CHRONOBIOLOGICAL ASPECTS OF CELLS. CHRONOLOGICAL CHANGES IN THE MORPHOLOGY OF HEPATOCYTES

YASUO UCHİYAMA

Department of Anatomy, Tohoku University School of Medicine, Sendai 980, Japan

REVIEW

In common textbooks of histology, hepatocyte substructures are described as being, for the most part, static. For some time, however, glycogen content in hepatocytes of several species has been known to exhibit a dramatic circadian variation. In recent years, electron microscopic morphometric studies have revealed statistically significant circadian changes in volume densities of various cytoplasmic organelles of rat hepatocytes. The changing patterns in cytoplasmic volume densities also differ strikingly between hepatocytes in peripheral and central parts of liver lobules. Furthermore, histochemistry has also been applied to chronobiological studies of hepatocytes and has shown dramatic circadian changes in enzyme localizations and activities, e.g., in those of various lysosomal enzymes, thiamine pyrophosphatase, glucose-6-phosphatase, and phosphorylase. This emphasizes the necessity of the chronobiological approach to the study of histochemistry.

The results of chronobiological studies on the cytoplasmic volume densities of ultrastructural elements and various histochemically indicated enzyme activities in hepatocytes may be accounted for by a difference of function between perilobular and centrilobular hepatocytes rather than by a difference of phasing during the 24-hour span.

From these studies it can be concluded that time is a fundamental factor in the understanding of the morphology and function of the hepatocyte.

INTRODUCTION

Variations related to the time of day have been demonstrated in cellular, organ, and blood systems of living organisms. These changes, which may be quite dramatic, can be detected in metabolism, function or structure. The field of study examining such time-related changes in life function is called chronobiology. Several reviews have contributed to the establishment of the concept of chronobiology (21, 33, 34, 60, 65).

Many rhythmic variations have a 24 hour (circadian) duration. Circadian variations have been recorded in body temperature, respiratory rates, leucocyte numbers, blood hormone concentrations (10, 25, 27, 28, 73), blood glucose concentrations (34), electrolyte and metabolite levels in blood and urine (4, 7, 42), contents of glycogen and various enzymes in the liver (20, 32, 34, 36, 37, 52, 59), enzyme and hormone levels in

the brain and pineal body (3, 30, 54, 63, 64, 81, 82), and mitotic index in the liver, bone marrow and corneal cells (1, 43, 66). Corresponding to these metabolic and/or functional rhythms in various animals, circadian variations in cellular structures have been revealed. Such changes have been reported in hepatocytes (44, 46, 61, 74, 78), parotid gland cells (2), gut epithelial cells (84), pinealocytes (5, 25, 55), synaptic ribbons (69), visual cells (49) and renal tubular cells (51). Histochemical studies have also demonstrated circadian variations in the activities of various enzymes in the digestive tract (62, 83), leucocytes (31) and hepatocytes (6, 8, 24, 33, 35, 41, 44, 45, 47, 48, 75-77, 79).

Other types of chronobiological variation include ultradian (from one to several hours), infradian (more than one day, i.e. 2 or 3 days), circatrigintan (monthly) and circannual (yearly),

all of which are well reviewed by Halberg (21) and Scheving *et al.* (65). Many variables in animals showing circadian rhythms also exhibit more complex types of rhythmicities when the span of the observations is increased. For example, glycogen content in the liver of rats showing a dramatic circadian variation also exhibits a seasonal change (32, 34, 36). DNA concentrations in the liver of rats, demonstrating a clear circadian rhythm with a magnitude of over 100%, continue to increase rhythmically throughout the life-span (1).

According to von Mayersbach (34, 39, 40) and Philippens (53), the factors influencing the circadian variations include sex, strain, sampling sequence, stress, feeding, lighting, and season. In chronobiological experiments animals are usually housed in well ventilated chambers or boxes and acclimated to constant environmental conditions (e.g. temperature and humidity) for several weeks. The light-dark change is often adjusted to a 12:12 hour cycle (standard LD). Food and tap water are available *ad libitum*. Even when housed under these idealistic conditions, sex, strain, and seasonal differences should not be ignored. Fortunately, the sex and strain characteristics may often be of minor significance and may be minimized by the use of homogeneous animal groups. Halberg (21) described the 'serial effect' which is considered to be the result of a disturbance of the colony caused by repeatedly removing groups of animals from a common chamber during the 24-hour span. This phenomenon manifests itself in end values of a circadian study which do not correspond to (are higher or lower than) the values at the beginning of the experiment.

