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CIRCADIAN RHYTHMS OF PRESUMPTIVE STEM CELLS IN THREE DIFFERENT EPITHELIA OF THE MOUSE

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ABSTRACT

Variation in the percentage of labelled cells (LI), mitoses (MI) and apoptosis (AI: i.e. shrinkage necrosis) have been studied throughout a 24 hr period (40 min after labelling with ^{3}H-TdR) for tongue epithelium, epidermis and intestinal epithelium in the mouse. A room with reversed light cycle was used to obtain data for half of the 24 hr period. All three tissues showed marked variations in LI with peak values between 24.00 and 03.00 hours. In the intestine a maximum value for MI was observed 3–6 hr after that for LI and with a maximum value for AI slightly later.

In all three epithelia the circadian rhythm was most striking in cells at positions which can be correlated with presumptive stem cell activity; e.g. in the crypts the labelling and mitotic peaks reflecting a circadian rhythm were most clearly distinguishable at the basal part of the crypts. These observations are discussed in relation to the validity of various proliferative models.

In this report we consider three different epithelial regions, all from the same groups of mice, and compare the variations in the percentage of cells in mitosis (MI) and per cent labelled cells (LI) in relation to the position which these cells may occupy within these highly organized epithelia. In these epithelia, regions of presumptive stem cell activity can be identified (Potten, 1974, 1975a, 1976a, b; Allen & Potten, 1974; Hume & Potten, 1976, Cheng & Leblond 1974; Leblond & Cheng, 1976) allowing a comparison to be made between the circadian rhythm of presumptive stem cells and that of committed or differentiated proliferative cells.

Circadian rhythms in proliferative activity occur in many tissues. Various mechanisms for controlling these rhythms have been suggested but these all assume that the proliferative cells are a fairly homogeneous cell population. If there are different classes of proliferative cells

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then each class may have its own specific control factors. In terms of the overall circadian rhythms it seemed possible that it could be explained by a marked rhythm in a vital precursor (stem) cell population with a subsequent loss of synchrony in the derived cell population. This idea was investigated and some evidence which supports this hypothesis is presented and discussed.

MATERIALS AND METHODS

Animals

Thirty-eight female BDF_1 (C57B1/6 × DBA-2) mice varying in age between 6 and 8 weeks were acclimatized for 4 weeks in temperature, light and humidity controlled animal rooms. Half the number of animals were housed in a room with a 12 hr light cycle running from 06.00 to 18.00 hours (normal cycle) while the others were placed in a room with the light cycle reversed (18.00–06.00 hours) (reversed cycle on graphs). The animals had a continuous supply of food and water and were housed four to a box, each box being an experimental group. Removal or handling of animals during the dark phase was accomplished with the help of a dark red photographic safe-light. Any animals with hair in the growth phase were discarded. It was assumed that 4 weeks was sufficient time for complete acclimatization to the reversed light cycle and that the procedures involved in pulse labelling with ^{3}H-TdR 40 min before sampling had no effect on the circadian rhythm.

Labelling procedures

Every 3 hr starting at 09.00 hours a group of animals from both normal and reversed light cycle rooms received 50 μCi in 0·2 ml of ^{3}H-TdR (5 Ci/mmol) intraperitoneally. The injections were conducted in the animal rooms and the mice were killed 40 min after the injections.

Histological techniques and autoradiography

Samples of dorsal skin, tongue and small intestine were taken from each animal. Epidermal sheet preparations were stained using the Feulgen technique before coating with melted Ilford K5 emulsion and subsequent exposure for 2 weeks (Hamilton & Potten, 1972; Potten & Allen, 1975). Carefully orientated tongue sections also stained by the Feulgen technique were coated with photographic emulsion and exposed for 10 days (Hume & Potten, 1976). Autoradiographs were prepared from crypt squashes (Wimber *et al.*, 1960, as modified by Kovacs & Potten 1973). Other samples of ileum (about 10 cm in length) were removed and fixed (Carnoy's) in a petri dish for 30 min prior to storage in 70% ethanol. These were cut into 1 cm lengths, six to ten of which were placed together enclosed in surgical tape, trimmed, embedded in paraffin and sectioned transversely at 6 μm. These sections were stained with haematoxylin and eosin and used to score mitoses and dead or apoptotic cells (Kerr, 1971; Kerr, Wyllie & Currie, 1972; Searle *et al.*, 1975). Discrete apoptotic bodies may contain either single pyknotic nuclear fragments, or several tiny fragments giving the appearance of karyorrhexis.

