of RER (cf. Fig. 8) is shown. Standard errors are indicated.
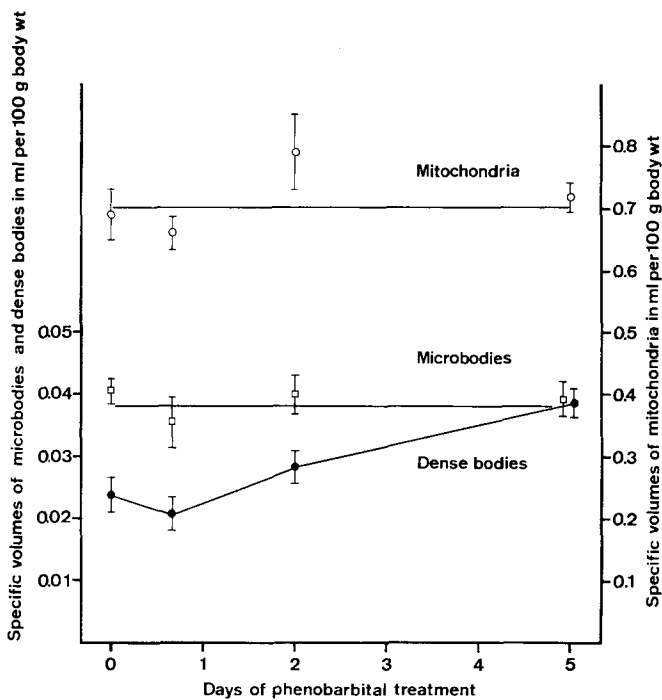

FIGURE 10 Changes in specific volume of mitochondria, microbodies, and dense bodies. The value of dense bodies after 5 days of treatment is significantly ($P < 0.01$) higher than the control. Standard errors are indicated.

surfaced variety—is more relevant from the point of view of function than the volume of the ER. The reasons for this are as follows:

1. Over the entire period of treatment the specific surface of the ER increased by approximately 25 m² (per 100 g body weight); of this

value, some 24 m² was accounted for by the SER alone (Fig. 8). In agreement with the findings reported by other authors (10, 22, 29, 52–58), this proliferation of the ER was reflected in an increase in microsomal proteins (Fig. 5). In common with Ernster and Orrenius (11), we
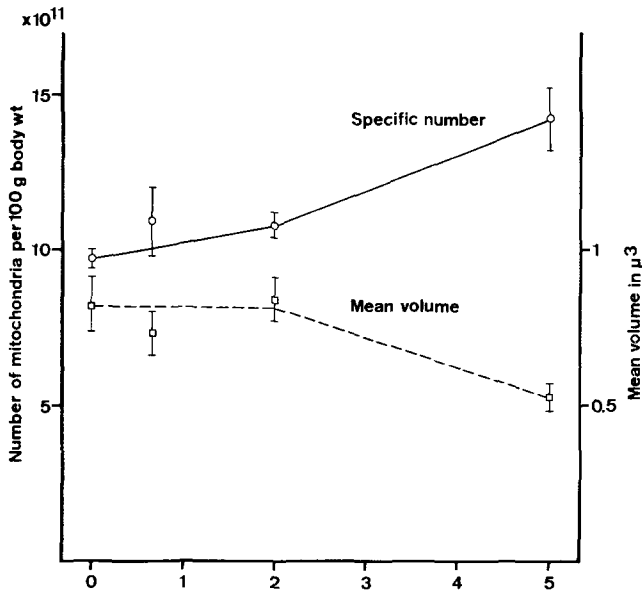
FIGURE 11 Changes in specific number and mean volume of mitochondria. After 2 and 5 phenobarbital doses the values of specific number differ significantly from the controls ($P < 0.05$ and $P < 0.01$, respectively). Standard errors are indicated.
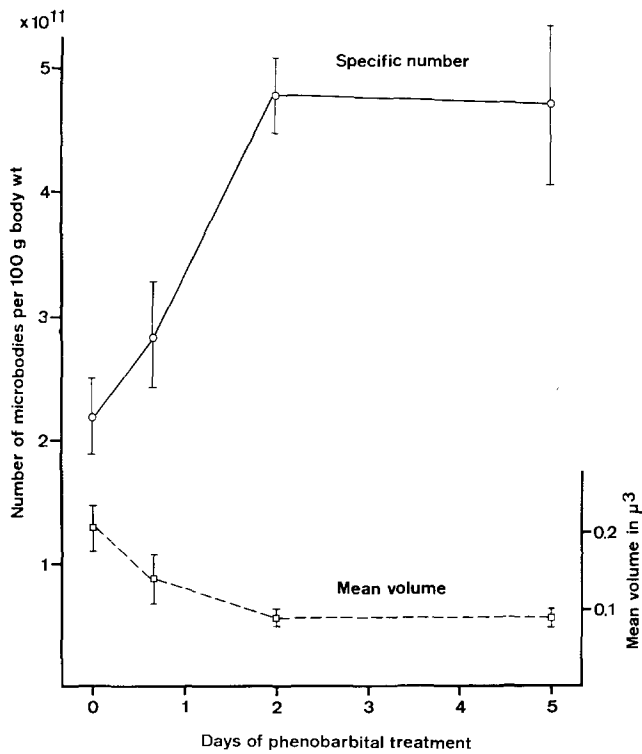

