CIRCADIAN PHASE RELATIONSHIPS OF THYMIDINE-^{3}H UPTAKE, LABELED NUCLEI, GRAIN COUNTS, AND CELL DIVISION RATE IN RAT CORNEAL EPITHELIUM

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ABSTRACT

Significant differences in the uptake of thymidine-^{3}H, percentage of labeled cells, numbers of grains per labeled nucleus, and mitotic rate were noted in rat corneal epithelium along a 24-hr time scale. These were demonstrated by injecting subgroups of five animals every hour during a 24-hr period with thymidine-^{3}H, sacrificing them 2 hr later, and analyzing the corneal epithelium by scintillation counter and radioautographic techniques. The increase in uptake during specific periods of the 24-hr time scale is attributed to an acceleration in the rate of DNA synthesis by individual cells and to an increase in the percentage of cells in the population actively synthesizing DNA.

INTRODUCTION

The rate of cell division in many plant and animal tissues is rhythmic over a 24-hr period. This rhythmicity is manifested by the occurrence of maximum and minimum values of the mitotic index which do not occur at random but rather are regulated along a 24-hr time scale. Once determined, the specific hours of occurrence of the peaks or troughs in mitotic rate are thereafter predictable for a specific tissue of a particular organism under comparable rigidly standardized conditions.

In the rat and mouse, the corneal epithelium is a tissue having a marked rhythmic pattern of mitosis which has been demonstrated in several laboratories (1–5). A question naturally arises whether the synthesis of deoxyribonucleic acid (DNA) that occurs before observable mitosis also is rhythmic in rate over the same 24-hr period. It seems probable that, if the cell division rate in a tissue is rhythmic, the rate of DNA synthesis also would show correlated variations. Studies of immature mouse liver (6) using P^{32} as a tracer show a circadian rhythm in nucleic acid metabolism that can be correlated with a similar rhythm in the mitotic rate. Radioautographic studies (7, 8) also have demonstrated that the percentage of labeled nuclei (tritium index) shows periodic fluctuations in mouse tissues that have a rhythmicity in their mitotic rate. Also, it has been reported that the amount of uptake of thymidine-^{3}H by urodele larval epidermis showed circadian fluctuations (9).

The objective of the present study was to determine whether or not a circadian rhythm occurs in thymidine-^{3}H uptake over a 24-hr period by rat corneal epithelium. Radioautographs also were prepared to determine whether more cells and individual nuclei were labeled more heavily at certain hours than at others.

TABLE I

*Hourly Mean Values for (1) Counts/Min/Mg Dry Weight, (2) Percentage of Labeled Nuclei, (3) Number of Grains/Nucleus, and (4) Mitotic Indices in Corneal Epithelium in the Rat Injected with Thymidine-^3H at Hourly Intervals over a 24-Hr Period and Sacrificed 1 Hr after Injection**

Hour of sacrifice	Mean counts/min/mg	Mean % of labeled nuclei	Mean No. grains/nucleus	Mean Mitotic index
0600	514	4.0	10.2	18.8h
0700	621	5.2	11.6	16.9
0800	463	3.8	13.0	13.3
0900	458	5.0	12.9	14.8
1000	639	3.2	13.5	14.6
1100	539	4.1	12.0	15.1
1200	643	4.3	10.9	15.4
1300	697h	4.8	12.9	14.4
1400	564	3.8	12.5	12.9
1500	591	6.1h	13.8h	13.4
1600	639	5.6	12.5	8.2
1700	498	5.2	13.6	8.2
1800	618	6.0	12.9	9.9
1900	544	4.4	13.6	3.7
2000	461	3.8	12.2	3.7l
2100	331l	2.8	10.6	6.0
2200	337	2.5	9.9	4.8
2300	334	2.4	10.1	8.8
2400	415	4.1	9.9	8.1
0100	428	1.5l	9.6l	10.6
0200	475	5.5	11.5	8.6
0300	546	3.8	10.3	8.7
0400	452	4.9	11.6	12.9
0500	628	4.1	12.5	12.4
Over-all 24-hr mean	518	4.2	11.8	11.0

* h, indicates peak hour.
l, indicates lowest hour.