Scoring

Epidermis. More than 1000 basal nuclei from four randomly selected fields (each of about 250 cells) on each mouse were scored for labelling index (LI). The epidermal cell columns

defining the epidermal proliferative units (EPU) can be identified using phase contrast microscopy (Potten, 1974). Forty EPUs with one or more labelled nuclei were studied from each mouse. The number and position of the labelled nuclei relative to the column boundaries were recorded, i.e. whether it was peripheral, non-peripheral or central. From these figures the average number of labelled nuclei of individually labelled EPUs, designated as EPU(L) and thus also the fraction of labelled EPUs (P) could be determined (P = LI.A/L).

The following nomenclature and symbols will be used:

The overall labelling index for the basal cells = LI.
The LI for peripheral part of the EPU = PLI.
The LI for the non-peripheral part of the EPU = NPLI.
The LI for the central cell of the EPU = CLI.
The average number of basal cells per EPU = A = 10·6 (Potten, 1974).

The average number of peripheral basal cells per EPU = PN = 6·6.
The average number of non-peripheral basal cells per EPU = NPN = 4.
(There is only one central cell per EPU.)

The average number of labelled cells per labelled EPU = L.
The average number of labelled peripheral cells per EPU(L) = PL.
The average number of labelled non-peripheral cells per EPU(L) = NL.
The average number of labelled central cells per EPU(L) = CL.

Values for the fraction of labelled nuclei at the periphery, the non-periphery and the centre (LI(P), LI(NP) and LI(C)), respectively, were calculated according to the following equations:

$$PLI = \frac{PL}{PN} \cdot LI \cdot \frac{A}{L}; \qquad NPLI = \frac{NL}{NPN} \cdot LI \cdot \frac{A}{L}; \qquad CLI = CL \cdot LI \cdot \frac{A}{L}$$

It has been suggested that the more centrally positioned cells might act as stem cells in this system (Potten, 1974).

Tongue. After extreme care in orientation, sections were selected for scoring if they contained filiform papillae well sectioned longitudinally. The overall labelling index was determined for the dorsal and ventral surfaces by scoring 1000 consecutive basal nuclei in both regions from each tongue. From the dorsal surface fifty filiform papillae on alternate 5 μm sections from the area immediately distal to the sulcus terminalis (the groove separating proximal and distal aspects of the tongue) were selected from each mouse for detailed scoring (Hume & Potten, 1976). The position and labelling of each basal cell was noted in relation to the filiform papilla and its dermal component, with the cells at the base of the dermal papilla being numbered position 1 (see Fig. 4). It has been suggested that this lowest cell position is the origin of the cell flow patterns in this highly organized tissue and may thus be the site of the stem cell component (Hume & Potten, 1976).

Intestine. In transverse sections crypts were selected for scoring if they showed a lumen open at the top, some evidence of the Paneth cell population at the base and seventeen or more nuclei up one side (counting from the mid-point at the base to the crypt neck). The average number of crypt cells up one side, in the crypts scored was 20·7. The total number of mitoses and apoptotic bodies was determined in forty crypt sections per mouse.

Each crypt was subdivided into three regions, each delineated by a third of the total number of cells up one side. The distribution of mitotic and apoptotic figures in these three regions was determined.