Days of phenobarbital treatment

FIGURE 12 Changes in specific number and mean volume of microbodies. For specific number, the level of significance is $P < 0.001$ at 2 and 5 days. The mean volume, as compared with the controls, is significantly decreased ($P < 0.05$) by the 2nd and 5th day of treatment. Standard errors are indicated.

found that the microsomal phospholipid content increased at a faster rate than the protein (Fig. 5). This disproportionate rise in the phospholipid content was no doubt due to the preferential ac-

cumulation of SER elements in hypertrophied livers. The assumption is supported by the observations of other authors (22, 29) who have reported that the phospholipid content of en-

riched SER fractions of phenobarbital-induced livers increases more markedly than that of the corresponding RER fractions.

2. The electron-transfer chain, which participates in the oxidation of foreign substances (and probably also of endogenous substrates [9]), is a constituent component of the ER membranes and probably also develops a greater activity in the SER than in the RER (39, 59, 60). Furthermore, the extent to which substrates for hydroxylation are bound is proportional to the concentration of the terminal oxidase in this
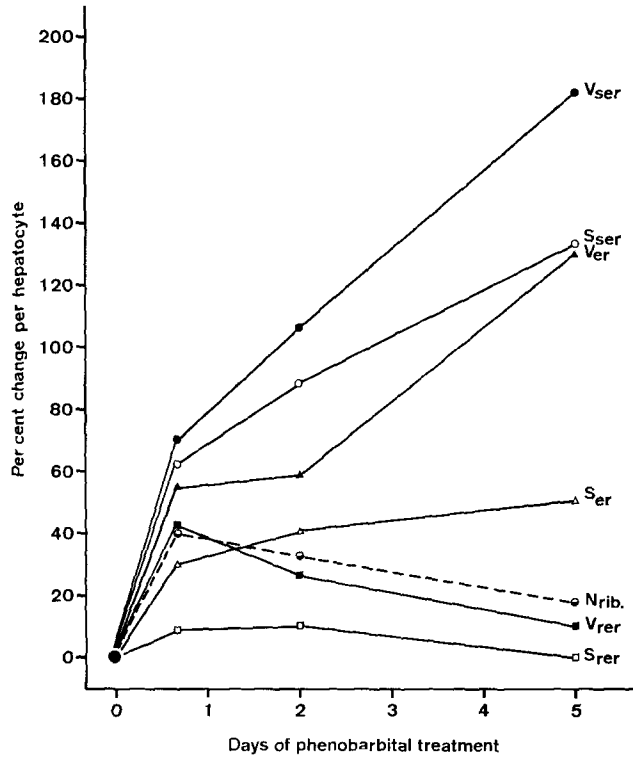