MATERIALS AND METHODS

Male Sprague-Dawley rats averaging 190 g in body weight were used. For 4 wks prior to the study, the animals were housed, two to a cage, in a room maintained at 23 ± 2°C. The room was illuminated artificially from 0600 to 1800 (CST) and completely darkened from 1800 to 0600 (CST). Rockland rat chow and water were available *ad libitum*. The room was entered only three times per wk, on Monday, Wednesday, and Friday, at exactly 1400 (CST) for cage cleaning and replenishing of food and water.

A record of the daily motor activity of the colony shown in Fig. 4 was obtained by placing a sensitive microphone in the room. Noises emanating from the colony through feeding, scratching on wire cages, running, vocalizing, or fighting were picked up by the microphone, amplified, and fed into a capacitor which, in turn, discharged every 2 sec. This discharge drove a galvanometer in a strip chart recorder which operated 24 hr a day.

Every hour during one 24-hr period, a subgroup of five rats was injected intraperitoneally with thymidine-H^3 diluted with isotonic saline to a concentration convenient for injection. Each animal was given a dosage equivalent to 0.8 μc/g of body weight. Each subgroup was sacrificed by rapid decapitation 2 hr after injection, and the whole animal was fixed in Bouin's fluid. A total of 120 rats was used in the study.

After fixation, one of the corneas from each rat was dehydrated completely by drying overnight in an oven at 66°C. Each dried cornea then was weighed and placed in an individual vial containing Hyamine 10× and kept overnight in an oven at 66°C; during this time it completely dissolved. To each vial was added 15 ml of scintillation fluid consisting of: 3.8 liters of toluene, 19 g of PPO (2, 5 diphenyloxazole), 1.14 g of Dimethyl POPOP-1,4-bis-2-(4 methyl-5-Penyloxazolyl)-Benzene. Because the objective of our experiment was to determine a relative or differential uptake of thymidine-^3H during different hours and not the actual value of radioactivity of newly synthesized DNA, no attempt was made to remove the acid-soluble fraction of the homogenate. Radioactivity was measured in a Packard Tri-carb Scintillation Spectrometer, as previously described (9). The final results are expressed as the mean counts/min/mg of corneal epithelium.

In addition, whole mounts of one-half of the one remaining cornea obtained from the same animal were prepared for histological study. In each specimen, at least 5000 cells and the number of mitotic figures were counted. The mean mitotic index of each hourly period was expressed as the number of mitoses per 1000 cells. The other one-half of the cornea was serially sectioned at 4 μ, and radioautographs were prepared by standard procedures with the use of NTB-3 emulsion. After 4 wks of exposure, all sections were developed and stained at the same time in order to make the grain counts strictly comparable. In each specimen, at least 1000 cells and the numbers of labeled nuclei were counted. Calculations were made so that the results could be expressed as percentages of labeled cells. From the radioautographs of each hourly group, 35 to 40 nuclei, which appeared to have been sectioned through their greatest diameter, were selected randomly and the grains over each nucleus were counted. The mean number of grains/labeled nucleus for each hour was determined, and the data are shown in Table I.