In the wide scope of chronobiological literature, morphological and histochemical works are few in number. The reason for this lag in the progress of chronomorphology may be found in the intricacies of specimen preparation techniques and the difficulties of data analysis. A case in point is that numerous artifacts are conceded to be inevitable in morphological and histochemical studies. These problems, however, can be reduced by setting up standardized conditions, e.g. methods of fixation, staining and enzyme detection and by measuring data using morphometric techniques introduced by Weibel (80), thereby minimizing subjective interpretations of the data.

Before entering the main subject of this review, the chronobiological changes in hepatocytes, it is necessary to consider briefly topographical variations in cellular structures and functions of

various tissues according to site in the organ, especially with regard to location in the microcirculatory system of the organ, historical views on chronobiological studies of hepatocytes, and some other points.

Rappaport *et al.* (58) and Rappaport (57) proposed that the liver lobule could be subdivided into three zones with respect to function and structure. According to this proposal which is based on the sinusoidal current in the hepatic lobule, perilobular hepatocytes belong to the first zone, midzonal hepatocytes to the second and centrolobular hepatocytes to the third. By stereological analysis Loud (29) demonstrated that the volume densities of subcellular structures were considerably different in hepatocytes obtained from these three zones. Similar observations on the appearance of hepatocytes at the subcellular level were reported by Müller (44). In the pancreas the insulo-acinar portal system takes up the hormone supply from the islet cells and delivers it to acinar cells (17-19). Due to this microcirculation, cell size, structure, and zymogen granule content of the exocrine acinar cells may conspicuously differ depending on the distance from the islet (personal communication by Professor Fujita). Excellent suggestions may be found in the above-mentioned works on methods of studying the morphology of liver cells.

The study of changes in subcellular structures during the 24-hour span developed after Forsgren (15, 16) described a strong circadian change in rabbit hepatocyte glycogen content at the light microscopic level. Similar variations in the glycogen content of hepatocytes were histologically shown in mice by Deane (9) and in rats by Ekman and Holmgren (12) and von Mayersbach and Leske (36). Electron microscopically, marked variations in glycogen granules and other subcellular structures of hepatocytes were first revealed by Müller *et al.* (46) using rats raised under highly standardized conditions. Such studies of circadian changes in the ultrastructure of hepatocytes have been continued by several investigators (24, 44, 61, 74, 78, Uchiyama *et al.* (in preparation)).

Adding to chronobiological works using laboratory animals, some investigators (50, 71, 72) studied seasonal variations in hepatocytes of wild animals which differ from laboratory animals by not having been raised under standardized environmental conditions. In amphibia, glycogen content, mitochondrial profiles, and ribonucleic acid (RNA) substance (ergastoplasm) dramatically exhibited seasonal changes (50, 72).

Similar observations were also reported in chiroptera (71). These studies examining the relationships of lipid substance and glycogen granules during hibernation and breeding also demonstrated seasonal changes in the glycogen and lipid content of Ito cells (71, 72).

With recent advances in techniques, it has been possible to correlate studies of hepatocyte morphology and enzyme activity at the light and electron microscopic levels.

CHRONOBIOLOGICAL CHANGES IN THE SUBCELLULAR STRUCTURES OF RAT HEPATOCYTES

This section deals with circadian variations in the ultrastructure of hepatocytes in rats raised under highly standardized conditions.

Cell Volume

According to Müller (44), the cell volume of rat hepatocytes exhibits a significant fluctuation during the 24-hour period. In semithin sections of Epon embedded, toluidine blue-stained tissue, the mean value of the volume density of hepatocytes in the early morning is clearly larger than in the early evening. This change is exactly synchronized with the variation in glycogen content of hepatocytes. Related to the variation in the cell volume is a prominent fluctuation in the area of the biliary canaliculi during the 24-hour span (47). In electron microscopic and histochemical studies it has been demonstrated that biliary canaliculi have a maximal width and surface area at the beginning of nocturnal feeding, and minimal luminal and surface areas in the morning after the completion of the food intake. A circadian change in cell size is also seen in the pineal cells of mice kept under standardized conditions (20). In pineal cells the variation in cell size does not completely synchronize with glycogen content. The maximal cell size occurs 2 hr after the beginning of the light cycle and the minimal size 2 hr after the end of the light cycle.

