Virchows Arch. Abt. B Zellpath. 8, 309–317 (1971)

Circadian Variations of DNA Synthesis, Mitotic Activity, and Cell Size of Hepatocyte Population in Young Immature Male Mouse Growing Liver*

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Received April 26, 1971

Summary. Circadian variations in DNA synthesis after tritiated thymidine and autoradiography, mitotic activity with the Colcemid method, and cell size, represented inversely by the number of cells per microscopic field, are reported for the hepatocyte population of the young immature growing liver of the male mouse. The peak of DNA synthesis is at 04:00 hours and that of mitotic activity at 12:00 hours. The 8-hour interval between the two peaks is considered, in agreement with other authors, a good estimation of the lag period between DNA synthesis and the initiation of mitotic activity.

The minimum cell number per microscopic field, which is considered to correspond to the maximum cell size, coincides with the starting of the circadian mitotic wave. The significance of this coincidence and the relationships of the circadian curves of the parameters controlled with the feeding pattern and food consummatory curve of standardized mice, are discussed.

Circadian variations in liver tissue growth parameters were reportet many years ago (for references see Echave Llanos, 1967a, b). Although extensively studied as a problem of ontogenesis (Doljanski, 1960), liver tissue growth has not been analysed thoroughly enough from the point of view of circadian variations of its growth parameters. This point is of major practical as well as theoretical importance. The existence of a circadian rythm in mitotic activity has been reported for normal mouse immature growing liver (Wilson, 1948; Jardetzky *et al.* 1956; Halberg, 1957), regenerating liver (Barnum *et al.*, 1957; Bade, 1962; Russo and Echave Llanos, 1964), and hepatomas (Echave Llanos and Nash, 1970).

The existence of a circadian rhythm in DNA synthesis has been reported, measured through P32 incorporation into DNA fraction for immature growing liver (Jardetzky *et al.*, 1956), regenerating liver (Barnum *et al.*, 1957) and, through the incorporation of tritiated thymidine into DNA fraction, for Morris Hepatoma 7793 (Potter *et al.*, 1966).

The method used by these authors, who have measured DNA synthesis by radioactivity determinations in whole homogenates or their fractions, is not specific enough to provide information about the participation of the different cell populations of the liver in the values of radioactivity obtained. Hepatocytes constitute only 60% of the whole cell population of the liver, and littoral cells

* Supported by grants from the Consejo Nacional de Investigación Científicas y Técnicas de la Republica Argentina and the Comisión de Investigación Cientifica de la Universidad Nacional de La Plata. Thanks are due to the members of the technical Staff of the Institute for their technical assistance.

are the major constituent of the remaining 40% (Abercrombie and Harkness, 1951). In addition Kuppfer cells can come in or go out from the liver (Wilson, Leduc, and Corner, 1950; Easton, 1952; Fabrikant, 1968) and their number can show circadian variations (Echave Llanos *et al.*, 1971). This variability of littoral cell population adds a new uncertainty to its participation in the values recorded with total determinations of radioactivity. These are the reasons why the autoradiographic method after the incorporation of tritiated thymidine can be considered the method of choice in the study of DNA synthesis. It is more specific and makes it possible to define, precisely, the participation of the different cell populations of the liver.

With this method circadian variations in DNA synthesis have been reported in regenerating liver (Bade *et al.*, 1966) and hepatoma (Nash and Echave Llanos, 1971). The phase difference between the circadian curves of DNA synthesis and mitotic activity has been considered a good estimate of the lag period between both processes (Jardetzky *et al.*, 1956; Nash and Echave Llanos, 1971). The interval found fits quite well with that reported by Pelc and Howard (1955). However, other authors (e.g. Scheving, 1970) have not found this time relationships between the circadian curves of DNA synthesis and mitotic activity, working on several rat tissues. This lack of agreement between different authors and the need for direct information about DNA synthesis in normal immature growing liver, obtained with the autoradiographic method, decided us to design the experiments reported here.

Material and Methods

C3H-S inbred, male mice (from Wilson in 1966) were used. The pregnant females and their male partners were placed in standard conditions for periodicity analysis (Halberg *et al.*, 1958; Nash and Echave Llanos, 1969; Vilchez and Echave Llanos, 1971), caged singly in a room especially designed for this purpose, at a temperature of $22 \pm 1°$ C, illuminated (fluorescent light, 40 W) from 06:00 to 18:00 alternating with 12 hours darkness. They were given water and food ad libitum. They were controlled twice a day and the litter age was determined with a maximal error of 12 hours. After birth the young grew in these standard conditions until weaning, 21 days later. Some of the mice were used at this age and the others were caged singly, remaining in the same standard conditions until they were 28 days old, the age at which they were used.

