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The length of S-phase and G_2-phase of epithelial cells is regulated during growth and morphogenesis in *Hydra attenuata*

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Summary

Feeding and sectioning of the freshwater polyp hydra generates signals that (1) shorten the G_2-phase of epithelial cells and (2) prevent epithelial cells from entering mitosis for a short period of time. The latter effect appears to be mediated by inhibitor I, an endogenous morphogenetically active compound of hydra tissue. At least after feeding, the S-phase is shortened as well.

Key words: *Hydra attenuata*, epithelial cell cycle, growth, regulation, feeding, sectioning, mitosis, inhibitor I.

Introduction

The freshwater polyp *Hydra* has a tube-shaped body bearing a head, a hypostome surrounded by a ring of tentacles at one end and a foot, a sticky patch, at the other end. The body consists of two layers of epithelial cells, separated by a basement membrane. Interspersed with the epithelial cells are some other cell types including stem cells of nerves and nematocytes. Upon feeding the animals grow and form buds: the number of epithelial cells doubles within 3 to 4 days, whereas upon starvation the budding rate and the epithelial cell number decreases.

The cell cycles of epithelial cells are partially synchronous in daily fed animals: fed regularly in the morning a peak of mitotic index was found at night. The S-phase lasts 12 to 15 h and the G_2-phase 24 to 72 h. Mitosis (1·5 h) and G_1-phase (1 h) are very short (David & Campbell, 1972, Otto & Campbell, 1977; Bosch & David, 1984).

Park, Ortmeyer & Blankenbaker (1970) found that sectioning causes the mitotic index of epithelial cells to decrease (in *Hydra pseudoligactis*). In a preliminary study we found that an endogenous substance, enriched from crude extract, termed inhibitor I, is also able to decrease the mitotic index (Berking, 1974).

Starting from these observations we investigate how the cell cycle of epithelial cells is controlled during growth and morphogenesis.

Materials and methods

All experiments were done with *Hydra attenuata* at 20°C. Animals were obtained from a mass culture fed daily at 9 a.m. for at least 14 days prior to the start of the experiment to reach a steady state in epithelial cell proliferation (Otto & Campbell, 1977). For all experiments large budless animals were chosen, which, if not specified otherwise, were collected from the culture 24 h after the last feeding. Animals developing a bud during the experiment were discarded.

Sectioning

Animals were bisected at two positions: just below the tentacle ring and at a position one-quarter the body length below the head. The piece of tissue between both cuts was termed the distal quarter.

Cell preparation and staining

Hydra tissue was macerated into single cell suspension in glycerol:acetic acid:water, 1:1:6·5 (David, 1973, modified). Cells were fixed with a drop of formaldehyde, spread on gelatin-coated microscope slides and dried overnight. Cells were stained with either orcein–acetic acid or carmine–acetic acid (Romeis, 1968) and analysed with a phase-contrast microscope. Recently fed hydra containing lipoid droplets and other digestion compounds were analysed by means of DAPI (4′,6-diamidino-2-phenyl-indol, Serva, Heidelberg, FRG) (Russel, Newman & Williamson, 1975). This compound, which preferentially stains DNA, was applied on top of the cell preparations on the slides (10^{-5} M in distilled water). The cells were analysed immediately with a fluorescence microscope (Zeiss IM 35, Oberkochem, FRG).

Determination of cells and mitotic figures

A sample of five tissue pieces was dissociated into single cells and the suspension was spread on the slide to form a rectangle. With an ocular diaphragm of given width the cell number was determined in stripes orthogonal to the length of the rectangle at positions 20, 40, 60 and 80 % of this length. From these values the number of epithelial cells per piece of tissue was calculated. At the time points given in the figures three samples (each of five tissue pieces) were prepared. The mean of these three samples and the standard deviation of the mean were calculated. Mitotic figures from metaphase through anaphase to telophase were scored. Prophase figures were excluded because they are sometimes difficult to distinguish from interphase figures. Thus the absolute numbers of mitotic figures we present may be lower than those reported by others. At least 1000 cells were scored for each value. The bars in the figures represent the standard deviation (S.D.) calculated by use of the binomial distribution.