Nuclear Size

It has been noted that biochemically demonstrated DNA concentrations in the liver are exactly correlated with the clock hour (1). Hováth (22), Eling *et al.* (14), Eling (13), and Echave Llanos *et al.* (11) showed a significant variation in DNA concentrations, with a ratio of maximal and minimal DNA concentrations of

approximately 2:1 during the 24-hour span. Jerusalem *et al.* (24) examined the volume of nuclei in perilobular and centrolobular hepatocytes using thick paraffin sections treated by the Feulgen reaction on glass slides. The volume of nuclei both in perilobular and centrolobular hepatocytes exhibited clear circadian variations although the mean values at each time-point in perilobular and centrolobular hepatocytes did not synchronize with each other. The maximal value of nuclear volume in perilobular hepatocytes was found to be about $250 \mu\text{m}^3$ and the minimal value $125 \mu\text{m}^3$. Jerusalem *et al.* also pointed out that the incidence of binuclear hepatocytes clearly varied, with an amplitude of more than 50%. Abicht and Philippens (1) reported that in addition to variations in the DNA concentration and binuclear cell numbers, the mitotic activity also showed a significant circadian change. They determined from long-term experiments that these rhythmic variations are strictly consistent, and further, that the rhythmic variation in the DNA concentration in the liver homogenates was not the result of a 'dilution effect' caused by the fluctuating amount of non-nucleic acid components.

Glycogen Content

Glycogen content in hepatocytes exhibits a strong circadian change, especially in perilobular hepatocytes. As shown in Table 1, amplitudes of variations in glycogen content differ clearly between perilobular and centrolobular hepatocytes. Usually, the cytoplasm of perilobular hepatocytes is occupied by glycogen granules during the early light cycle, whereas the granules can rarely be seen during the early dark cycle (Fig. 1, a and b). Thus, glycogen content in perilobular hepatocytes reaches a peak at the early

Fig. 1 Parts of perilobular hepatocytes of adult male Wistar rats (AF/Han, ca. 300 g) raised under standardized conditions (light period: 0600h–1800h). a. At the maximal glycogen time-point. Large glycogen areas can be seen. Packages of rER are well developed and round or oval mitochondria are scattered through the cytoplasm. b. At the minimal glycogen time-point. Glycogen granules and packages of rER are not seen in the cytoplasm. Mitochondria are somewhat voluminous and take a round or oval form. Note the increment of polysomes (arrows). G: Golgi apparatus, GA: glycogen area, L: lysosome, Li: lipid droplet, M: mitochondrion, N: nucleus, P: peroxisome. $\times 8,000$

Fig. 1

Table 1 Volume Densities of Cytoplasmic Organelles

		glyl	mit ²	sER ³	rER ⁴	lys ⁵	per ⁶	lip ⁷	pol ⁸
Perilobular hepatocytes	1100	28.5	16.3	17.6	21.6	0.61	0.73	0.63	0.39
		(1.34)	(0.99)	(1.14)	(1.39)	(0.17)	(0.21)	(0.08)	(0.07)
	2300	3.54	24.6	30.8	21.2	1.48	0.85	0.73	2.96
		(0.19)	(1.23)	(1.39)	(1.23)	(0.27)	(0.18)	(0.26)	(0.21)
Centrolobular hepatocytes	1100	17.4	12.6	17.3	15.1	1.17	0.66	0.62	0.76
		(0.81)	(1.48)	(1.36)	(1.34)	(0.28)	(0.17)	(0.15)	(0.10)
	2300	8.21	16.6	32.3	20.6	0.69	1.57	0.77	0.90
		(0.70)	(1.38)	(1.58)	(1.19)	(0.16)	(0.26)	(0.19)	(0.19)

(): standard error, 1: glycogen granule, 2: mitochondrion, 3: smooth surfaced endoplasmic reticulum, 4: rough surfaced endoplasmic reticulum, 5: lysosome 6: peroxisome, 7: lipid droplet, 8: polysome, unit: % volume

it decreases. In the early evening, the glycogen content displays a trough, and during the dark cycle it increases again. Compared with the perilobular hepatocytes, the variation in glycogen content in centrolobular hepatocytes is not as prominent. Thus, during the early light period the glycogen-occupied area in the cytoplasm of centrolobular hepatocytes is clearly smaller than in perilobular hepatocytes, whereas during the early dark period a considerable amount of glycogen granules in centrolobular hepatocytes is still seen; α -glycogen (aggregated) granules are scattered through the cytoplasm (Fig. 2, a and b). It seems worthy to add that a circadian change in glycogen content is clearly demonstrated in pineal cells of mice kept under highly standardized conditions (26). The maximal glycogen content in these cells occurs at the end of the dark cycle, the minimal at the end of the light cycle.