Amongst the crypt squashes only relatively undersquashed crypts were selected for scoring (Kovacs & Potten, 1973). These selected crypts were remarkably constant in size (cell numbers). Amongst the under-squashed crypts only those where the top and bottom could be reliably identified were used for scoring. The features used in determining the crypt orientation were: (1) the general flask shape of the crypt with a rounded base and a broken top; (2) the higher grain density of the labelled cells at the base (mean grain count for 200 crypt base cells was 35·7 while in the same crypts 200 cells higher up provided a mean value of 20·8); (3) the relatively larger cytoplasmic component (and thus greater inter-nuclear distance) of the basal Paneth cells compared to the cells of the upper crypt. Crypts were rejected if the orientation was in doubt, if they contained numerous superimposed cells or if they were squashed to an extent that cells were grossly displaced. Suitably squashed crypts show a band of labelled cells (LI $\simeq$ 40–50%) across the middle of the crypt). The number of labelled cells in these crypts was previously demonstrated to be the same as in totally squashed crypts where topography could not be recognized (Kovacs & Potten, 1973).

The crypts were divided into three regions based on the presence or absence of a high LI; the basal Paneth region (PA) containing the Paneth cells; the central proliferative region (PR) containing most of the labelled cells; and the upper maturation region (MA). Ten crypts from each mouse were scored for the number of labelled nuclei (LN), unlabelled nuclei and mitotic cells (M) in each of the three regions and also in the crypt as a whole. Thus about 2500 cells were scored from each mouse.

In all cases the mean values were determined for each mouse and the mean of the mouse values with its standard error are presented in the figures and table. The data points are

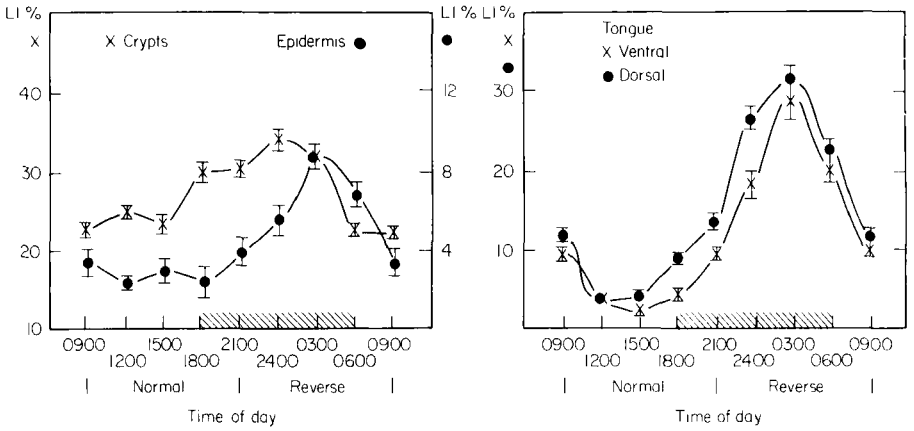

FIG. 1. LI values throughout a 24 hr period for ileal crypts (×, left-hand ordinate) and dorsal epidermis (●, right-hand ordinate) (left-hand graph). LI values for the dorsal (●) and ventral (×) surfaces of the tongue (right-hand graph). For each data point at least 1000 basal cells were scored per mouse, the mean and standard error for four to eight mice being determined (09.00 hours consisted of eight mice and 21.00 hours of six mice). The period of the day when the lights were switched off is shown shaded. The data points obtained from the mice under the normal cycle (09.00–21.00 hours) are indicated by the range of times marked 'normal'. Those from mice under a reversed cycle (21.00–09.00 hours) are indicated by 'reversed'.

plotted against the time of day in the graphs; those data points from animals in the reverse-cycle room have had the time adjusted to the normal light cycle. The points obtained from the normal and reverse cycle rooms are indicated on the graphs.

RESULTS

Overall LI

Fig. 1 shows that variations in the overall values for LI, throughout the 24 hr period are similar in four epithelia, with high values at 24.00–03.00 hours. These data do not account for any tissue topography. The 24 hr average of all the individual values throughout the 24 hr period are shown in Table 1.