Labelling

Cells were labelled with methyl-[^{3}H]thymidine by injecting the isotope directly into the gastric cavity. A single injection resulted in a pulse of about 1 h duration (David & Campbell, 1972; Bosch & David, 1984). One hour after injection of the label the animals were fixed. Labelled cells were identified by autoradiography using Kodak AR 10 stripping film exposed for 10 days at 4°C. Each time five animals were labelled. At least 400 cells were scored per sample. The experimental error, S.D., was determined by use of the binomial distribution.

Crude extract, inhibitor I

Crude extract was obtained by sonication of animals in distilled water. An OD_{280} of 0·033 of crude extract was defined arbitrarily as one Biological Unit (1 BU) per ml (Berking, 1977). The inhibitory agent was enriched from the crude extract according to Berking (1977, 1983), yielding inhibitor I. Animals were treated with inhibitor by applying the agent to the culture medium.

Results

In head-regenerating tissue the mitotic index of epithelial cells increased one day after head removal

Head regeneration was started by bisecting animals just below the tentacle ring (at position SI, Fig. 1A). At 24, 28 and 32 h, respectively, 5, 50 and 90 % of the animals have regenerated tentacles. At various times following decapitation samples were taken from the pool and sectioned at position SII to isolate the distal quarter. Some of these distal quarters were prepared immediately for cell counting. The others were allowed to develop for up to 72 h.

The number of epithelial cells was found to decrease in the distal quarter (Fig. 1B), as expected because in head-regenerating and starved animals the epithelial cell number decreases with similar kinetics (Otto & Campbell, 1977; Berking & Schindler, 1983). This decrease may be caused by cell loss at the cut surface and phagocytosis (Bosch & David, 1984).

However, hidden behind this decrease in the distal quarter was a period of epithelial cell proliferation: the mitotic index was much higher by the second day than on the day before and the day after (Fig. 2A). Distal quarters isolated during the second day contained far more cells after 72 h than at the time of isolation (Fig. 1B). This was not observed with distal quarters isolated earlier or later.

In undisturbed starved animals (without decapitation at zero time) the mitotic index of the distal quarter remained constant at a level of 0·2 to 0·4 % for 72 h (not shown).

Sectioning within the proliferation period causes a rapid decrease followed by a large increase of the mitotic index of epithelial cells

Isolating the distal quarter when the mitotic index was high, 28 h after decapitation, caused a rapid decrease of the mitotic index from 1·7 to 0·3 % within 3 h (Figs 2B, 3). Then the mitotic index increased tenfold within 27 h. Without this cut it decreased continuously from 28 h onwards (Fig. 2A).

The effect of the second cut seemed to depend on the developmental state of the animal: isolating the distal quarter during the period of high proliferation rate caused a large increase in cell number, whereas a second cut in a period of low proliferation rate (either at early or late times) did not cause such an increase.

Both an injury and treatment with inhibitor I decrease the mitotic index but do not cause a subsequent overshooting increase

Animals were decapitated and 28 h later either injured at the position of the second cut or treated with inhibitor I for various times (Fig. 3)

Both treatments caused a rapid decrease of the mitotic index (Fig. 3). For 14 h an injury had apparently the same influence on the mitotic index as a complete excision of the distal quarter. However, subsequently the mitotic index increased in excised pieces, whereas it decreased in injured pieces. Treatment with inhibitor I for 3 h mimicked the fall and rise of the mitotic index caused by an injury.

To compare the mitotic activity of undisturbed head-regenerating animals with the activity of injured ones the cumulative mitotic indices were estimated. We determined the area below the respective mitotic index curve between 28 and 48 h (Figs 2A, 3). We found no differences: in injured animals the activity is 1·03-fold higher than in control animals. Thus treatment with inhibitor or an injury seemed to block immediately and reversibly the mitotic activity. In contrast, a second cut that completely isolated the