Von Mayersbach *et al.* (32, 34, 46) demonstrated a seasonal difference in circadian changes of glycogen content of the liver by biochemical as well as by morphological means. Even under standardized environmental conditions, e.g. temperature, humidity, light, food, and electromagnetism, whose effects were minimized by the Faraday properties of the sound-proofed climate room, circadian changes in the glycogen content of rat liver exhibited a clear seasonal difference. The seasonal variation of glycogen content has also been seen in hepatocytes of wild animals, especially amphibia and chiroptera (50, 71, 72).

Müller (45) demonstrated that glucose-6-phosphatase activity was well localized in rER. The enzyme displayed a topographical difference in the liver lobule which was correlated to glycogen content in hepatocytes. This suggests that the activity of the enzyme is increased when glycogen content in hepatocytes reaches its maximum, whereas it falls when the glycogen is

decreased.

The difference of glycogen content between perilobular and centrolobular hepatocytes may be explained by the nutrient concentration in the sinusoidal stream between them. However, the fact that a considerable amount of glycogen can still be seen in centrolobular hepatocytes at the minimal glycogen time-point, may suggest a functional difference between these two cell populations.

Mitochondria

The mitochondrial volume densities in hepatocytes vary significantly during the 24-hour span, i.e. they are high during the early active phase of rats and low during the early resting phase (44, 61, 74). As shown in Table 1, the mitochondrial volume density in perilobular hepatocytes is clearly higher than in centrolobular hepatocytes. The reason is that mitochondria in perilobular hepatocytes are significantly larger than those in centrolobular cells. In both cell locations, mitochondria take round, oval, and often cylindrical forms (Figs. 1a, b and 2a, b). Müller (44) and I (74) reported that mitochondria in perilobular hepatocytes were provided with poorly organized cristae in the early light cycle and distinct cristae during the early dark cycle. Jerusalem *et al.* (24)

Fig. 2 Parts of centrolobular hepatocytes (the same conditions as in Fig. 1). a. At the maximal glycogen time-point. Glycogen areas are definitely small in comparison with Fig. 1a. b. At the minimal glycogen time-point. A certain number of glycogen granules can still be seen. No clear variation in the distribution pattern of rER can be discerned, in contrast with Fig. 2a. Densely ramified profiles of sER are increased throughout the cytoplasm. $\times 8,000$

Fig. 2

reported that variations in the electron density of the mitochondrial matrix and in mitochondrial cristal profiles were also dependent upon the season.

Rough Surfaced Endoplasmic Reticulum

The variations in the distribution pattern and volume density of rER in perilobular hepatocytes prominently differ from those of centrolobular hepatocytes. The distribution pattern of rER in perilobular hepatocytes dramatically fluctuates during the 24-hour period (44, 78, Uchiyama *et al.* (in preparation)). At the maximal glycogen time-point, strands of rER pile up making packages which are distributed throughout the cytoplasm (Fig. 1a). At the minimal glycogen time-point, packages of rER are rarely seen and a single strand of rER often envelops mitochondria (Fig. 1b). The volume density, however, of rER in perilobular hepa-

toocytes does not vary significantly between time points (61, 74). On the other hand, in centrolobular hepatocytes almost no variation in the distribution pattern of rER is discerned, and packages of rER are often scattered through the cytoplasm. The volume density of rER, however, varies significantly during a 24-hour span.