TABLE 1. Average circadian labelling, mitotic and apoptotic index values for skin, tongue and intestinal epithelia

		Peak-to-trough ratio*	24 hr average†
Skin:	Overall LI‡	3·8	4·5 ± 0·8
	Central LI	6·9	2·2 ± 0·6
	Peripheral LI	3·7	6·0 ± 1·1
	Labelled EPU (%)	3·3	41·0 ± 6·6
Tongue:	Ventral LI§	14·3	11·7 ± 3·3
	Dorsal LI§	9·5	15·0 ± 3·7
	Anterior aspect 1	14·6	32·9 ± 7·1
	2	23·8	15·0 ± 5·4
	3	23·0	5·8 ± 2·6
	4	—	1·9 ± 0·8
	Posterior aspect 1	13·8	27·7 ± 6·4
	2	14·5	25·7 ± 6·7
	3	14·7	14·8 ± 4·5
	4	—	5·4 ± 2·3
Crypts:	Overall LI	1·5	27·6 ± 1·7
	Proliferative LI	1·4	42·0 ± 2·0
	Paneth LI	2·0	7·6 ± 0·7
	Overall M (mitoses/crypt)	2·3	3·4 ± 0·4
	Proliferative M	2·8	2·4 ± 0·3
	Paneth M	6·5	0·3 ± 0·05
	Overall A (apoptoses/crypt)	1·8	0·17 ± 0·01
	Proliferative A	3·0	0·06 ± 0·01
	Paneth A	2·8	0·08 ± 0·01

* Ratio of highest to lowest values. † 24 hr average values are the mean and standard errors for the eight time points (i.e. an overall average of thirty-two mice). ‡ LI values are expressed as %. § Based on 1000 consecutive basal cells. For explanation of tongue scoring see text and Fig. 4.

Skin

Figure 2 summarizes the results obtained when the LI is scored in relation to cell position within mouse dorsal EPUs. There was no evidence of any clear circadian variation in the number of basal cell/mm^2. The curve with the largest difference between the peak and trough was that for the central cells of the EPUs (the presumptive stem cell area) (Table 1). There

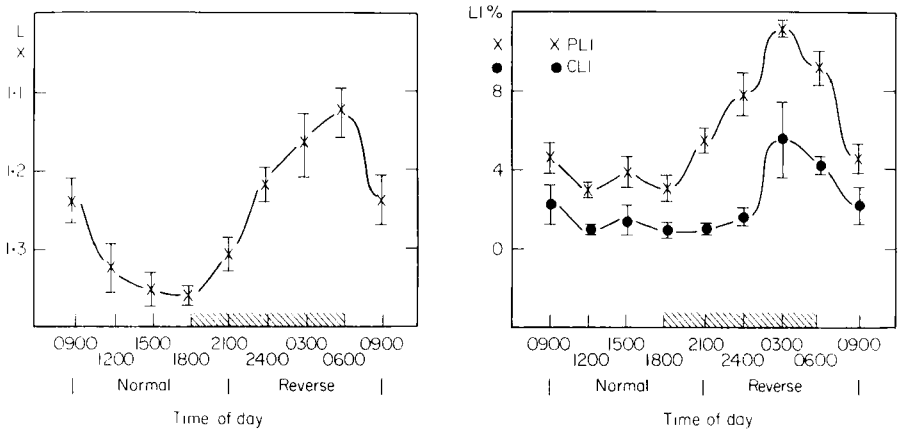

FIG. 2. Details of labelling characteristics for dorsal epidermal proliferative units (EPUs); LI values were calculated from data of forty EPUs scored per mouse. The number of labelled nuclei/labelled EPU (L, ×) is shown together with values of LI for the central (CLI, ●) and peripheral cells (PLI, ×) per EPU.

was a marked rhythm in the fraction of labelled EPUs, 75% being labelled at 03.00 hours with 25% labelled between 12.00 and 18.00 hours.