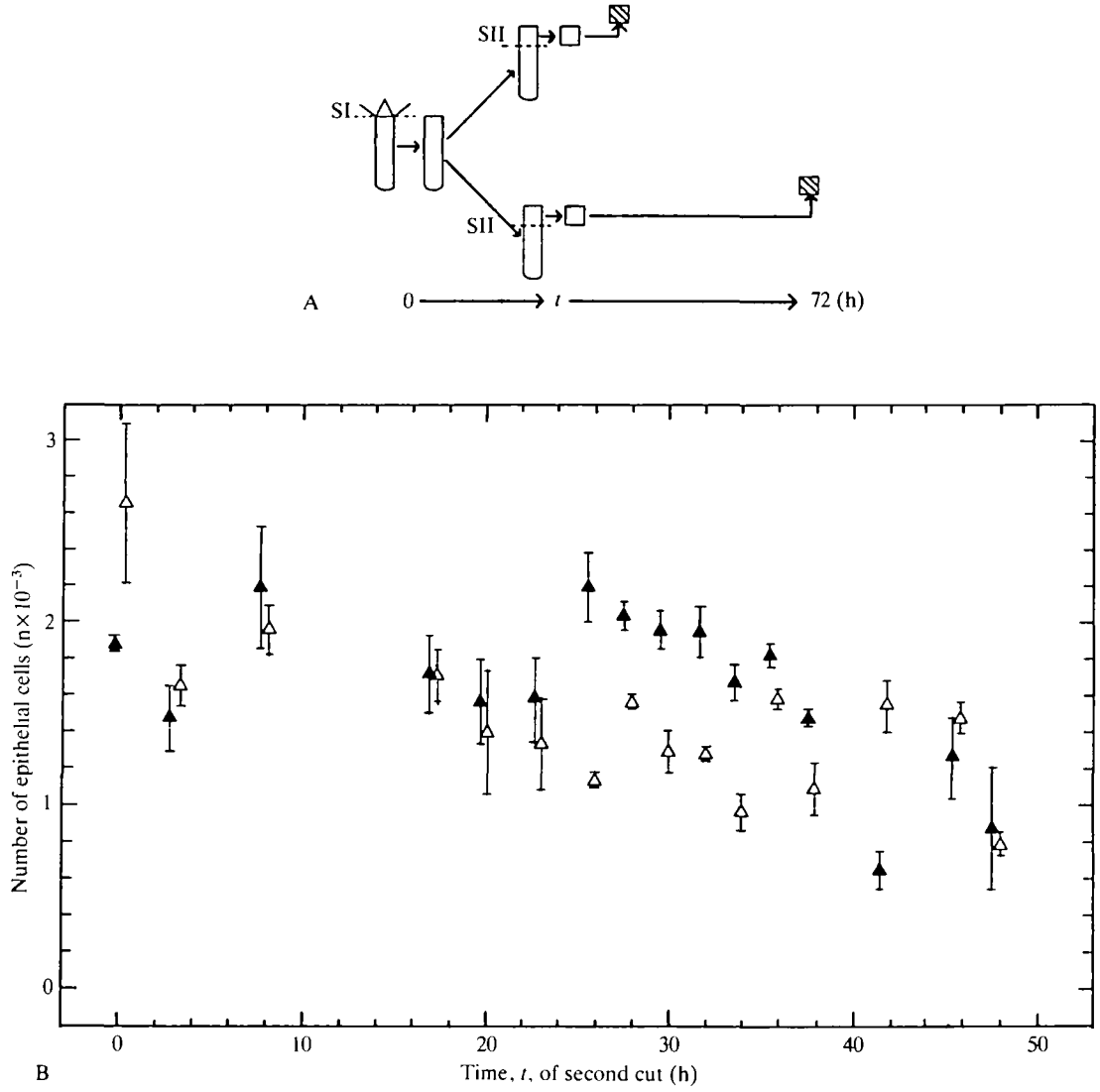

Fig. 1. Number of epithelial cells of distal quarters. (A) Outline of the experiment. All animals were decapitated (position SI) between 11 a.m. and 1 p.m. (zero time). At various times, *t*, the distal quarter was isolated by a second cut (SII) and prepared for cell counting (dashed rectangle) either immediately or 72 h after decapitation. (B) The number of epithelial cells of distal quarters isolated and prepared at time *t* (△) and the number of those isolated at time *t* and prepared at 72 h (▲) are plotted against the time of isolation. The vertical bars indicate the S.D. of the mean from three independent experimental groups of animals.

distal quarter stimulated additional activity: the cumulative mitotic index of sectioned animals as compared to injured ones was 2·4-fold higher between 28 and 64 h.