Considering the possibility of membrane transformation between rER and sER which has been suggested from the examination of rat hepatocytes receiving phenobarbital treatment by Stäubli *et al.* (70), it is certainly of interest that similar findings reflecting the same phenomenon have been obtained by chromomorphological approaches. During the late dark and late light period endoplasmic reticulum, which is definitely continuous with rER including the nuclear envelope, and is not lined by ribosomes, frequently appears in the cytoplasm (Figs. 3 and 4). The appearance of endoplasmic reticulum having both rough and smooth parts at certain time

Fig. 3 At the late light. Some of the rERs lose ribosomes from their cisternal ends (arrows). $\times 22,000$
 Fig. 4 Higher magnification of Fig. 3 and its adjacent area. a. The smooth part of the endoplasmic reticulum branching from rER can be seen (arrow). b. The endoplasmic reticulum having rough and smooth parts is clearly demonstrated. At the cisternal ends of rER, sER is discernible (suggesting a separation from rER) (arrow). $\times 70,000$
 Figs. 3, 4 and 5 Parts of hepatocytes obtained from adult male Wistar rats (AF/Han, ca. 300 g) raised under standardized conditions (light period: 0600h–1800h)

points, which may be correlated with the reorganization of the endoplasmic reticulum during the 24-hour period, suggests the possibility of membrane transformation between them. The membrane transformation may occur by flowing- and branching-off accompanied by the attachment or detachment of ribosomes.

In addition, Müller (44) counted the number of membrane-bound ribosomes which changed clearly with the clock hour. At the minimal glycogen time-point, the number was decreased prominently; that is, cisternal membranes of rER adjacent to outer mitochondrial membranes often lost ribosomes.

Smooth Surfaced Endoplasmic Reticulum

The cytoplasmic volume densities of sER both in perilobular and centrilobular hepatocytes exhibit dramatic circadian changes, i.e. both are high at the minimal glycogen time-point, and low at the maximal glycogen time-point (44, 61, 74, Uchiyama *et al.* (in preparation)). At the maximal glycogen time-point sER is chiefly

Fig. 5 At the minimal glycogen time-point. A part of a centrilobular hepatocyte. A marked increment of sER is shown in the cytoplasm. $\times 22,000$

located in mitochondrial, perinuclear, and peripheral areas of the cell, whereas at the minimal glycogen time-point, it is densely distributed throughout the cytoplasm (Figs. 1a, b, 2a, b and 5).

Müller (41, 48) demonstrated histochemically the correlation of variation in glycogen content with phosphorylase activity in mouse hepatocytes. At the maximal glycogen time-point, phosphorylase activity was weak in the endoplasmic reticulum, whereas it reached a peak when glycogen content decreased. Müller concluded that the degradation of glycogen granules by phosphorylase and intake into sER at the minimal glycogen time-point are highly corre-

Fig. 6 The rhythmic variation in TPPase activity of the Golgi apparatus ($n=100$, $P<0.01$). At the mid-dark point TPPase activity in the Golgi apparatus is markedly decreased. Black bar: the reaction product can be seen strongly in two or more layers of the Golgi lamellae; dotted bar: in the innermost layer; white bar: very weak or negative reaction.

Fig. 6-10 These figures show rhythmic variations in TPPase and lysosomal enzyme activities detected cytochemically in perilobular hepatocytes of adult male Wistar rats (AF/Han, ca. 300 g) raised under highly standardized conditions (light period: 0900h-2100h).

lated with the marked increment of sER. However, since the increment of sER at the glycogen minimal time-point is more prominent in centrolobular hepatocytes showing a smaller amplitude of variation in glycogen content than perilobular hepatocytes, the marked increment of sER does not seem to correlate simply with the decrease of glycogen content.

Golgi Apparatus

The Golgi apparatus in hepatocytes is generally

well developed. I have investigated the change in thiamine pyrophosphatase (TPPase) activity of perilobular hepatocytes during the 24-hour span (76, 79). TPPase activity demonstrates a significant rhythmic change (Fig. 6). Peaks of the activity occur at the transition from the light to the dark and from the dark to the light, and troughs occur at the late dark and late light time points. Paralleling the rhythmic change in TPPase activity, profiles of the Golgi apparatus also vary considerably (Fig. 7, a, b and c). The Golgi apparatus at the time of higher activities

Fig. 7 Electron micrographs of hepatocytes after the histochemical reaction of TPPase. a. At 1300h. TPPase activity is clearly demonstrated in the Golgi apparatus (arrow), endoplasmic reticulum, and biliary canaliculi. The Golgi apparatus exhibiting the activity takes a circular form. B: bile canaliculus. b. At 2100h (the transition from the light to the dark). The Golgi apparatus showing a strong activity of TPPase takes an expanded form. c. At 0500h. TPPase activity can scarcely be seen in the Golgi lamellae which are somewhat shrunken (arrow). Biliary canaliculi and some lysosomes also weakly show the activity. The thin section is weakly stained with uranyl acetate to show the Golgi apparatus. $\times 15,000$

appears clearly expanded and distended, whereas at mid-dark and mid-light sampling points lower enzyme activities are often combined with a shrunken or rounded appearance of the Golgi apparatus. The correlation of TPPase activity with specific profile shapes of the Golgi apparatus remains unexplained. However, it may be that the rhythmic variation in profile shapes of the Golgi apparatus may reflect changes in its function during the 24-hour span. Furthermore, it is interesting that at the late dark time-point almost no Golgi apparatus exhibits TPPase activity with the methods used, although TPPase is regarded as a marker enzyme of the Golgi apparatus.