Tongue

The overall LI values for the dorsal and ventral surface of the tongue are shown in Fig. 1 and Table 1. Fig. 3 shows the rhythms observed in cell positions 1–4 for the anterior and posterior aspects of the filiform papilla (anterior being nearest the tip of the tongue). All positions show peak values at 03.00 hours, with the highest LI values at the lowest cell positions (presumptive stem cell region). The LI value for cell position 1 reaches 55–65% at

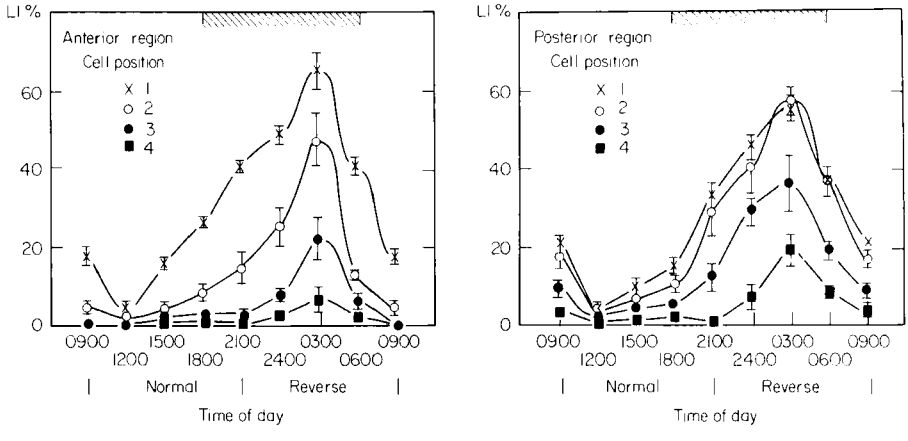

FIG. 3. Labelling characteristics (LI) for cell positions 1–4 of the anterior and posterior regions of the filiform papilla; individual points shown were calculated from results of fifty papillae scored per mouse. For details of the structural organization and the methods employed for scoring of the mouse filiform papilla see Hume & Potten (1976).

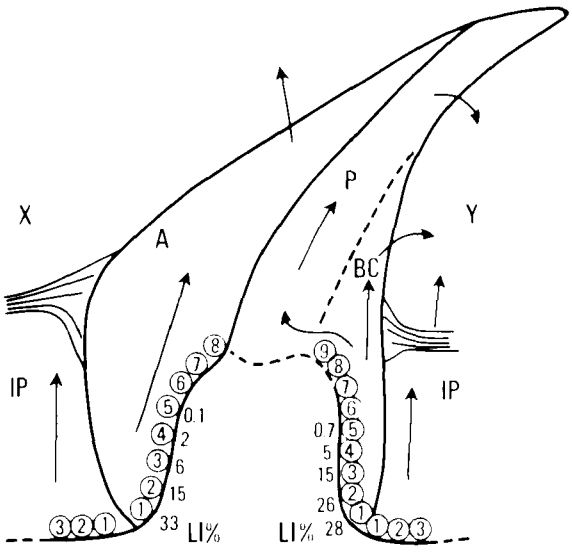

FIG. 4. Summary of the organization, cell flow patterns, cell numbering technique and average LI values for all samples throughout a 24 hr period for the mouse filiform papilla. IP = interpapillary regions: XY is the longitudinal plane of sectioning (i.e. along the length of the tongue): A = anterior aspect; P = posterior aspect; BC = posterior buttressing column of cells. The arrows suggest probable cell flow patterns. The basal cells are numbered 1–8 or 9 starting at the border between the papilla columns and the interpapillary region. The average LI values for each cell position are shown (see also Table 1).

03.00 hours. The overall average LI values for the entire 24 hr are shown together with the suggested cellular organization and cell flow patterns (Hume & Potten, 1976) in Fig. 4. The overall LI (Fig. 1) obviously results from the combination of the data shown in Fig. 3 together with the interpapillary labelling patterns and a considerable contamination by the relatively unlabelled cells at higher cell positions on the dermal papilla. The average LI values for all eight time points are shown in Table 1.

Intestine

The overall crypt LI values are shown in Fig. 1 and Table 1. Fig. 5 shows the number of mitoses per crypt (sections and crypt squashes) with the labelling index shown for comparison. The peaks in both mitoses per crypt and LI are broad and both occur in the early hours of the morning. It appears from the curves that the LI curve is 3–6 hr in advance of the mitotic peak while the apoptotic curve appears to be 1–3 hr after the mitotic curve.