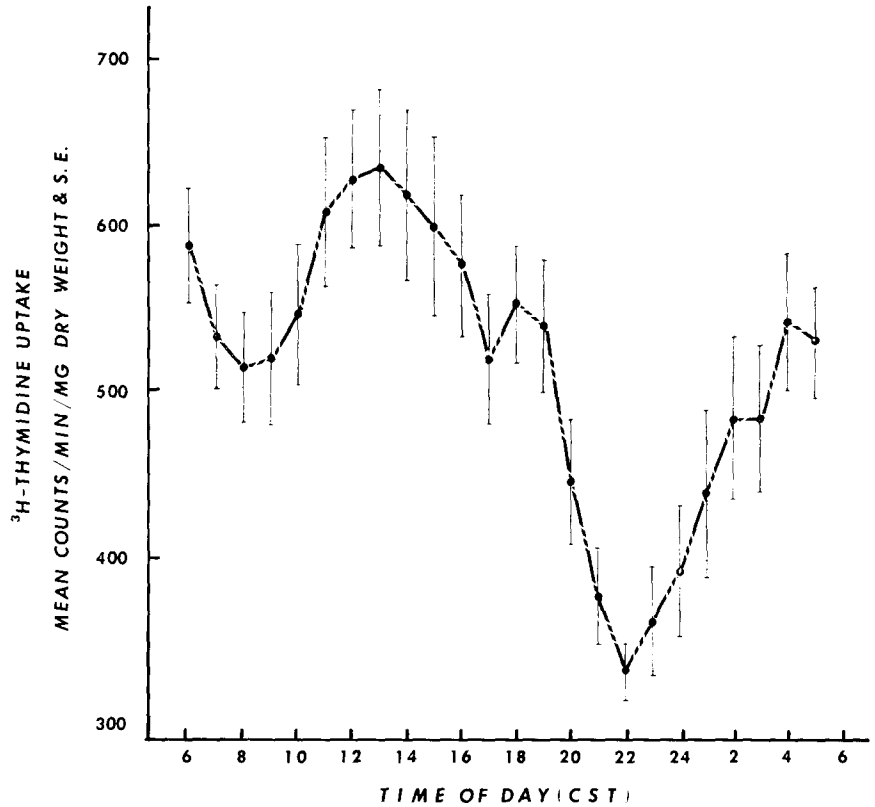

FIGURE 1 The individual plotted values, calculated as 3-hr moving averages, are the means (with standard errors) which indicate thymidine-^{3}H uptake by corneal epithelial cells 2 hr subsequent to injection.

It was of interest and importance to determine what relationships existed between the fluctuating or rhythmic patterns for (1) uptake of thymidine-^{3}H, (2) mitotic indices, (3) percentage of labeled nuclei, and (4) the number of grains per labeled nucleus. Although plotting the hourly data resulted in graphs which clearly indicated phase relationships between the four sets of data, it was decided to use 3-hr moving averages. Thus, some of the hourly fluctuations that may be of little significance in the over-all circadian pattern were eliminated; and the basic trends over the 24-hr period were better illustrated graphically. The 3-hr moving averages and their standard errors for the 4 sets of data are shown graphically in Figs. 1–4. Table I contains the mean hourly raw data from which the 3-hr moving averages were calculated. The values of a particular hour in Table I actually indicate the activity over the preceding 120 min, since the times listed are when the animals were sacrificed and not when injected.

The statistical significance of the differences between the high and low values was determined by calculating the standard error of the difference between the particular hourly means, determining the t value, and finding the p value from standard t tables. The 0.05 level was selected as the criterion for significance. Although differences between high and low values frequently are expressed as percentages in this paper, all of the statistical evaluations are based on the raw data found in Table I.

RESULTS

During a 24-hr period, there was an overt rhythm in the uptake of thymidine-^{3}H by the corneal epithelium of rats. The mean values of counts/min/mg, calculated as 3-hr moving averages, indicate that a major sustained crest occurred between 1000 and 1500, with the minimum value at 2200 (Fig. 1). When the highest mean during the 24-hr period (at 1300), based on 3-hr moving averages, was compared with the lowest mean (at 2200), there was a 90% increase. When a similar comparison was made between the hour (1300) with the high mean and the hour (2100) with the low

mean, based on the hourly data (Table I), there was a 110% increase ($p < 0.001$). The 3-hr moving averages dampen the more acute peaks and troughs; consequently, more of a trend analysis is obtained.