In addition, TPPase activity is also found in the endoplasmic reticulum, lysosomes, and biliary canaliculi. In these cellular components the activity varies considerably depending upon the clock hour.

It is still unknown whether the number of Golgi apparatuses varies during the 24-hour span. Since the Golgi apparatus is a cellular element indicating the state of activity of cells, it may be important to understand variations in the incidence of the Golgi apparatus.

Lysosomes

The lysosomal volume densities in perilobular and centrolobular hepatocytes significantly fluctuate during the 24-hour period. At the maximal glycogen time-point, the volume density of lysosomes in perilobular hepatocytes is decreased, while it is markedly increased at the minimal glycogen time-point. The variation in centrolobular hepatocytes shows an inverse pattern in comparison with that in perilobular hepatocytes.

The rhythmic changes in lysosomal enzyme activities have been examined during the 24-hour span using histochemical techniques at the light (6, 8) and electron (75, 77) microscopic levels. Enzyme activities of acid phosphatase, β -glucuronidase, arylsulphatase and A-esterase were detected displaying strong circadian changes. However, the changing patterns of these marker enzyme activities did not synchronize with each other, i.e., each of these marker enzymes demonstrated maximal and minimal activities at different clock hours. Furthermore, simultaneous topographical differences of activities among marker enzymes were also observed in the liver lobule. In the electron microscopic study the number of lysosomes revealed by each marker enzyme per hepatocyte

was counted (Fig. 8). Irrespective of the standardization of histochemical techniques at any time-point, while the classical localization of enzyme activities was realized, acid phosphatase activity was also found in the ground plasma at other time points (Fig. 9, a and b). It remains unknown whether the activity in ground plasma originates from lysosomes or other organelles, or is really localized. In the case of arylsulphatase, when the activity in lysosomes was considerably decreased, it was well demonstrated in the Golgi apparatus (Fig. 10, a and b). This may reflect the cytogenic pathway of arylsulphatase in hepatocytes during the 24-hour span.

Fig. 8 Circadian changes in incidences of lysosomes displaying acid phosphatase, arylsulphatase, and β -glucuronidase activities per hepatocyte ($n=250$, $P<0.01$). The phasings of each lysosomal enzyme activity are not synchronized. Each enzyme exhibits its maximal and minimal activities at different clock hours. Standard errors are marked on each line. —: acid phosphatase; ---: arylsulphatase; ···: β -glucuronidase

Fig. 9 Acid phosphatase activity histochemically demonstrated in perilobular hepatocytes. a. 1300h. The activity is clearly localized in lysosomes located near the biliary canaliculi. b. 1700h. The activity is localized not only in lysosomes, but also in the ground plasma. $\times 6,000$

Fig. 10 Arylsulphatase activity histochemically demonstrated in perilobular hepatocytes. a. At 1300h. The activity is well localized in the lysosomes. b. At 1700h. Lysosomes exhibiting the activity are scarce. However, the activity is clearly demonstrated in the Golgi lamellae. $\times 10,500$

From these studies, it is concluded that lysosomal enzymes in hepatocytes may appear to be heterogeneous with respect to their regulation and possibly also to their distribution in functionally different subpopulations of the lysosome compartment. Furthermore these studies may indicate that the detection of enzyme localization in histochemistry should be performed at various hours of the day, and that a single marker enzyme is not always an adequate representative of the lysosome.

Peroxisomes

As shown in Table 1, the peroxisomal volume density in centrolobular hepatocytes varies significantly during the 24-hour span, whereas in perilobular hepatocytes it does not exhibit a clear variation. This may also reflect the difference of function between perilobular and centrolobular hepatocytes.