Fig. 6 shows the variations in the number of mitoses, apoptoses and labelled cells for the crypt base, mid-crypt and total crypt cell population. There is a marked rhythm in mitotic and apoptotic cells in the crypt base region, the presumptive stem cell region. The labelling data are also suggestive of a marked rhythm in the presumptive stem cell region. The proliferative region shows about twenty additional labelled nuclei and a similar increase in total nuclei at a time when the Paneth region has decreased in size by about twenty cells. This is because many cells in the upper part of the Paneth region become labelled at 03.00 hours, thus raising the local LI to above 50% in this region, making it technically impossible to differentiate these

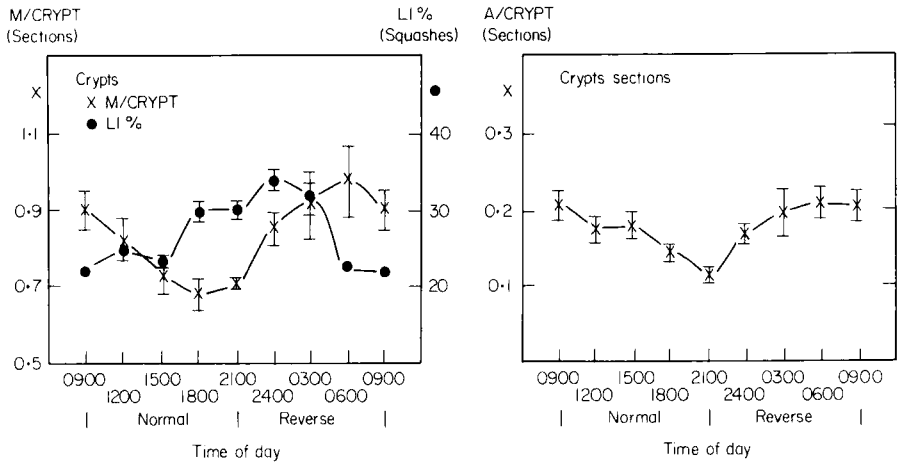

FIG. 5. Mitotic (M, ×) and apoptotic (A, ×) cells per ileal crypt section throughout a 24 hr period shown with the LI (●) obtained from crypt squashes.

cells from those in the proliferative region. The apoptotic counts for the Paneth region are again displaced slightly to the right of the mitotic points.

DISCUSSION

It was clear from the early work on proliferative circadian rhythms that epidermis showed significant mitotic (Carleton, 1934; Cooper, 1939; Bullough, 1948) and labelling rhythms (Messier & Leblond, 1960; Pilgrim, Erb & Maurer, 1963; Blenkinsopp, 1968). The early reports also showed rhythms for tongue (see also Gasser, Scheving & Pauly, 1972; Burns *et al.*, 1976) but little evidence for rhythms in intestine (see also Leblond & Stevens, 1948). Comparison of the present results with data from the literature is difficult because of the variability in peak size, peak time, and even the number of peaks observed. These differences are due, in part, to differences in species, strain, sex, age, housing, handling conditions, technical procedures and the timing of the sampling (Potten *et al.*, 1971; Sigdestad, Bauman & Lesher, 1969).

Even recent studies of epidermal LI rhythms show variability in time, size and shape of the peak (Grube, Auerbach & Brues, 1970; Hegazy & Fowler, 1973; Tvermyr, 1969, 1972); however, peak activities generally occur between 24.00 and 05.00 hours. Amplitude variations occur even within our own colony of mice (Potten, 1975b; Figs 1 and 2 and Table 1).

Intestinal rhythms have recently been demonstrated (Sigdestad *et al.*, 1969; Sigdestad & Lesher, 1970, 1971) which may be correlated with food intake (Sigdestad *et al.*, 1974) though both may be due to other factors (Tutton, 1973, 1975; Tutton & Helme, 1973).