The per cent of labeled nuclei in the radioautographs also showed an overt rhythm along the 24-hr time scale. The crest hours occurred between 1500 and 1800 (Fig. 2), whereas the minimum mean values were recorded between 2200 and 2400. The maximum mean value, based on 3-hr moving averages, showed an increase of 131% over the minimum mean value. A similar comparison based on hourly mean data (Table I) showed a 306% increase over the lowest mean value. The peak mean value in either instance is significantly different from the low mean value ($p < 0.001$).

The mean number of grains/labeled nucleus showed trough and crest hours over the 24-hr time cale (Fig. 3). The peak hourly mean, based on 3-hr moving averages, occurred at 1800 and represented a 35% increase over the lowest hourly mean recorded at 2400. A similar comparison based on the hourly mean data (Table I) showed a 44% increase between the peak mean value (at 1500) and lowest mean value (at 0100). The difference was statistically significant ($p < 0.001$).

The minimum period of mitotic activity was observed between 2000 and 2300; the maximum activity occurred between 0600 and 1100 (Fig. 4). When the hour having the highest recorded mean value, based on 3-hr moving averages, was compared with the hour having the lowest mean value, the mean value was found to have increased by 250%. When a similar comparison from the hourly data (Table I) was made, the highest mean value was 408% greater than the lowest value ($p < 0.001$). Again, this demonstrates the blunting effect when data are presented as 3-hr moving

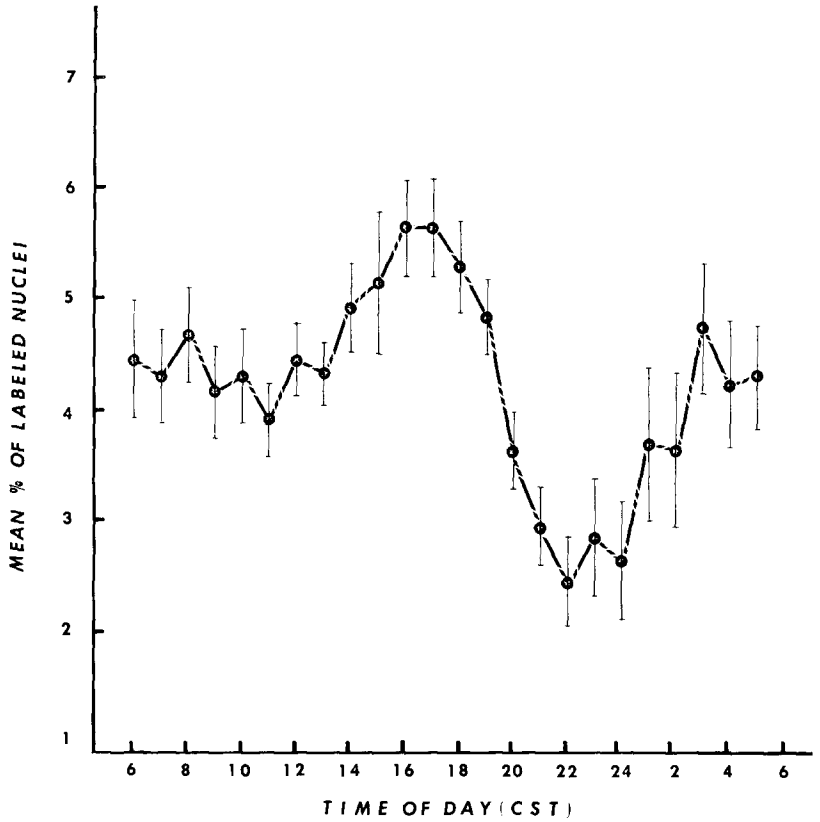

FIGURE 2 The plotted values, calculated as 3-hr moving averages, are the means (with standard errors) representing the percentage of labeled cells in a population of rat corneal cells 2 hr subsequent to injection with thymidine-^{3}H.

