Table 1. Mobilization of polymorphonuclear (PMN) leukocytes in the uterine or abdominal activities in response to metallic copper; IU, intrauterine; IA, intraabdominal.

Device	Site	Time in place (mo)	PMN*
	Rats		
None	IU		0
Copper	IU	~1/3	+++
Polyethylene	IA	3	0–+
Polyethylene + Cu	IA	3	++++
	Monkeys		
None	IU		0
Polyethylene	IU	7	0–+
Polyethylene + Cu	IU	7	+++
Polyethylene	IA	3	+
Polyethylene + Cu	IA	3	++++

* PMN infiltration or exudation at or near device as estimated from microscopic examination.

abdominal or the uterine cavities of both rats and monkeys induced an impressive accumulation of leukocytes on the surface of the device and in the neighboring tissues. These leukocytes were mainly neutrophils; small numbers of eosinophils and macrophages were also present. In contrast, leukocyte accumulation in response to plain polyethylene devices was scanty or absent. The cellular response to copper persisted for at least 7 months, without detectable local tissue damage or systemic effects on the host. The mechanism by which copper induced the local accumulation of leukocytes is not clear. Previous studies (*4*) have shown that metallic copper is very slowly dissolved in the uterine cavity; presumably the cuprous ions then interacted with tissue components to produce conditions chemotactic for neutrophil leukocytes.

The means by which polymorphonuclear leukocytes or products liberated from these cells exert an antifertility effect in the uterine lumen is not known. In some situations products liberated from disrupted leukocytes, or other cells, have been shown to interfere with maturation or even with survival of the newly fertilized egg (*5*). Thorough studies have not yet been made of other possible antifertility effects, such as spermicidal action or interference with implantation.

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References

1. J. A. Zipper, H. J. Tatum, M. Medel, L. Pastene, M. Rivera, *Amer. J. Obstet. Gynecol.* **109**, 771 (1971); C. C. Chang and H. J. Tatum, *Contraception* **1**, 265 (1970).
2. E. L. Parr, R. W. Schaedler, J. G. Hirsch, *J. Exp. Med.* **126**, 523 (1967).
3. S. El Sahwi and D. L. Moyer, *Fert. Steril.* **22**, 398 (1971).
4. J. Zipper, M. Medel, L. Pastene, M. Rivera, H. J. Tatum, *Nobel Symp.* **15**, 199 (1970).
5. E. L. Parr, *Biol. Reprod.* **1**, 1 (1969); D. M. Smith, S. El Sahwi, N. Wilson, D. L. Moyer, *ibid.* **4**, 74 (1971).

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Diurnal Rhythm in Endoplasmic Reticulum of Rat Liver: Electron Microscopic Study

Abstract. *Electron microscopy of rat hepatocytes revealed a diurnal variation in the relative amounts of endoplasmic reticulum structures and regional differences in their distribution within the hepatic lobule. The diurnal changes in smooth and rough endoplasmic reticulum structures were compared with the diurnal changes in the hepatic microsomal enzyme hexobarbital oxidase. In the control group, at the time when enzyme activity was maximum, the amount of smooth endoplasmic reticulum was also maximum and vice versa. When the enzyme rhythm was abolished, as in blinded rats, the diurnal rhythm in the endoplasmic reticulum was also abolished.*

Many drug-metabolizing enzymes are localized in the microsomal fraction of liver homogenate (*1*). The microsomal fraction is composed of fragments of endoplasmic reticulum (ER), which, by electron microscopy, appear to consist of smooth- and rough-surfaced structures. The development of pharmacologic procedures that alter the activities of these drug-metabolizing enzymes has permitted electron microscopic studies of the associated changes in the morphology of the ER. For example, agents such as 3,4-benzopyrene, 3-methylcholanthrene, phenobarbital, aminopyrine, and phenylbutazone, when given to rats, produce a rapid increase in the activity of many enzymes, as determined chemically in the microsomal fraction (*2*), and, concomitantly, produce ultrastructural changes in the ER (*3*) of intact liver cells. In phenobarbital-treated rats, Remmer and Merker found an increase in smooth ER, and this increase reached a maximum when the enzyme activity was highest (*3*). Similar results were noted with other enzyme inducers—tolbutamide, nikethamide, phenylbutazone, and meprobamate (*4*).