The present studies have shown that all three regions possess a clear circadian rhythm with a common peak in LI at 24.00–03.00 hours. This suggests that the factors determining the circadian rhythm are more likely to be systemic than local. Thus feeding activity and the possible attrition of villus height by food is unlikely to be the cause of the rhythm elsewhere in the body (Sigdestad *et al.*, 1969, 1974). Hormones secreted during periods of activity might

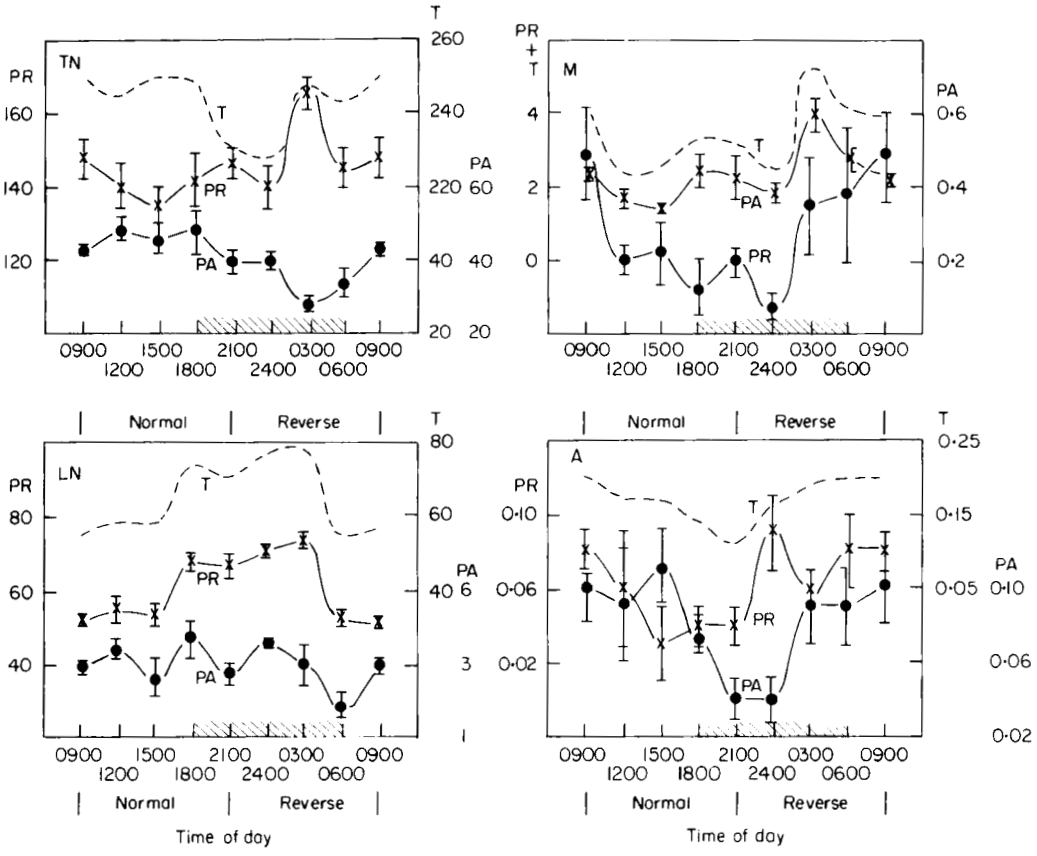

FIG. 6. Mitotic (M), apoptotic (A), labelled (LN) and total (TN) cells per whole crypt (T), per proliferative region (PR) or per Paneth region (PA) throughout a 24 hr period. The proliferative region scale is on the left-hand ordinate while the total crypt scale is on the right (with the exception of the mitotic data graph where the PR and T scales are identical). The Paneth region scale is on the lower right ordinate. Apoptotic counts obtained from section material.

be involved (Bullough & Laurence, 1961; Epifanova & Tchoumak, 1963; Tutton, 1973; Tutton & Helme, 1973).

Though the peaks and troughs of the curves representing the variation in LI for the three tissues shown in Fig. 1 are coincident, the amplitude of the cycle in LI or M activity was variable (Table 1); The curves obtained for the tongue show very large peak-to-trough ratios. The significance of this is also obscure at present.

Time relationships between various parameters

Unlike some earlier studies, the peaks in LI and M for crypts were not coincident (Sigdestad *et al.*, 1969). The LI peak was broader and peaked about 6 hr earlier than the mitotic peak (Fig. 5).