FIGURE 13 Percentage changes, in terms of an average hepatocyte, in volume (V) and surface (S) of the various components of the endoplasmic reticulum. N_{rib}: Number of membrane-bound ribosomes.
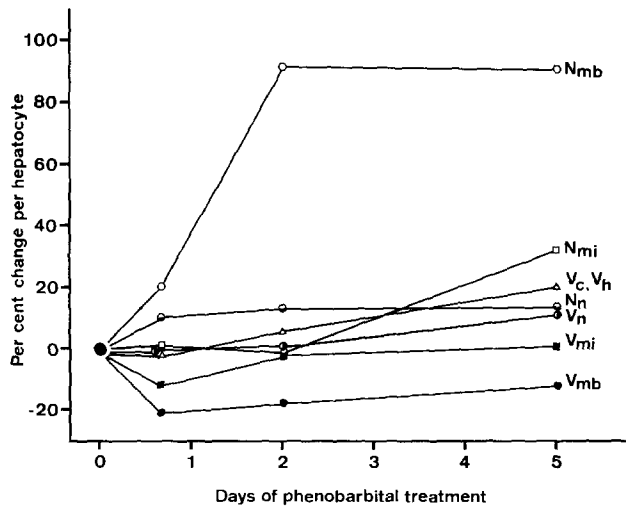

FIGURE 14 Percentage changes, in terms of an average hepatocyte, in volume (V) and/or number (N) of hepatocytes (h), cytoplasm (c), cell nuclei (n), mitochondria (mi), and microbodies (mb).
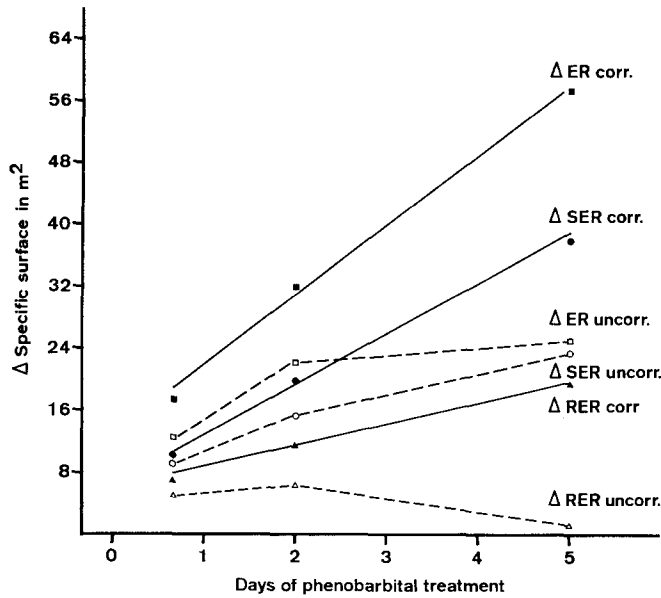
Figure 15   Correction of the specific surface of total (ER), rough-surfaced (RER), and smooth-surfaced (SER) endoplasmic reticulum for membrane catabolism. As explained in the text, the increments in the various ER membranes in relation to the controls (Δ uncorrected) can be linearized on the assumption that the increments recorded in response to phenobarbital (Δ corrected) are the result of accelerated synthesis and reduced break down operating simultaneously.
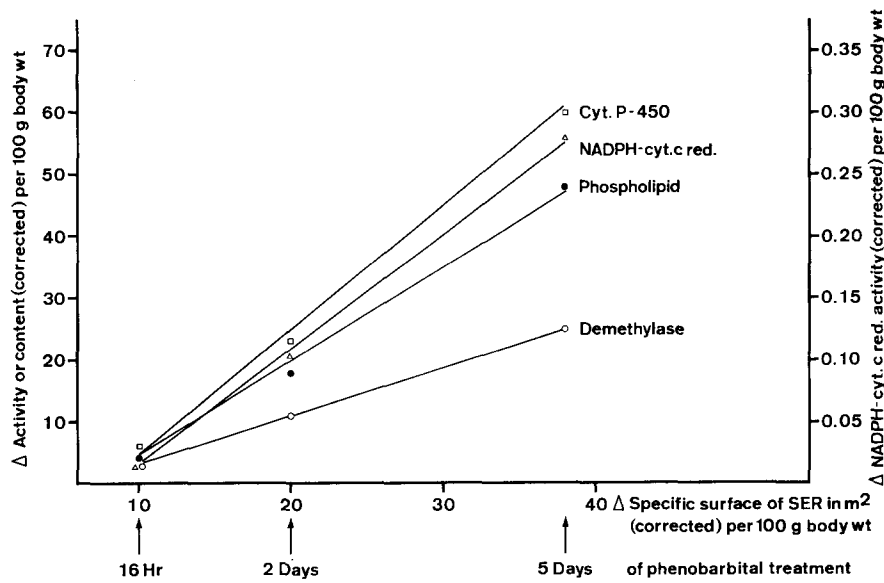