Feeding affects the mitotic index and the labelling index

The mitotic index of epithelial cells is not uniform throughout the day in animals fed regularly (David & Campbell, 1972). If the animals were fed in the morning the mitotic index and the labelling index peaked at night.

To study the influence of feeding on both indices, we scored animals that were either starved for 10 days, or starved and refed, or daily fed, or daily fed and refed after 8 h. In all cases whole animals were analysed. The mitotic index was not different and very low both in starved and daily fed animals 24 h after the last feeding (Fig. 4A, zero time). The mitotic index remained at this low level in starved and daily fed animals that were not fed the day of the experiment. But feeding raised the mitotic index about 9 h later with similar kinetics in starved and daily fed animals. However, the mitotic index did not increase 9 h after the first feeding in animals fed twice (0 h and 8 h) (Fig. 4B), the index remained low for at least 15 h. Thus feeding prevented an increase of the mitotic index in close temporal vicinity. A pulse

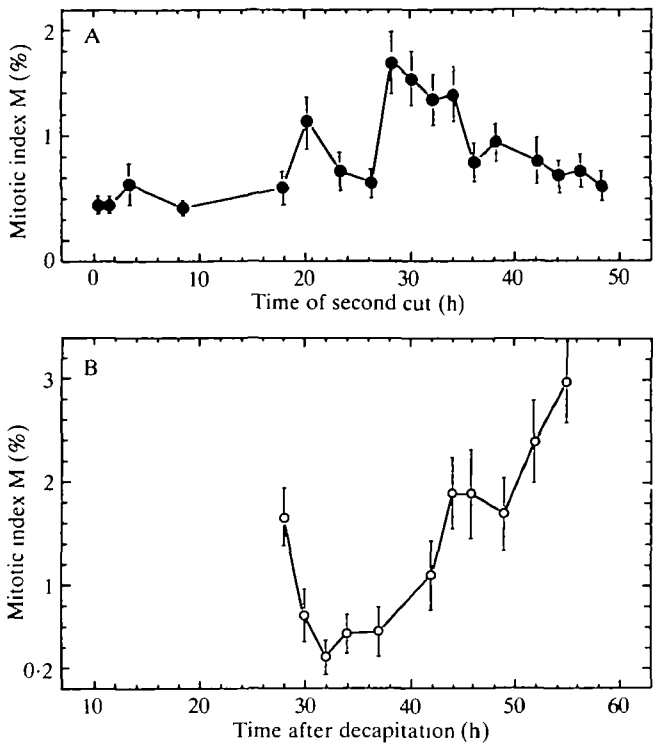

Fig. 2. Mitotic index of epithelial cells of distal quarters. (A) The cell preparations from the experiment shown in Fig. 1B were analysed. The mitotic index at the time of isolation of the distal quarter is plotted against the time of isolation. (B) All animals were decapitated at zero time and the distal quarters isolated after 28 h. At various times the mitotic index was determined. The bars indicate the S.D. as determined by use of the binomial distribution.

treatment with inhibitor I within the first hours after feeding had no influence on the increase of the mitotic index, whereas a later treatment delayed the time of the increase (Fig. 4B).

The labelling index of daily fed animals increased about 10 to 12 h after feeding and 2 to 3 h after the increase of the mitotic index (Fig. 4A), supporting the idea that G_1-phase is short (David & Campbell, 1972). Feeding of starved animals caused a decrease of the labelling index to one-quarter of its original value within 6 h, whereas it remained constant without feeding (Fig. 4A).

Both application of inhibitor I (8–10 h) and refeeding after 8 h prevented the increase of the labelling index (Fig. 4B), as expected from the respective mitotic index curves.

Discussion

With respect to volume hydra tissue consists almost completely of epithelial cells. Thus an important task of the pattern-controlling system consists of controlling their proliferation during growth and morphogenesis.