Others have also found a relation between the induced enzyme synthesis and the stimulated formation of ER membranes (*5*, *6*). Dallner *et al.* have studied the relation between microsomal enzyme activities and ER membranes in early development (*7*). The activity of many of these enzymes is low at birth and increases at different rates soon afterward. Extensive synthesis of microsomal membranes occurs in the livers of rat fetuses toward the end of gestation. These appear first as rough-surfaced ER. After the fetus is born, two different processes take place: (i) the production of a large amount of smooth ER membranes and (ii) the appearance of membrane-bound enzyme activities in a characteristic temporal sequence. The implication of the findings of Dallner *et al.* (*7*) is that the ER can be a dynamic rapidly changing structure.

A daily rhythmicity has been observed for some of the hepatic drug-metabolizing enzymes (*8–10*). For hexobarbital oxidase (HO) and *p*-nitroanisole-*O*-demethylase (OD), Nair and Casper (*9*) found that, in rats, the enzyme activities were high in the night (10 p.m. and 2 a.m., respectively) and low during the day (2 p.m. and 6 p.m., respectively). For HO, the values at 10 p.m. were 44 percent higher than those at 2 p.m. and for OD, the 2 a.m. values were 52 percent higher than those at 6 p.m. The results from measurements in vitro of HO activity in the microsomal fraction have been further supported by parallel determinations in vivo of the duration of sleep induced by hexobarbital. Furthermore, Nair *et al.* (*10*) noted that the rhythmicity of enzyme activity is abolished in animals exposed to continuous illumination, continuous darkness, or blinding. In these experiments, animals were killed every 4 hours.

Thus studies by electron microscopy have revealed changes in the endoplasmic reticulum parallel to the alterations in hepatic microsomal enzyme activity. Previous investigators have examined the changes associated with (i) the normal developmental increase in enzyme activity taking place during growth and (ii) the increase in activity brought about by chemical inducers. The findings of a diurnal variation in enzyme activity provide another experimental