Figure 16   Linear relationship between the increments in specific surface of smooth-surfaced endoplasmic reticulum (SER) and in activity or content of microsomal enzymes. As described in the text, not only the surface increments of the SER can be corrected for inhibited breakdown in response to the various doses of phenobarbital (arrows) (Δ specific surface of SER (corrected)), but also the increase in the activity of the constituent ER enzymes in relation to the control value (Δ activity or content [corrected]).

chain, cytochrome P-450 (61, 62), and hence to the membrane surface of the ER. In view of these considerations, the surface of the ER or SER was selected as reference parameter in our

attempts to correlate morphometric and biochemical findings.

Ernster et al. (11, 29), and Orrenius and Ericsson (41) have obtained evidence that the

increase in cellular membranes induced by phenobarbital treatment can be attributed to *de novo* synthesis. On the other hand, Shuster and Jick (57), in the light of kinetic studies on liver microsomes of phenobarbital-treated mice, came to the conclusion that the accumulation of membranes must be regarded as the result of a simultaneously operating acceleration of synthesis and inhibition of catabolism of ER membranes. It can be deduced from Fig. 8 that the specific surface of the ER and of its two structural components does not increase linearly in function of the time. We have converted the data on which Fig. 8 is based in order to shed some light on the characteristics of membrane development; for this purpose, the following assumptions were made:

1. For each phenobarbital dose we used the increments (denoted by the symbol $\triangle$ in Figs. 15 and 16) in the specific surface of the different ER membranes with respect to the controls, instead of the absolute values.

2. In line with the findings of Shuster and Jick (57) we assume a phenobarbital-induced prolongation in half-life from 3.5 to 7 days for the preexisting and newly formed ER membranes. The catabolic decay of membranes was assumed to be of constant rate. The results of this correction are illustrated in Fig. 15. It can be seen that, in agreement with Fig. 8, the absolute increase ($\triangle$ uncorrected) in the specific surface of the ER membranes is not linear in function

of time; if, on the other hand, the surface increment is regarded as the result of *de novo* synthesis and catabolism, a reasonable linearization of the surface increment ($\triangle$ corrected) is obtained. In other words, during the first 5 days of phenobarbital treatment the ER membranes appear to be synthesized at an approximately constant rate.

We then corrected the increments of the other ER parameters—e.g. microsomal content of phospholipids (Fig. 5) and of cytochrome P-450 (Fig. 6), and the activities of the other constituent membrane enzymes (Fig. 6)—in the same way as for the ER membranes in order to allow for degradation (63). As the enzyme activities were determined in the total microsomal fraction (SER and RER), the activity or content increments had to be distributed among the corrected SER and RER surface increments. Finally, the surface increments (corrected) could be plotted against the activity increments (corrected); Fig. 16 shows that, on the premises we selected, a colinearity between proliferation of the ER membranes (as measured by morphometry) and synthesis of their constituents (as assayed by biochemical methods) becomes apparent. This kinetic agreement suggests that with these two methods one and the same process—e.g. membrane synthesis—is being observed at two different levels of organization (individual constituent molecules versus assembly of those molecules to form a membrane).
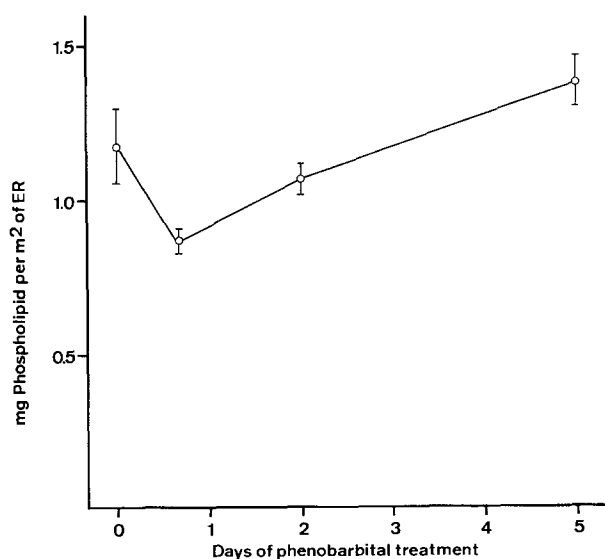

FIGURE 17 Change in loading of membranes of the endoplasmic reticulum (ER) with phospholipids. The value at 16 hr is significantly ($P <$ 0.05) smaller than the control value. Standard errors are indicated.