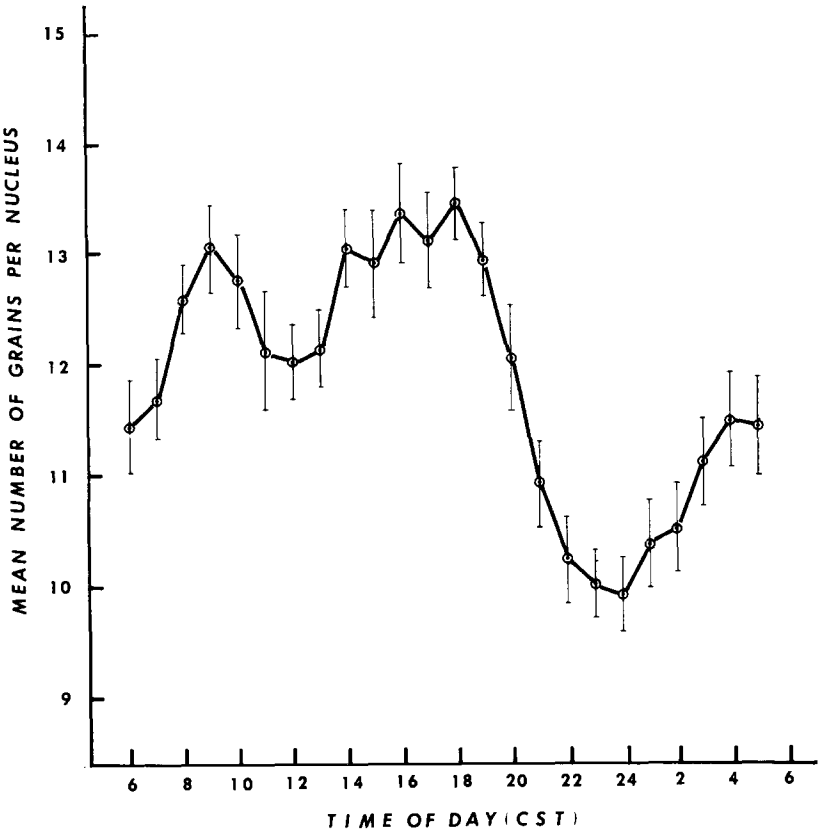

FIGURE 3 The plotted values, calculated as 3-hr moving averages, are the means (with standard errors) which indicate the number of grains/labeled nucleus in rat corneal epithelium 2 hr subsequent to injection with thymidine-^{3}H.

averages. Fig. 4 also shows the typical motor activity of a colony of rats for one 24-hr period. This is not relevant necessarily to this investigation, but it is included so that the motor activity may be compared with the physiological variables being measured.

DISCUSSION

The data on the percentage of labeled nuclei (Table I; Fig. 2) show that the fraction of cells taking up thymidine-^{3}H, and therefore synthesizing DNA, varied from hour to hour over a 24-hr period. The greatest number of labeled nuclei was found at a time which corresponded, in general, to the time when the uptake of thymidine-^{3}H was also greatest, as measured by scintillation counter methods (Table I; Figs. 1 and 2). This indicates that the variation in the amount of thymidine-^{3}H uptake, at least in part, results from fluctuations in the numbers of cells synthesizing DNA. Therefore, it appears that more cells are simultaneously in the S phase of the mitotic cycle at certain hours than at other hours. Pilgrim et al. (7, 8) found circadian variation in the numbers of labeled cells in such mouse tissues as epidermis, the epithelium of the esophagus, forestomach, and tongue. Typically, they recorded only one peak in the numbers of labeled nuclei in each tissue during a 24-hr period. Scheving and Chiakulas (9) reported two peaks or a bimodal rhythm in the uptake of thymidine-^{3}H by urodele epidermis; later (10), they found a related bimodal rhythm in the number of labeled nuclei as well as in the number of grains/labeled nucleus in the same urodele epidermis.