The 21-day-old mice were divided into two groups, one of them for control of DNA synthesis and the other one for control of mitotic activity. The first group of animals was injected intraperitoneally with tritiated thymidine (New England, Chicago. Specific Activity: 3 C/mM) in a dose of 1 μg per gram of body weight, and 60 minutes later they were killed at 00:00, 02:00, 04:00, 06:00, 08:00, 12:00, 16:00, 18:00. 20:00, and 22:00 hours. The different time groups were killed on different days in order to avoid the serial effect (Halberg, 1959).

After sacrifice by decapitation and exsanguination, the liver of the animals was removed and fixed in buffered formaldehyde (Echave Llanos and Sadnik, 1964) after addition of cold thymidine. They were embedded in paraffin, sectioned at 5 μ, and processed for autoradiography.

The DNA synthesis index was determined in the autoradiograms, counting, at 1000×, the labeled nuclei in the hepatocyte population, in 140 microscopic fields. The number of hepatocytes was determined in 14 fields (every ten fields) and was considered an indirect measure of hepatocyte cell size. It was also used to establish the number of cells controlled and to reduce the labelled nuclei counted as DNA synthesis index (labeled nuclei/1000 nuclei).

The second group of animals was injected intraperitoneally with Colcemid (Ciba) (1 μg in 0.01 ml of destilled water) in a dose of 1 μg per gram of body weight (Echave Llanos

and Balduzzi, 1970) and killed 4 hours later at 00:00, 02:00, 04:00, 06:00, 08:00, 12:00, 16:00, 18:00, 20:00, and 22:00 hours.

After sacrifice the liver was processed as reported above and the sections stained with hematoxylin and eosin. In the slides the arrested metaphases were counted in hepatocyte population at 1000×, in the same way as described above. The mitotic index was expressed as arrested metaphases /1000 nuclei.

A third group of 28-day-old animals was used to control with greater accuracy the position of the mitotic peak, and to check if the level of mitotic activity was maintained a week later. The animals were injected with Colcemid, as described, and killed 4 hours later at 06:00, 08:00, 10:00, 12:00, 14:00, 16:00, and 18:00. The rest of the procedure was the same as that described for the second group. The results were analysed with the Student's t test.

Results

DNA synthesis shows circadian variations in the young growing immature liver of 21-day-old animals (Table 1 and Fig. 1). The maximal value, which is 215% of the 24-hour mean, is seen at 04:00, in the second half of the activity (dark) period. The minimum value, found at 16:00, the second half of the rest (light) period, is only 34% of the 24-hour mean. The difference between peak and trough is highly significant ($p < 0.01$).

In 21-day-old animals, the circadian curve of mitotic index presents a maximum value at 12:00, in the middle of the rest (light) period. This value is 376% of the 24-hour mean. The minimum value, found at 22:00, during the

Table 1. *Circadian variations of DNA synthesis, mitotic activity, and number of hepatocytes per microscopic field in the growing liver of immature male mice*

Group	Time of day	21 days old mice DNA S Index			21 days old mice Mitotic Index			28 days old mice Mitotic Index			21 days old mice Hepatocytes/field		
		$\bar{x} \pm$ SE	(N)	%	$\bar{x} \pm$ SE	(N)	%	$\bar{x} \pm$ SE	(N)	%	$\bar{x} \pm$ SE	(N)	%
1	00:00	26.0 ± 11.2	(5)	100	0.9 ± 0.1	(7)	41	—	—	—	24 ± 1.8	(14)	114
2	02:00	22.0 ± 1.9	(7)	84	0.6 ± 0.2	(9)	27	—	—	—	22 ± 1.1	(21)	104
3	04:00	56.0 ± 14.0	(7)	215	1.0 ± 0.2	(6)	45	—	—	—	19 ± 0.6	(18)	90
4	06:00	—	—	—	0.8 ± 0.3	(3)	36	0.4 ± 0.1	(3)	—	21 ± 0.3	(5)	100
5	08:00	27.0 ± 5.5	(5)	103	1.4 ± 0.7	(4)	63	0.9 ± 0.6	(4)	—	17 ± 0.5	(13)	81
6	10:00	—	—	—	—	—	—	2.8 ± 0.8	(5)	—	—	—	
7	12:00	29.0 ± 5.3	(5)	111	8.3 ± 1.4	(6)	376	6.2 ± 1.9	(5)	—	17 ± 0.4	(11)	81
8	14:00	—	—	—	—	—	—	7.9 ± 3.8	(5)	—	—	—	—
9	16:00	9.0 ± 3.6	(6)	34	5.2 ± 1.3	(23)	235	1.9 ± 0.4	(6)	—	21 ± 0.8	(34)	100
10	18:00	20.0 ± 12.4	(2)	76	2.4 ± 1.1	(5)	109	0.2 ± 0.1	(6)	—	25 ± 2.1	(7)	119
11	20:00	28.0 ± 6.6	(3)	107	1.0 ± 0.2	(10)	45	—	—	—	21 ± 0.5	(13)	100
12	22:00	24.0 ± 5.9	(4)	92	0.5 ± 0.1	(7)	23	—	—	—	24 ± 0.9	(13)	114
P groups (Student's t test)		2–3 < 0.05 3–9 < 0.01			2–7 < 0.01 4–7 < 0.01			4–7 < 0.05 8–10 < 0.05			5–10 < 0.001 5–12 < 0.001		