The effect of sectioning on the cell cycle length

First we discuss the effect of an injury. Our test system was the distal quarter of head-regenerating animals. Animals were injured when the mitotic index was high (28 h after decapitation). The mitotic index immediately decreased, about 5 h later it increased and after 14 h it decreased again. It decreased

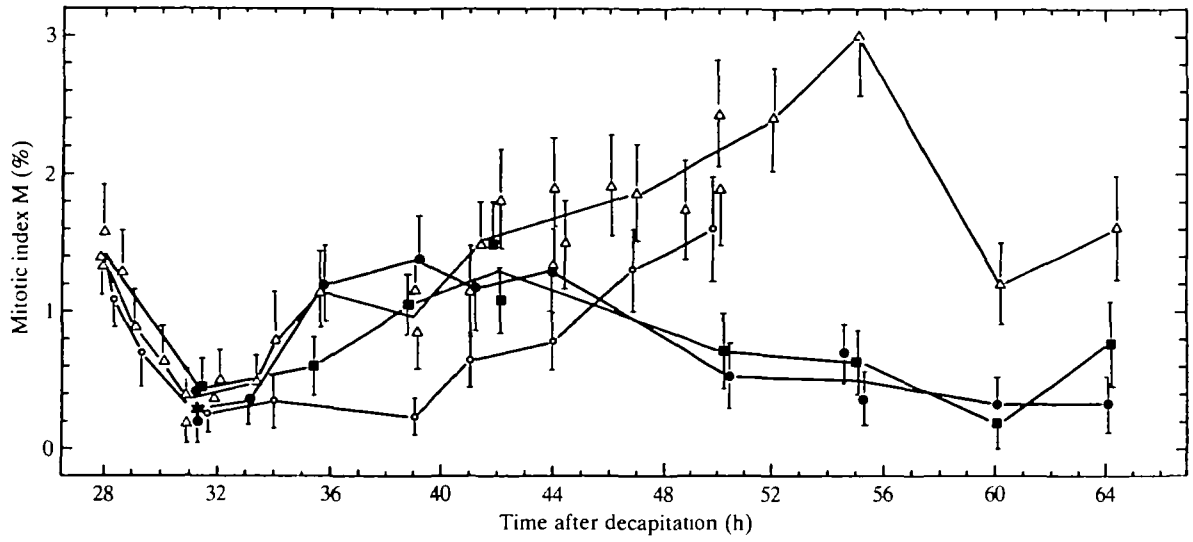

Fig. 3. Mitotic index of distal quarters following an injury or treatment with inhibitor I. 28 h after decapitation animals were either sectioned at position SII to isolate the distal quarter (△) or injured at position SII (■) or treated with inhibitor I (45 BU ml^{-1}) for 3 h (●) or 10 h 40 min (○) or treated with crude extract (84 BU ml^{-1}) for 3 h (*). At various times samples were removed and the mitotic index of the distal quarter was determined and plotted against the time of preparation. The bars indicate the S.D. as determined by use of the binomial distribution.