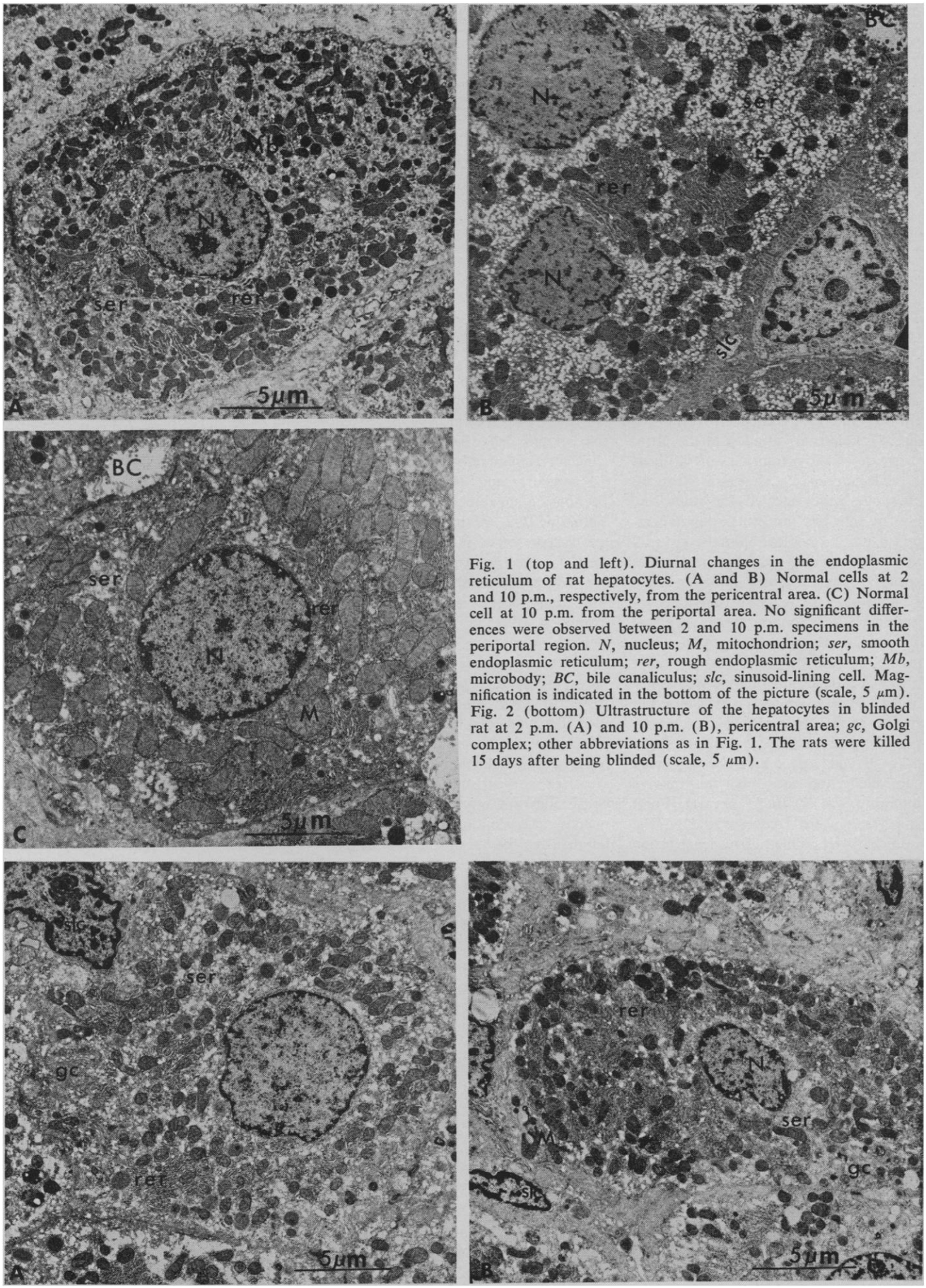

Fig. 1 (top and left). Diurnal changes in the endoplasmic reticulum of rat hepatocytes. (A and B) Normal cells at 2 and 10 p.m., respectively, from the pericentral area. (C) Normal cell at 10 p.m. from the periportal area. No significant differences were observed between 2 and 10 p.m. specimens in the periportal region. *N*, nucleus; *M*, mitochondrion; *ser*, smooth endoplasmic reticulum; *rer*, rough endoplasmic reticulum; *Mb*, microbody; *BC*, bile canaliculus; *slc*, sinusoid-lining cell. Magnification is indicated in the bottom of the picture (scale, 5 μm). Fig. 2 (bottom) Ultrastructure of the hepatocytes in blinded rat at 2 p.m. (A) and 10 p.m. (B), pericentral area; *gc*, Golgi complex; other abbreviations as in Fig. 1. The rats were killed 15 days after being blinded (scale, 5 μm).

Table 1. Semiquantitative estimates of the smooth (SER) and rough (RER) endoplasmic reticulum structures in rat hepatocytes. The numbers in parentheses represent the number of cells examined in each area and those for SER and RER represent the mean values of the positives obtained from the total number of cells examined (for details see text). The number of animals in each time period ranged from three to six. The data were analyzed by the Mann-Whitney U test; Cen, central; Mdz, midzonal; Por, portal.

Item	2 p.m.			10 p.m.		
	Cen	Mdz	Por	Cen	Mdz	Por
			Normal			
No.	(33)	(24)	(35)	(48)	(12)	(32)
SER	2.7+*	2.5+	2.1+	3.4+*	3.0+	2.1+
RER	2.4+	2.1+	1.9+	2.0+	2.6+	2.2+
			Blinded			
No.	(11)	(9)	(12)	(9)	(6)	(18)
SER	3.0+	3.2+	2.5+	2.9+	1.8+	2.2+
RER	1.5+	2.0+	1.8+	2.3+	2.2+	2.5+
			Phenobarbital treated			
No.	(12)	(7)	(12)			
SER	4.9+	5.0+	3.4+			
RER	2.8+	2.7+	2.6+			

* $P < .009$.

situation to study the relation between ultrastructure of ER and microsomal enzyme activity. We now present evidence for diurnal variation in the ultrastructure of ER membranes of rat liver.

Male Sprague-Dawley rats, 70 to 80 days old, were selected for study and divided into two groups. Group 1 comprised normal control animals; group 2 rats were rendered blind by enucleation; group 3 consisted of animals (30 to 40 days old) treated with four daily injections of phenobarbital (75 mg/kg) and were included as a control for our method since the ER changes after phenobarbital stimulation are well established (*3*). Two animals were housed in each cage in a room in which the temperature (72° to 75°C) and light (light on at 6 a.m. and off at 6 p.m.) were controlled, and in which the relative humidity was 50 to 55 percent. Food and water were freely available. The animals were killed by decapitation (group 2 at 15 days after blinding, and group 3 at 24 hours after the last injection) either 2 p.m. or 10 p.m., the previously observed periods of low and high enzyme activity, respectively, for HO (*8–10*). For enzyme assay, a part of the liver was removed from the periphery of the right lobe, blotted free of blood and tissue fluids, weighed, frozen on a bed of Dry Ice, and stored at −20°C until assayed (freezing and storage had no influence on the assays). Tissue from contiguous areas was used for electron microscopy. Hexobarbital oxidase activity was determined in a whole liver homogenate system as described (*9, 10*).