$\bar{x} \pm$ SE = Arithmetic mean ± 1 Standard error.
(N) = Number of mice.
% = Percent of the 24-hour mean.
Light = 06:00–18:00.
Dark = 18:00–06:00.

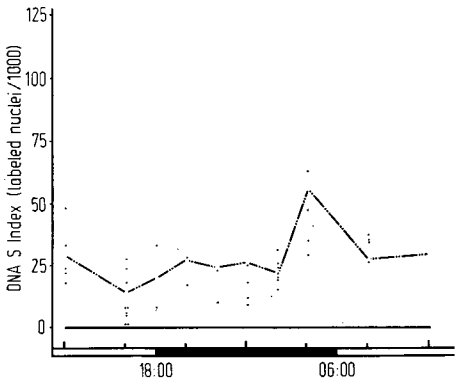

Fig. 1

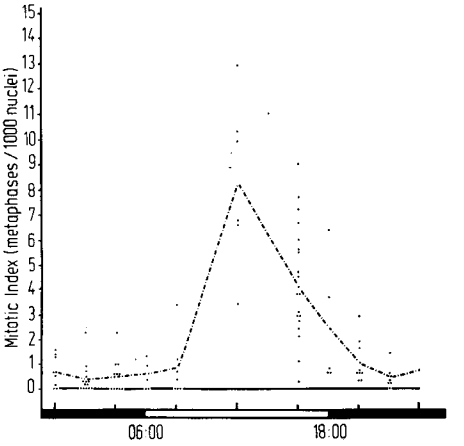

Fig. 2

Fig. 1. Circadian variation in DNA labeling index in immature growing liver of 21 days old male mice. In the bottom line is indicated the illuminating regimen: Light 06:00 to 18:00, Dark: 18:00 to 06:00

Fig. 2. Circadian variation in mitotic index in immature growing liver of 21 days old male mice

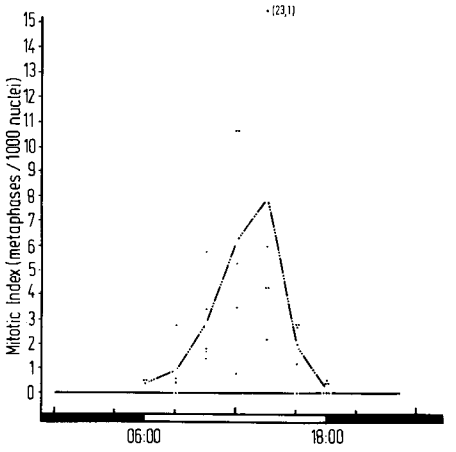

Fig. 3

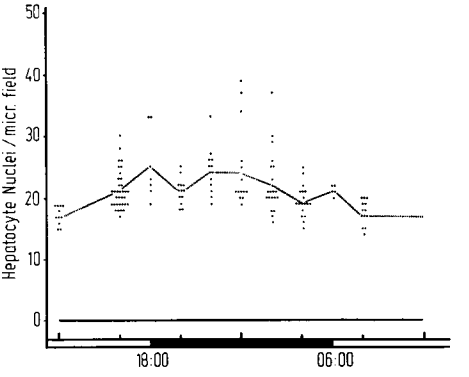

Fig. 4

Fig. 3. Mitotic wave and peak position of the circadian curve of mitotic activity in 28 days old male mice

Fig. 4. Circadian variations in hepatocyte number per microscopic field

activity (dark) period, is 23% of the 24-hour mean. The difference between peak and through, is highly significant ($p < 0.01$) (Table 1 and Fig. 2).