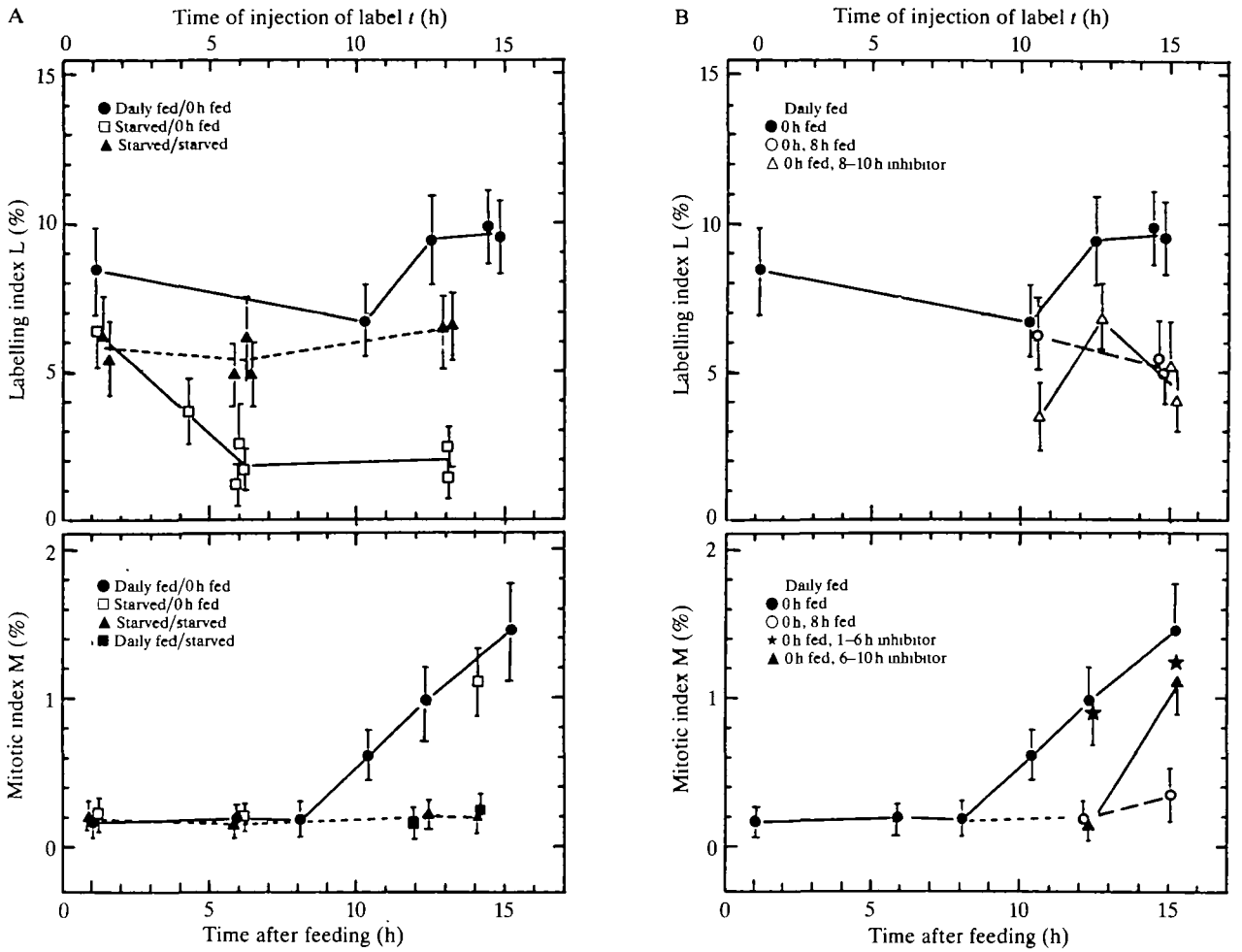

Fig. 4. Mitotic index and labelling index following feeding. Animals starved for 10 days and daily fed animals were used. Zero time was 9 a.m. (A) Effect of feeding on starved and daily fed animals. (B) Effect of feeding and inhibitor I on daily fed animals. The bars represent the S.D. as determined by use of the binomial distribution.

slowly but constantly if animals were not injured. There was no difference between the respective cumulative mitotic indices: the same number of epithelial cells seemed to have divided between 28 and 48 h. The simplest explanation is that an injury caused a signal that transiently prevents epithelial cells from leaving G_2-phase but not from cycling through the last hours of their G_2-phase. Though we have not distinguished between ectodermal and endodermal epithelial cells the degree of the decrease of the mitotic index indicates that both cell types are affected.

Application of crude extract of *Hydra* tissue or a fraction thereof, termed inhibitor I (Berking, 1977, 1983), also prevented epithelial cells from entering mitosis. A pulse treatment for 3 h had the same influence on the fall and rise of the mitotic index as an injury. Thus we argue that the injury increased the concentration of inhibitor within the tissue for a period of 3 h. The inhibitor may be released from its stored form which contains 1000 times more than is freely diffusible in the tissue during normal growth (Berking, 1977).