In addition to the circadian fluctuations in the numbers of labeled nuclei, the data of Table I and the graphs (Figs. 2 and 3) reveal that the mean numbers of grains/labeled nucleus show maximum values at approximately the same time

when the uptake of thymidine-^{3}H and the numbers of labeled cells are maximum. The increases in numbers of grains/labeled nucleus at certain times during the 24-hr time scale indicate that, at those hours, the rate of synthesis for individual cells is somehow accelerated. Scheving and Chiakulas (9) explained the peaks in thymidine-^{3}H uptake in urodele tissue by suggesting, first, that at certain hours of the 24-hr period more cells in a standard cell population are synthesizing DNA and, second, that at these same times the individual cells are synthesizing at an accelerated rate. The results of the present investigation support their conclusion, since also in the case of the rat cornea both processes contributed to the observed peak of measured thymidine-^{3}H uptake.

Although we found periodic variations in the amount of labeling in nuclei of the corneal epithelium of rats, Pilgrim et al. (7, 8) did not find this in the mouse tissues they studied. They concluded that the rate of synthesis and, therefore, the duration of the S phase of the mitotic cycle of the cells is constant. The results of this present investigation suggest that the synthesis rate of rat corneal epithelial cells may be accelerated at certain hours, and, therefore, the duration of the S period may vary at certain periods of the day.

In general, the findings of this investigation demonstrate the innate rhythmic nature of several processes or events involved in the total mitotic cycle of the cell. We feel that a careful analysis and correlation of the circadian characteristics of the various phases of the mitotic cycle and the determination of the effects of DNA, RNA, and protein synthesis inhibitors on these rhythms would lead to a further elucidation of the basic controlling mechanism of cell division.

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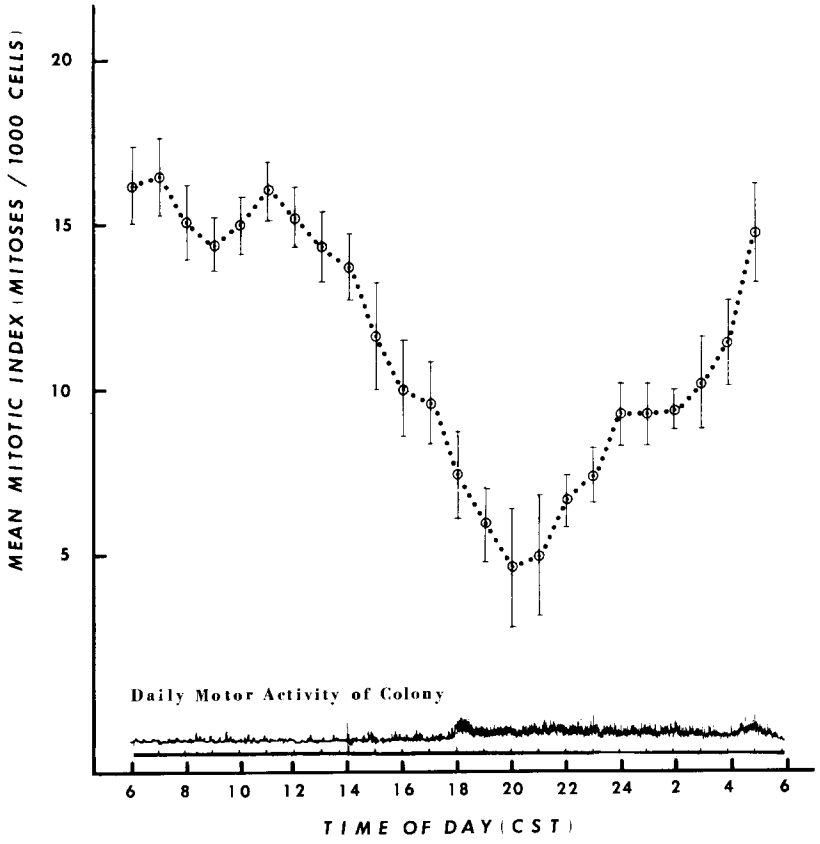

FIGURE 4 Mean mitotic indices (with standard errors), based on 3-hr moving averages, in rat corneal epithelium. Also, activity pattern of a colony of rats over a 24-hr period based on noise emitted by the colony.

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