Lipid Droplets

No significant variation in the cytoplasmic volume density of lipid droplets can be observed in either perilobular or centrolobular hepatocytes. However, according to unpublished data by the author the stainability of lipid

substance in the Golgi lamellae revealed by the osmium impregnation method exhibits a significant variation during the 24-hour period. It should also be taken into consideration that the appearance of lipid droplets in hepatocytes is markedly influenced by the rats' age and feeding conditions.

Polysomes

Müller (44) demonstrated an increase of free ribosomes in hepatocytes at the minimal glycogen time-point. The polysomal volume density in perilobular hepatocytes reveals a significant variation during the 24-hour span, i.e., at the maximal glycogen time-point it is low, and at the minimal glycogen time-point, it is markedly high. Polysomes are often located around the Golgi apparatus and rER, although at the maximal glycogen time-point they are located only near the rER and at the minimal glycogen time-point they are distributed more widely throughout the cytoplasm (Fig. 11). On the other hand, in centrolobular hepatocytes a variation in polysomal volume density is not significantly discernible. Since polysomes are one of the sites of protein synthesis, there probably exists a difference in the ability of perilobular and centrolobular hepatocytes to

Fig. 11 A part of a perilobular hepatocyte obtained from adult male Wistar rat (AF/Han, ca. 300 g) raised under highly standardized conditions (light period: 0600h–1800h). At the minimal glycogen time-point, many polysomes are seen in the cytoplasm (arrows). $\times 20,000$

synthesize proteins. It remains an open question whether this difference reflects simply a difference of phasings in protein synthesis between perilobular and centrolobular hepatocytes, or an actual difference in the proteins they produce.

Thus, it is clear that most cellular components of rat hepatocytes show dramatic circadian changes with respect to the volume density and distribution pattern. The rat hepatocyte is not functionally and morphologically static, but dynamic. In order to know the dynamism of hepatocytes, more detailed analysis of cytoplasmic organelles during the 24-hour period is necessary. Moreover, though many differences between perilobular and centrolobular hepatocytes are detectable at various circadian phases, it remains to be seen whether these differences are caused simply by a difference of location in the liver lobule, or by different cell populations.

Finally, relationships among various cytoplasmic organelles, particularly transformations of membranes among them, have not been discussed in detail in this review. However, considering the marked circadian variations in the volume densities of cytoplasmic organelles, it is important to examine their reorganization and degradation during the 24-hour span, with particular reference to the membrane dynamics. The membrane dynamics in hepatocytes during the 24-hour span may take place by synthesis and degradation of membranes, or by membrane transformation among the organelles. In the course of my chronobiological studies, I tried to study the membrane transformation among cytoplasmic organelles. Continuities can be discerned not only between sER and rER, but between the endoplasmic reticulum and the outer mitochondrial membranes. This observation is also supported by Sottocasa *et al.* (67, 68) who showed that the biochemical characteristics of the outer mitochondrial membranes differed from those of the inner membranes, and that the outer membrane might be a differentiation and specialization of the endoplasmic reticulum. Furthermore, membrane continuities of the Golgi lamellae to rER and the direct engagement of rER having a large smooth part to the Golgi lamellae on the cis side have also been discerned. Such a membrane association is exactly observed during the late dark and late light period when the reorganization of cytoplasmic organelles in hepatocytes occurs. Further study will be necessary in order to understand the possible

membrane transformations among cytoplasmic organelles in hepatocytes during the 24-hour period.

CONCLUSIONS

In this communication, circadian changes in the morphology of rat hepatocytes housed under highly standardized conditions with a mainly 12:12 hour light-dark cycle are reviewed. In order to further understand the chronobiological aspects of the morphology of hepatocytes, it is also necessary to examine modifications in the morphology of hepatocytes of animals raised under standardized conditions, e.g. drug administration, novelty stress, manipulation of food and light-dark schedules, etc. The chronobiological approach to the pre- and postnatal development of hepatocytes may give us excellent insights into these cells.

From the chronobiological studies of the morphology of hepatocytes the following conclusions and suggestions can be made:

1. Perilobular and centrolobular hepatocytes differ considerably in cellular appearance at any circadian phase.
2. Most subcellular structures demonstrate dramatic circadian changes with respect to cytoplasmic volume densities, profiles and distribution patterns in the cytoplasm.
3. Judging from the fact that enzyme localizations and activities are revealed histochemically (e.g. lysosomal enzymes, TPPase, phosphorylase, glucose-6-phosphatase, etc.), histochemical studies, as well as biochemistry, are needed to elucidate rhythmic variations in enzyme activities.

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