In the 28-day-old mice, with sampling along the mitotic wave period, the peak is at 14:00 (Fig. 3). The differences between the peak values and the values recorded at the start and end of the mitotic wave are significant ($p < 0.05$). The level of the peak is not different from that of 21-day-old animals.

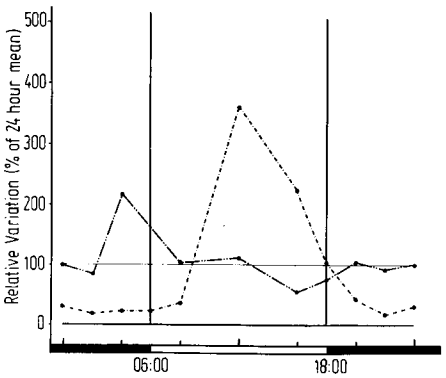

Fig. 5

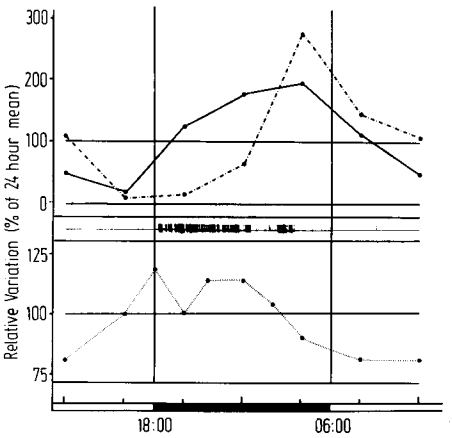

Fig. 6

Fig. 5. ●–·–·–● DNA synthesis rhythm in immature growing liver of 21 days old male mice. ●–—–● Mitotic rhythm in immature growing liver of 21 days old male mice. Each point corresponds to the mean values given in the Table, expressed as percent of the 24-hour mean. Illuminating regimen: Light: 06:00 to 18:00. Dark: 18:00 to 06:00

Fig. 6. ●–·–·–● Liver glycogen concentration (Echave Llanos and Epele de V., 1971). ●——● Gastric content rhythm (Nash and Echave Llanos, 1969). ●·····● Hepatocyte number per microscopic field. Each point represents the mean values given in the Table, expressed as percent of the 24 hour mean. In the middle part of the figure is illustrated a record of the feeding activity (Vilchez and Echave Llanos, 1971) of normal standardized mice. Illuminating regimen: Light 06:00 to 18:00. Dark: 18:00 to 06:00

The number of hepatocytes per microscopic field is considered the indirect measure of hepatocyte cell size. In Table 1 and Fig. 4 it can be seen that the number of hepatocytes per microscopic field presents a circadian variation with higher values during the activity (dark) period and lower values during the rest (light) period. The higher values are seen at 18:00 and 22:00 and they are highly significantly different ($p < 0.001$) from the lower values observed at 08:00. The variation is from 81 to 119% of the 24-hour mean.

The peaks of DNA synthesis and mitotic activity are separated by a lag period of 8 hours, from 04:00 to 12:00 (Fig. 5).

The maximum cell size values (minimum number of hepatocytes per microscopic field) are reached at the time point in which the mitotic wave starts (Figs. 5 and 6).

Discussion

The time position of the peak of incorporation of tritiated thymidine into hepatocyte nuclei, measured in the present series by autoradiography, is just the same as that reported by Jardetzky *et al.* (1956) for the peak of incorporation of P32, measured in DNA fractions by scintillation counting. The different technical approaches make this coincidence more valuable and the technique

here used adds more precision and specificity to the former data, in relation with the cell type involved.

The peak position for mitotic activity found in our experiment with 21-day-old mice also coincides with one found by Jardetzky *et al.* (1956) and later by Halberg (1957) in young NH mice, without use of the Colcemid method. The objections raised by the last author to the Colcemid method, based on the circadian variation in the action of Colcemid reported by Mülemann *et al.* (1956) are not supported by the coincidence between our results and those obtained by himself. The resulting 8-hour phase difference between the curves of DNA synthesis and mitotic activity in the present experiments coincides with the phase difference reported by Jardetzky *et al.* (1956) and is approximately similar to that reported by Pelc and Howard (1955). This adds further support to the statement by Jardetzky *et al.* (1956) that this interval can be considered an estimation of the lag period between DNA synthesis and the initiation of mitotic activity in young immature growing liver. If the peak position of mitotic activity is really at 14:00 hours, as it appears to be from our experiment on 28-day-old mice, this lag period would be longer, but, in this experiment, the scatter of the peak values does not permit certainty on this point and, furthermore, we have still not checked the peak position of DNA synthesis with more frequent sampling.