If attention is directed not so much to the increments in the individual ER parameters as to their whole amount, a comparison of Figs. 5, 6, and 8 shows that, in the first 16 hr after the injection of phenobarbital, for example, the specific surface of the entire endoplasmic reticulum (ER in Fig. 8) increases more rapidly than that of the other membrane components (Figs. 5 and 6). If the ratio mg phospholipid per unit of ER surface is taken as a measure of surface loading for the various phenobarbital doses, the degree of loading will be found to be significantly reduced 16 hr after injection (Fig. 17). If the surface loading of the ER membranes with enzymes is calculated in a similar way, a more or less pronounced deficit—except in the case of N-demethylase—will likewise be found to occur 16 hr after the administration of phenobarbital.

Assuming a loss of about 50% of microsomal phospholipid during differential centrifugation (64, 65) and a mean molecular weight of 800 for an average phospholipid molecule, we can calculate that the latter would occupy an area of approximately 100 A^2. This value is of the same order of magnitude as that reported for natural or synthetic phospholipid membranes (66–68). In membranes with a relative phospholipid deficit (Fig. 17), the area occupied by a phospholipid molecule would have to increase to about 150 A^2; a similar situation is encountered in soaps in which, as their concentration decreases, the area occupied by their polar groups increases (69).

Any discussion about the extent to which the occurrence of ER membranes relatively depleted of structural elements and enzymes (Fig. 17) has a bearing on membrane structure and development (phase transitions, cf. references 70, 71) and drug metabolism (binding of the substrates and removal of the more hydrophilic reaction products; interactions between constituent enzymes and lipids [72–74]) will continue to be highly speculative until relevant biochemical data are available. In addition, it is conceivable that the deficits we observed are due to the fact that the distribution of the ER membranes among the individual subcellular fractions differs quantitatively at various points in time during phenobarbital induction; morphometric analysis on the other hand is invariably based on the entire ER present in situ.

As already mentioned, the number of membrane-bound ribosomes increased most markedly during the first 16 hr of phenobarbital treatment (Fig. 9). It is striking to note that this time is also the time at which the ER membranes underwent their sharpest increase (Figs. 7 and 8). As the specific surface of the RER (Fig. 8), increased to a lesser extent than the number of ribosomes (Fig. 9), the RER surface must be more densely loaded with ribosomes. Kato et al. (56) found in the livers of phenobarbital-treated rats a greater amount of membrane-bound ribosomes which was similar in extent to our values. Findings from the same (54, 56, 75) and another laboratory (76) revealed that the amino acid incorporation by liver microsomes is stimulated by phenobarbital; this observation, too, could be accounted for by a more dense loading of the enlarged specific surface of the RER (Fig. 8) with ribosomes.

Membrane Synthesis and Conversion

The model of membrane synthesis which has been most extensively studied to date is the postnatal proliferation of the ER in rat hepatocytes. As regards our observations, the relevant results of these investigations conducted by Omura et al. (77) and Dallner et al. (38, 65, 78) are briefly as follows:

1. The increase in the ER membranes takes place in two phases, inasmuch as the enlargement of the RER portion precedes chronologically the increase in SER; this sequential appearance of RER and SER and the large measure of agreement between their respective chemical compositions suggests that the mechanism of smooth-membrane generation is based on a possibly reversible detachment of ribosomes from the RER.

2. The ribosomes on the rough-surfaced membranes are densest at the time when the RER is growing at its fastest rate.

3. The morphological and biochemical evidence as a whole is most readily compatible with a model of membrane synthesis based on the expansion of a preexisting membranous framework, the individual constituent components of which enter and leave the macromolecular assembly at varying rates (asynchronously).