For electron microscopy, cubes of liver tissue (~ 1 mm^3) were rapidly immersed in 2.5 percent cold glutaraldehyde. After 3 hours, they were transferred to a cold solution of sodium cacodylate containing 3 percent sucrose at *p*H 7.4, postfixed in osmium tetroxide for 2 hours, dehydrated with increasing concentration of ethanol (starting with 70 percent), and embedded in Epon. Sections, 2 μm thick, were stained with Toluidine Blue and used for selection of the best preserved blocks. Thin sections were obtained from at least two lobules for each animal; the grids were stained with uranyl acetate and lead citrate and examined with an RCA EMU 3612 electron microscope. In rat hepatocytes, the distribution and characteristics of the cytoplasmic organelles varied with respect to their location within the lobule. Because of this heterogeneous distribution of the organelles, we felt it necessary to delineate and specify the areas of comparison for purposes of consistency and reproducibility. For the purpose of evaluation within the hepatic lobule, the first seven layers of cells around the central veins and portal tracts were considered pericentral and periportal, respectively, whereas the intermediates were classified as midzonal.

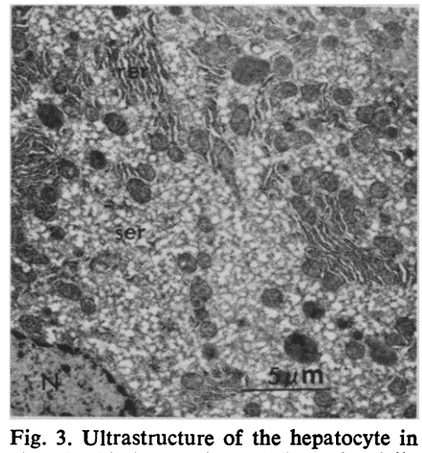

Fig. 3. Ultrastructure of the hepatocyte in phenobarbital-treated rat (75 mg/kg daily for 4 days and killed 24 hours after the last injection); pericentral area, 2 p.m. (scale, 5 μm).

An average of six cells per zone was studied, and only those cells with a visible nucleus indicating that the section passed approximately through the center of the cell were used for evaluation. When the position of a cell within the lobule could not be ascertained, this cell was not used. Electron micrographs were taken at an original magnification of ×2160 and enlarged photographically fourfold. Semiquantitative estimate of the amount of ER was done "blindly," that is, without the evaluator having prior knowledge of time when the animals were killed or to what group the animals under consideration belonged. No attempt was made to assess the total amount of ER in each cell, but rather the relative proportions of smooth and rough ER were estimated. A scale of 1+ to 7+ was used, the maximum value of 7+ corresponding to approximately 70 percent of the cytoplasmic surface (*11*), as follows: 1+ = < 10 percent; 2+ = 10 to 20 percent; 3+ = 20 to 30 percent; 4+ = 30 to 40 percent; 5+ = 40 to 50 percent; 6+ = 50 to 60 percent; 7+ = 60 to 70 percent or more. In order to answer the question of reproducibility, these estimations were performed independently by three different investigators, and the overall results were in good agreement. Taking into consideration the total number of cells examined within each lobular zone, we obtained an average value for each zone in each group of animals.

Our findings indicate that, in the normal control animals, there were regional differences in the distribution of smooth and rough ER within the hepatic lobule (Table 1). Furthermore, the amount of smooth ER varied with respect to the time of day (Fig. 1). In general, within the same lobule, there appeared to be a centro-portal gradient in the distribution of smooth ER. Smooth ER was greater in the pericentral and midzonal areas than in the periportal zones. A similar trend was observed for rough ER, although the regional differences were less striking. With respect to the diurnal changes, HO activity was highest at 10 p.m. and lowest at 2 p.m. (Table 2). Smooth ER was more abundant at 10 p.m. in the pericentral areas and to a certain extent in the midzones when compared to the same areas at 2 p.m. Rough ER, in contrast, showed less

Table 2. Time-dependent changes in hexobarbital oxidase activity of rat liver. The animals were the same as those used for electron microscopy. The values are expressed as micromoles of hexobarbital metabolized per gram of liver tissue (mean ± S.E.). A more detailed temporal distribution of the enzyme levels has been reported earlier (*9, 10*). The numbers in parentheses indicate the number of animals.