The positions in time of the peaks and troughs of the curve for total apoptotic figures per crypt (Fig. 5) are displaced to the right by 1–3 hr as compared to those of the corresponding

peak and trough of the mitotic curve. This displacement suggests that the dead cells may be aborted mitotic cells since earlier (unpublished) observations on drug or radiation-induced apoptoses indicate that these clearly recognizable dead cells appear within about 3 hr after treatment. These data may also lead to the conclusion that approximately 10% of normal mitoses are not successful and result in cell death. (The apoptotic index is approximately one tenth of the mitotic index.)

Circadian rhythm in relation to topography

In all three regions there is a particularly strong rhythm in LI for the regions postulated to contain the stem cell component, i.e. the central region of the EPU (Potten, 1974; Allen & Potten, 1974), the base of the dermal papilla in the filiform tongue papilla (Hume & Potten, 1976) and the crypt base, or more particularly the region immediately beneath the proliferative region (Cheng & Leblond, 1974; Leblond & Cheng, 1976; Potten, 1975a, 1976a; Potten & Hendry, 1975). The significance of this is not clear at present but it is possible that if all three regions do indeed operate via a two-compartment proliferative model (stem cells and stem cell-derived proliferative cells) then the overall circadian rhythm might be the consequence of a predominant stem cell rhythm. The derived cells, which probably only divide two to four times before terminal differentiation, would continue at least their initial divisions in reasonable synchrony. This would suggest that whatever the stimulus is for the circadian rhythm the stem cells alone respond.

The shape of the intestinal labelling or mitotic circadian curves suggests two distinct cell populations. The majority of crypt cells divide approximately every 12 hr and if these cells were susceptible to the factors that control the circadian rhythm one would expect two peaks in LI, LN or M probably separated by 12 hr. This is clearly not observed in the present or earlier experiments and can be explained only if the crypt contains two types of cells, one type susceptible to circadian triggers and one not. Thus it seems likely that the rhythm occurs in, and is largely generated by, a small subpopulation of stem cells that are cycling more slowly than the majority of cells. The present data and earlier cell kinetic studies suggest that the stem cells have cycle times of about 24 hr (Potten, 1975a; Potten, Kovacs & Hamilton, 1974; Al-Dewachi *et al.*, 1975). The tongue data (Fig. 3) also suggest that the cells at position 1 (presumptive stem cells) cycle once a day. The lower absolute values at higher cell positions suggest that the cells at these positions are either cycling more slowly, or that many of them are post-mitotic differentiating cells.

The skin central cells also appear to have a 24 hr cycle; however, since the absolute LI values only reach 5% and there is considerable error inherent in this type of determination the data may be more correctly interpreted to indicate that when the slowly cycling EPU (Potten, 1974) central cells enter S they tend to do so during the early hours of the morning.

Although it is not entirely clear, there are indications that the tumour yield after skin is painted with a chemical carcinogen varies depending on the time of day when the carcinogen is applied (Iversen & Iversen, 1976; Iversen *et al.*, 1970; Frei & Ritchie, 1964). It is possible that this reflects circadian variations in behaviour of a crucial carcinogen-target cell population (stem cells?).

Burns *et al.* (1976) have recently noted that oesophageal and tongue per cent labelled mitoses (PLM) curves initiated at 09.00 hours showed a distinct second wave while curves initiated at 21.00 hours did not. This further indicates the presence of two distinct proliferative cell populations. This observation is quite consistent with, and is largely explained by, the

model suggested here; namely that epithelial tissues contain two types of proliferative cells: stem cells and derived cells committed to differentiation with a limited division potential. The stem cells respond to factors (possibly systemic) that control their cyclic behaviour. The derived cells are not susceptible to these factors and exhibit less circadian rhythm the further they are in time from their birth from stem cells (progressive desynchronization with each division step). Thus a PLM curve started when many stem cells are in S will show a second peak when the stem cells and early derived cells enter a second division, while a curve started when few stem cells are in S will tend to select more terminal derived cell divisions and thus will not show a second peak.

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