Before we compare the events occurring in phenobarbital induction with those encountered in hepatocytic differentiation, reference must be made to two further points. As already explained in the previous paper (24), in our morphometric

determinations of the volume and surface of the ER we adopted the convention that measuring points falling on ribosome-free stretches of RER[3] should be allocated to the SER. In other words, our measurements did not differentiate between ribosome-free zones belonging, as regards their structural appearance and subcellular localization, to the RER and "typical" SER which appears in the form of a tightly meshed network of smooth-surfaced tubules and vesicles. Owing to this convention, it is, for example, impossible to ascertain how much of the phenobarbital-induced increase in SER surface is accounted for by "intercalated" SER and how much by typical SER. As, however, ribosomal loading of the rough-surfaced membranes is significantly increased 16 hr and 2 days after the administration of phenobarbital (Fig. 9), the intercalated SER may not make a substantial contribution to the increment in the specific surface of the SER.

Phenobarbital-induced proliferation of ER in the hepatocytes of male mice (57, 63) and rats (79) is the outcome of a simultaneously operating stimulation of membrane synthesis and inhibition of membrane catabolism. This factor obscures the conditions obtaining during drug induction; it is, for example, not known whether degradation is slowed down to the same extent in both types of ER membrane. In addition, the studies conducted by Holtzman and Gillette (79) suggest that the half-life of microsomal phospholipids, which represents the net product of synthesis and catabolism, may not remain constant during phenobarbital induction.

After these remarks, we can now compare the two models of membrane proliferation—hepatocytic differentiation and phenobarbital induction. In contrast to the conditions obtaining in the differentiation model (38, 65, 77, 78), membrane growth following repeated administration of phenobarbital does not take place in two phases; on the contrary, RER and SER increase simultaneously in volume (Fig. 7) and surface (Fig. 8), the volume and surface of the entire ER rising towards a new steady state (Figs. 7 and 8). In the case of prenatal and postnatal differentiation of liver parenchymal cells, the sequential

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[3] To make these remarks easier to understand, we should like, for the purposes of the present study, to use the term "intercalated SER" to denote that portion of the SER which is inserted mosaic-like between rough-surfaced cisternal stretches.

increase in RER and SER clearly suggests that the RER plays the role of a precursor, with the transformation of RER into SER being brought about by the detachment of ribosomes from rough-surfaced cisternae within a relatively short time (a maximum of 3 days). In the case of phenobarbital induction, the morphogenetic correlations between RER and SER are less obvious, probably because of the influence exerted, as already mentioned, by membrane catabolism; the SER must develop as a result of a continuous process taking place at the same time as RER synthesis. This mechanism might consist of a budding-off (41) of smooth-surfaced elements on the cisternal ends of the RER, which would account for the continuities frequently observed (23, 41, 80, 81) between the two types of membrane (Fig. 18). If one accepts the hypothesis that the whole of the SER is derived from the RER as a result of ribosomal detachment, it can be calculated that this transformation may assume considerable proportions. On the assumption that the phenobarbital-induced inhibition of catabolism (57) affects RER and SER to the same extent, some 17 m^2 of RER per 100 g body weight must be formed in the first 16 hr after the administration of phenobarbital; of this value, 9.4 m^2 (or roughly 0.6 $m^2/100$ g body weight per hr) must be transformed into SER, 5.1 $m^2/100$ g body weight remaining in the RER as a net gain (Fig. 8). The remaining 2.5 m^2 would then be degraded.