Treatment	Hexobarbital oxidase activity	
	2 p.m.	10 p.m.
None	9.9 ± 1.2 (6)	14.5 ± 0.9* (6)
Blinded	12.4 ± 0.6 (4)	12.2 ± 1.9 (4)
Phenobarbital	19.4 ± 0.7 (4)	19.7 ± 0.8 (4)

* Significantly different from the 2 p.m. values ($P < .02$).

variability. In the blinded animals, the diurnal variation in enzyme activity was absent (Table 2). With respect to smooth ER, no significant differences were noted between 2 p.m. and 10 p.m. for central and portal regions (Table 1 and Fig. 2). There appears to be a decrease in smooth ER at 10 p.m. in the midzonal region, but in view of the small number of cells examined in this zone, the significance of this observation remains to be established. As in the case of normal controls, the changes in rough ER were less striking.

Since biochemical studies have revealed the absence of enzyme rhythmicity in the phenobarbital-stimulated rats (Table 2), only the 2 p.m. specimens were taken for examination by electron microscopy. In the phenobarbital group, there was a significant increase in smooth ER, mainly confined to the pericentral and midzonal regions but seen to a lesser degree in the periportal regions. The rough ER changes were less conspicuous, but the overall estimates were higher than in controls.

There have been many reports of diurnal variation in hepatic morphology and function (*12*), but no ultrastructural study has been reported, to our knowledge, relating the biochemical rhythms with subcellular morphological variations. In rat liver, mitotic activity was high during the morning hours, with a peak at 8 a.m., but gradually decreased to a minimum at 10 p.m. These changes paralleled the changes in the size of hepatocytes (*13*). In a study on liver cell regeneration, Fabrikant showed that DNA synthesis follows a temporal distribution in the normal resting liver, being high at 6 a.m. and low at 10 p.m. (*14*).

On the basis of phenobarbital-stimulation studies, it has been suggested that the smooth ER membranes originate from rough ER elements by ribosomal detachment once protein synthesis has been achieved; this view is supported by the possibility of preventing or delaying the smooth ER proliferation with simultaneous administration of actinomycin D or puromycin (*15*). The studies of Dallner *et al.* (*7*) also suggest that, in developing rat liver, there is a similar mechanism for the origin of smooth ER. It has also been observed that, in phenobarbital-stimulated rats, the increase in microsomal enzyme activity closely paralleled increase in smooth ER. In our studies, smooth ER was abundant at the time when enzyme activity was maximum, a finding consistent with the observations in phenobarbital-treated rats. It is of interest to recall that, in the normal rat liver, DNA synthesis and mitotic activity are highest in the morning hours and lowest in the night (*13*). If one assumes that these events precede the formation of ER membranes and enzyme proteins (*5, 15*), it is compatible with our findings of an abundant smooth ER at 10 p.m. and a lower amount at 2 p.m.

We also focus attention on the regional differences in the distribution of smooth and rough ER observed in our study. Fabrikant (*14*) earlier noted that, in regenerating liver, there is a porto-central gradient in the synthesis of DNA, RNA, and protein. The synthesis of DNA and RNA and cell division begin in the periportal regions first and progress in waves toward the pericentral regions. He suggested that, as new cells were formed, by rearrangement within the lobule, they were pushed from the peripheral zone to the more middle and central regions. The situation with respect to the normal adult liver is not known. This may be due to the technical difficulties in assessing the relatively smaller changes in the "resting liver" in comparison to those in the regenerating liver. If we assume, as is generally believed, that enzyme is synthesized in rough ER and transferred to smooth ER, this process being mediated by a DNA→RNA→protein mechanism, our finding of an increased amount of smooth ER in the pericentral regions at 10 p.m. is consistent with the high DNA synthesis and mitotic activity in the morning hours reported by other investigators (*12*) and the observations of Fabrikant (*14*).