Sectioning an animal had at least two effects on epithelial cells: (1) it decreased the mitotic index as an injury did; (2) in contrast to an injury it subsequently caused an overshooting increase of the mitotic index. We found a nearly 2·4-fold increase in the cumulative mitotic index (between 28 and 64 h) in sectioned animals than in injured ones. Because the cell number nearly doubled in the isolated distal quarter from 28 h to 72 h (Fig. 1B), the high mitotic index observed must be due to an increased number of cells performing mitosis rather than to an increase of mitotic length. We suggest that sectioning caused a signal that shortened G_2-phase of epithelial cells. Because no increase in cell number was observed when the distal quarter was isolated earlier (within one day after decapitation) or later (at the 3rd day) either the signal was not generated to the same strength at the various times or cells responded

differently to the signal depending on their developmental state. We are not able to answer this question by our assay system. And we cannot exclude the possibility that the signal in question is also generated by an injury at a low intensity.

The effect of feeding on the cell cycle length

A rapid effect of feeding starved animals was a decrease of the labelling index of epithelial cells. During the same period of time the mitotic index remained constant at a very low level. We suggest that feeding caused a shortening of the S-phase. The arguments are as follows. In steady-state animals mitosis was determined to last 1·5 h and S-phase to last 12 to 15 h, i.e. 10 times longer than mitosis (David & Campbell, 1972). In animals starved for 10 days the mitotic index was about 0·2 %. Thus one would expect a labelling index of 1·6 to 2 %. Instead we found a labelling index of 6 % (Fig. 4A). Upon feeding, the labelling index decreased to 1·7 % within 6 h. Obviously, this cannot be caused by preventing cells from entering mitosis. Thus we argue that the S-phase in starved animals is about three times longer than in animals fed daily, i.e. about 30 to 40 h (in agreement with Otto & Campbell, 1977), and that this length is shortened within a period of 6 h following feeding to about the length found in daily fed animals.

A late effect of feeding is an increase of the mitotic index (Fig. 4A). This increase cannot be due to the proposed shortening of the S-phase. G_2-phase must be shortened: assuming the length of mitosis not to be affected at all, the increase of the mitotic index by a factor of 5 means that five times more epithelial cells perform mitosis at this moment. Thus the length of the G_2-phase has shortened 5 times the length of mitosis at least. It appears that changes of the length of both the S-phase and G_2-phase also occur in animals fed daily (Fig. 4A).

Feeding prevents an increase of the mitotic index for a short period of time as was shown by feeding animals twice a day. We argue that inhibitor I is generated following feeding as it was proposed to be generated following sectioning. Application of inhibitor I for a period of 6 h after feeding did not interfere with the time course of the increase of the mitotic index, but treatment from 6 to 10 h did. Therefore inhibitor I (in the applied concentration) did not interfere with the generation of the signal that shortened G_2-phase.

Epithelial cell proliferation and nerve cell formation

Feeding and sectioning had not only an influence on the epithelial cell cycle but also on nerve cell development: feeding was found to cause multipotent stem cells (David & Murphy, 1977) of *Hydra* to develop into nerve cells. Only those stem cells become committed that are in the very middle of their S-phase at the time of feeding though a much larger part of the first half of the S-phase, the 'period of reversible commitment', PRC, is sensitive to the signal generated upon feeding. Inhibitor I applied during this sensitive period prevents the commitment. Stem cells remain stem cells. It was argued that at first a signal is generated that commits stem cells to nerve cells and then inhibitor I is released. The latter prevents those stem cells from entering the nerve cell pathway that are still in their PRC (Berking, 1979).

It has further been shown that decapitation causes a commitment of stem cells to nerve cells (Yaross, Baca, Chow & Bode, 1982). Thus soon after feeding and sectioning, signals are generated that cause both a commitment of stem cells to nerve cells and a shortening of the cell cycle of epithelial cells. The 'head activator', a substance isolated from *Hydra* tissue, was found to shorten G_2-phase, possibly by deblocking epithelial cells arrested in the G_2-phase (Schaller, 1976*a*) and a stimulation of commitment of stem cells to nerve cells in the course of head regeneration (Schaller, 1976*b*). Agents other than the head activator seem to be involved, too: we found a large increase in the mitotic index of epithelial cells following start of foot regeneration. The head activator was proposed not to be involved in this process (Schaller, 1976*a*). The inhibitory signal generated following feeding and sectioning is proposed to be an increase in the concentration of inhibitor I.

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