During the postnatal differentiation processes in the hepatocyte the loading of cisternal surface with ribosomes is at its maximum at the time when RER proliferation is proceeding at its fastest rate (65). Similar conditions are encountered in the phenobarbital-induced growth of the ER membranes (Fig. 9); this similarity suggests that the RER is expanded mainly by the mosaic-like incorporation of intercalated SER, which would have to be occupied by ribosomes immediately after insertion into the performed rough-surfaced cisternae. This process might be responsible for the enlargement in the RER surface and simultaneous increase in ribosomal loading observed in both models; the number of membrane-bound ribosomes would thus have to increase considerably (Fig. 9), and the increased demand for constitutive membrane proteins could then be met. In this case, the RER → SER transformation would have to be regarded as a
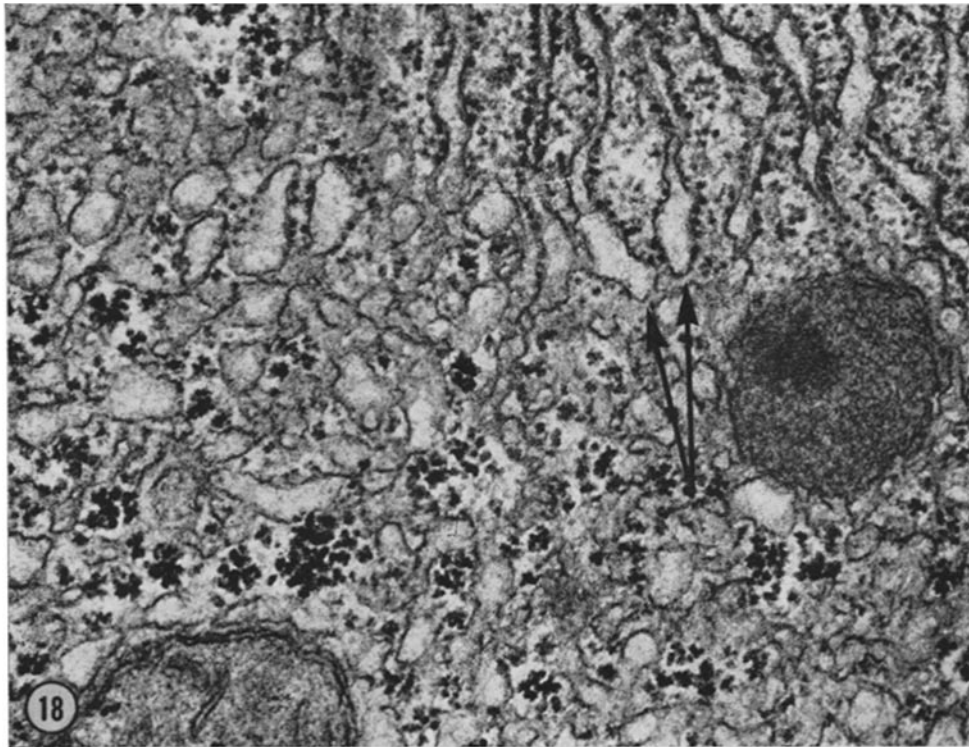
FIGURE 18   Part of the cytoplasm of a hepatocyte after 5 days of phenobarbital treatment, showing a zone of close spatial relationship (arrows) between RER and SER. × 60,000.

vectorial flowing-off of SER elements—accompanied at the same time by detachment of membrane-bound ribosomes—from the cisternal ends (Fig. 18). This hypothetical course of events would be compatible with the framework model designed by the group of Palade (38, 77) and discussed at the beginning of this section.

Cytoplasmic Particles

Phenobarbital stimulates certain oxidizing functions of the liver mitochondria (82). The specific volume of these mitochondria did not change throughout the duration of treatment, but their number increased between the 2nd and 5th day of treatment while the mean volume decreased. Those structural parameters of the mitochondria which might perhaps provide the most direct measure of their oxidizing function—such as, for example, the ratio between the surface of the internal membranes and the volume—did not undergo any substantial change. We demonstrated in a previous study that the ad-

ministration of chlorophenoxyisobutyrate is followed not only by SER proliferation but also by an increase in the peroxisomes' share of the volume (83, 84). Phenobarbital, on the other hand, did not affect the specific volume of the microbodies (Fig. 10); in line with morphological observations (40), all that could be seen was an increase in number accompanied by a decrease in mean volume. At present we are unable to explain these changes.

Of the cytoplasmic particulate structures, only the dense bodies showed, towards the end of treatment, an appreciable increase in specific volume (Fig. 10), which may possibly be connected with the intracellular break down of organelles (40, 85). Like other authors (21, 23), however, we were unable to find any major structural changes in our preparations. Schmid et al. (86) recently reported that cytochrome P-450 found in the ER membranes constitutes an important precursor of bile pigments and that phenobarbital markedly stimulates the trans-

formation of microsomal hemoprotein into bilirubin. In view of the occurrence of enzymes possessing a high phospholipase activity in rat liver lysosomes (87), these organelles might conceivably be involved in this process in a way suggested by the observation of Bruni and Porter (88).

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