Thus a diurnal rhythm exists in the endoplasmic reticulum of normal hepatocytes and there are regional differences in the distribution of ER within the hepatic lobule (*16*).

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References and Notes

1. B. B. Brodie, J. R. Gillette, B. N. La Du, *Annu. Rev. Biochem.* **27**, 427 (1958); H. Remmer, *Arch. Exp. Pathol. Pharmacol.* **238**, 36 (1960); J. R. Fouts, *Biochem. Biophys. Res. Commun.* **6**, 373 (1961).
2. A. H. Conney, E. C. Miller, J. A. Miller, *J. Biol. Chem.* **228**, 753 (1957); A. H. Conney, J. R. Gillette, J. K. Inscoe, E. R. Trams, H. S. Posner, *Science* **130**, 1478 (1959); A. H. Conney, C. Davidson, R. Gastel, J. J. Burns, *J. Pharmacol. Exp. Ther.* **130**, 1 (1960); J. R. Fouts, *Fed. Proc.* **21**, 1107 (1962); *Ann. N.Y. Acad. Sci.* **104**, 875 (1963).
3. H. Remmer and H. J. Merker, *Klin. Wochenschr.* **41**, 276 (1963); *Science* **142**, 1657 (1963); *Ann. N.Y. Acad. Sci.* **123**, 79 (1965).
4. H. Remmer, *Proc. Eur. Soc. Study Drug Toxicity* **4**, 57 (1964).
5. S. Orrenius, J. L. E. Ericsson, L. Ernster, *J. Cell Biol.* **25**, 627 (1965).
6. L. Ernster and S. Orrenius, *Fed. Proc.* **24**, 1190 (1965).
7. G. Dallner, P. Siekevitz, G. Palade, *Biochem. Biophys. Res. Commun.* **20**, 135 (1965); *ibid.* p. 142.
8. F. M. Radzialowski and W. F. Bousquet, *J. Pharmacol. Exp. Ther.* **163**, 229 (1968).
9. V. Nair and R. Casper, *Life Sci.* **8**, part 1, 1291 (1969).
10. ———, S. Siegel, D. Bau, *Fed. Proc.* **29**, 804 (1970).
11. It has been calculated [A. V. Loud, *J. Cell. Biol.* **15**, 481 (1962)] that ER, both rough and smooth together, occupy about 75 percent of the total cytoplasmic surface area. Considering that this calculation has not taken into account the space occupied by Golgi complex and lysozomes, we have used the value of 70 percent in our estimation.
12. R. L. Bramachary, *Int. Rev. Cytol.* **21**, 65 (1967); G. M. Higgins, J. Berkson, E. Flock, *Amer. J. Physiol.* **105**, 177 (1933); *ibid.* **102**, 673 (1932); H. W. Deane, *Anat. Rec.* **88**, 39 (1944); C. M. Blumenfeld, *ibid.* **72**, 435 (19[illegible] → *Science* **90**, 446 (1939); *Arch. Pathol.* **33**, 770 (1942); F. Halberg, H. A. Zander, M. W. Houglum, H. R. Muhlmann, *Amer. J. Physiol.* **177**, 361 (1954); J. W. Wilson, *Anat. Rec.* **101**, 672 (1948); C. P. Barnum, C. D. Jardetzky, F. Halberg, *Texas Rep. Biol. Med.* **15**, 134 (1957); *Amer. J. Physiol.* **195**, 301 (1958).
13. B. Jackson and C. H. Willey, *Anat. Rec.* **125**, 583 (1956); M. McKellar, *Amer. J. Anat.* **85**, 263 (1949); B. Jackson, *Anat. Rec.* **134**, 365 (1959); A. B. Novikoff and V. R. Potter, *J. Biol. Chem.* **173**, 223 (1948); J. J. Jaffe, *Anat. Rec.* **120**, 935 (1954).
14. J. I. Fabrikant, *Johns Hopkins Med. J.* **120**, 137 (1967).
15. S. Orrenius, *J. Cell Biol.* **26**, 713 (1965); —— → and J. L. E. Ericsson, *ibid.* **28**, 181 (1966); *ibid.* **31**, 243 (1966).
16. After this report was submitted for publication, an abstract titled "Circadian rhythmicity in response to barbiturates" was brought to our attention [O. Müller, *Naunyn Schmiedebergs Arch. Pharmakol. Exp. Pathol.* **270** (Suppl.), R 99 (1971)].
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