This lag period has been also estimated for liver neoplastic tissue (Nash and Echave Llanos, 1971) and, comparing the circadian curves of DNA labeling (Bade *et al.*, 1966) and mitotic activity (Bade, 1962; Russo and Echave Llanos, 1964), it can be seen that it is approximately the same in regenerating liver. However, negative results have been obtained in the estimation of this lag period through the comparison of the two circadian curves in different rat tissues (Scheving, 1970). The reason for these negative results does not appear to be very clear. The use of inbred animals can be of basic importance to our results as well as to those of Jardetzky *et al.* (1956). It is difficult to accept that liver tissue has some special characteristic in this sense. Our results with mitotic activity do not coincide with those reported by Wilson (1948), who was the first to call the attention on the circadian variation of this parameter in the liver. This author reports peak values at some time between 05:00 and 09:00 hours. This earlier position of the peak can be due to the strain of the mice or, more probably, to standardization differences between our animals and those of Wilson. He does not refer to any of these experimental details in his paper.

The number of hepatocytes per microscopic field is abviously in inverse ratio to their size. Consequently the circadian variation of this parameter can be considered the inverse of the circadian variation of cell size. Comparing Figs. 5 and 6, it can be observed that the time of maximum hepatocyte size (minimum cell number per microscopic field) is at 08:00, just the time at which mitotic activity starts to increase. This fact would appear to give support to the "critical mass hypothesis", which was propounded in its orginal form by the German biologist R. Hertwig (1903) and discussed recently by Johnson (1969). However, as the curves represent the mean values for all the hepatocyte population, and only a fraction of this population goes into cell division, one must arrive at the conclusion that there are cells that reach the maximum size and do not divide. This is actually seen in many microscopic fields with a very small number of

large hepatocytes which do not show signs of cell division. Furthermore, we have found (Nash and Echave Llanos, 1971) a lack of circadian variation of cell size in the hepatocyte population of a slow growing hepatoma, which presents a very importan circadian variation of mitotic activity (Echave Llanos and Nash, 1970). Consequently the coincidence between the point of maximum size (minimum cell number per microscopic field) and the starting of the circadian mitotic wave does not give direct support to the "critical mass hypothesis".

Very elegant but still unpublished experiments by Weinbren (1970) would indicate that the relation between cell size and the triggering of mitotic activity is not a causal but an external one. He achieved cell division in previously atrophied liver lobules and in cells which had not reached the critical size required by the "critical mass hypothesis".

The decision of the single cell to reproduce its DNA is taken at the end of G_1, during the dichophase (Bullough, 1965). This time would be (for the majority of the hepatocytes in our material that later enter cell division) at sone point between 00:00 and 04:00 hour, according to the peak position in the DNA labeling curve (Fig. 1). At this time the hepatocyte cell size is minimum (Figs. 5 and 6). Bearing in mind all this information it could be concluded that the "critical mass hypothesis" is not well founded.

The triggering of mitotic activity in hepatocytes that have doubled their DNA might be due to some other cause, like an adequate supply of energy in the anthephase (Bullough and Johnson, 1951). This might depend on an adequate reserve of glycogen in the cells. The glycogen content of hepatocytes is probably correlated with the cell size.

In order to analyse these relationships, we have compared (Fig. 6) the circadian variation in hepatocyte number per microscopic field (cell size) with the feeding pattern (Vilchez and Echave Llanos, 1971), gastric content (Nash and Echave Llanos, 1969) and circadian curves of liver glycogen concentrations (Echave Llanos *et al.*, 1971). It can be seen (Fig. 6) that hepatocytes are smaller (more numerous per microscopic field) at the beginning of the activity (dark) period. The size increases as the time advances, and the feeding activity of the standardized mice, which has startet at the time of switching off the light (18:00), is reaching the peak values of some of its parameters, i.e. gastric content. Some hours later, at 08.00, the maximal hepatocyte cell size is reached. It is interesting to observe that the increase in cell size is correlated with the increase in liver glycogen. After 04:00, and probably earlier, the gastric emptying starts and the food materials pass from the intestine through the portal circulation into the liver. The increase in glycogen is obviously related to this arrival of food. It is suggested that glycogen participates in the cell size increase and in providing the necessary energy in the antephase of those cells that go, some time later, into mitosis.

The results reported here add further support to the importance of the knowledge of the circadian variations of the growth parameters that are going to be measured in growth control experiments. This has been stated to be an important methodological aspect concerning the measurements of growth (Iversen, 1967).

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