J. Cell Sci. **20**, 29–46 (1976)
Printed in Great Britain

REGULATION OF INTERSTITIAL CELL DIFFERENTIATION IN *HYDRA ATTENUATA*

I. HOMEOSTATIC CONTROL OF INTERSTITIAL CELL POPULATION SIZE

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SUMMARY

Mechanisms regulating the population size of the multipotent interstitial cell (i-cell) in *Hydra attenuata* were investigated. Treatment of animals with 3 cycles of a regime of 24 h in 10^{-3} M hydroxyurea (HU) alternated with 12 h in culture medium selectively killed 95–99 % of the i-cells, but had little effect on the epithelial cells. The i-cell population recovered to the normal i-cell:epithelial cell ratio of 1:1 within 35 days. Continuous labelling experiments with [^{3}H]thymidine indicate that the recovery of the i-cell population is not due to a change in the length of the cell cycle of either the epithelial cells or the interstitial cells.

In control animals 60 % of the i-cell population undergo division daily while 40 % undergo differentiation. Quantification of the cell types of HU-treated animals indicates that a greater fraction of the i-cells were dividing and fewer differentiating into nematocytes during the first 2 weeks of the recovery after HU treatment. Therefore, the mechanism for recovery involves a shift of the 60:40 division:differentiation ratio of i-cells towards a higher fraction in division until the normal population size of the i-cells is regained. This homeostatic mechanism represents one of the influences affecting i-cell differentiation.

INTRODUCTION

The interstitial cell (i-cell) of hydra is considered to be a multipotent stem cell (e.g. Lentz, 1966) capable of giving rise to 4 types of nematocytes (Lehn, 1951; Slautterback & Fawcett, 1959), 1–3 types of nerve cells (Davis, 1974; Westfall, 1973) and 2 kinds of gametes (Brien, 1961). The evidence that the i-cell is, in fact, multipotent as opposed to each of the differentiated products arising from a separate stem cell is for the most part indirect. For example, *H. pirardi* upon entering the sexual phase produces gametes and ceases production of nematocytes (Burnett & Diehl, 1964). Regenerating animals produce more nerve cells at the regenerating apical tip than they would in this same region of a normal animal (Bode *et al.* 1973). Recently, David (personal communication) has provided direct evidence. By following the fate of individual i-cells in reaggregates of dissociated hydra cells (Gierer *et al.* 1972; Bode, 1974) he has shown that one i-cell can give rise to both nematocytes and nerve cells.

A culture of hydra fed daily will increase exponentially in number, which implies

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that the populations of each cell type will also expand exponentially. All cell types in the continuously growing body column of a hydra are capable of cell division except those which arise by differentiation from the i-cells. Hence, the i-cell population increases in size in concert with the other dividing populations, and in addition, produces nematocytes and nerve cells in the appropriate quantities (gametes are not made under laboratory culture conditions). Since the ratios of the cell types to one another remain reasonably constant (Bode *et al.* 1973) during this continual increase in number of animals and expansion of the several populations of cells over a period of many years, it is reasonable to assume that mechanisms exist for regulating the relative sizes of the several cell populations (Bode, 1973).

In *H. attenuata*, the i-cell has a mean cell-cycle time of 1 day and the ectodermal epithelial cells, which make up the outer cell layer in which the i-cells are interspersed, have a mean cell-cycle time of 3 days (David & Campbell, 1972; Campbell & David, 1974). These 2 cell types are found in roughly a 1:1 ratio (Bode *et al.* 1973). If all i-cells underwent cell division daily, this ratio could not be maintained. However, 60% of the i-cells divide, and the remaining 40% undergo differentiation (David & Challoner, 1974). This ratio of 60:40, division to differentiation, is very close to the calculated 61·5:38·5 ratio required to maintain a 1:1 ratio in expanding populations of i-cells and epithelial cells with the stated cell-cycle times. It seems highly unlikely that these ratios (1:1, 60:40) occur in the animal by happenstance (see Bode, 1973). In this paper we present evidence for the existence of a homeostatic mechanism that maintains the 1:1 ratio, and that the 60:40 ratio can be varied to restore the normal i-cell to epithelial cell ratio if perturbed from 1:1. These findings suggest the ability of an i-cell to 'measure' the density of other i-cells in its immediate surroundings. This measurement is one factor affecting the commitment of a multipotent cell to division or to differentiation.

MATERIALS AND METHODS

Culture of animals

Hydra attenuata were used for all experiments. They were maintained in a medium containing 1×10^{-3} M $CaCl_2$, $1{\cdot}25 \times 10^{-5}$ M Na_2EDTA, pH 7·5–8·0, in spring water (Loomis & Lenhoff, 1956) at a temperature of 19 ± 1 °C. Animals were fed *Artemia* nauplii (5–8/hydra) daily and washed about 6–8 h later.

Hydroxyurea treatment

Animals were exposed to 10^{-2} M hydroxyurea (HU) by adding the drug to the culture medium for varying lengths of time depending on the regime. Animals were fed and washed daily as usual and fresh HU added to the medium after washing.

Quantification of cell types

The number of each cell type was determined with the maceration procedure described by David (1973). The total number of cells/animal was measured with a Neubauer Cell Counter (0·1 mm depth) under phase optics. The cell composition was obtained by typing at least 1000 cells of a macerated preparation. From these 2 kinds of data the numbers of each cell

type/animal were calculated. In some experiments in which a particular cell type occurred at a low frequency and was of importance, the cell types of low frequency and a reference cell type (Big i-cell; see David [1973] for nomenclature) were counted until 100 cells of each type were counted.

Analysis of cells capable of DNA synthesis

Animal DNA was labelled with [^{3}H]thymidine (50 μCi/ml; 6Ci/mM; Schwarz–Mann) by injecting the compound through the animal's mouth into the gastric cavity using a 10-μl Hamilton syringe tipped with a polyethylene needle. For continuous labelling experiments this procedure was repeated 2–3 times per day at 8–12 h intervals. This was, in fact, a continuous labelling procedure since the *S*-phase of the cell types of interest (i-cells and epithelial cells) was $\leqslant$ 12 h (David & Campbell, 1972; Campbell & David, 1974). For analysis, periodically 5 animals were washed, then macerated, the resulting cell suspension dried down on a glass slide and prepared for autoradiography. The slides were dipped in Kodak NTB3 Nuclear Track Emulsion, dried, exposed 7–14 days, and developed. One to two hundred cells of a cell type were counted to determine the fraction of labelled cells.

Colchicine treatment and formation of reaggregates

Animals were subjected to 0·4 % colchicine in culture medium for 8 h at 19 °C. Thereafter, they were placed in normal culture solution, starved, and washed every other day for 7 days. These hydra were dissociated into cells according to the procedure described by Gierer *et al.* (1972). The dissociation medium consisted of: 5×10^{-3} M $CaCl_2$, 1×10^{-3} M $MgSO_4 \cdot 7H_2O$, $2{\cdot}8 \times 10^{-2}$ M KCl, $1{\cdot}1 \times 10^{-2}$ M TES, $6{\cdot}7 \times 10^{-4}$ M Na_2HPO_4, $4{\cdot}4 \times 10^{-4}$ M KH_2PO_4, 5×10^{-3} M sodium pyruvate; 5×10^{-3} M trisodium citrate. Normal hydra were dissociated into cells in the same manner. Cells of colchicine-treated animals and cells of normal animals were mixed in a 10:1 ratio, centrifuged, and allowed to reaggregate and develop as described by Gierer *et al.* (1972). Every 5 days, 4–6 reaggregates were macerated and the total number of cells of each type/reaggregate determined as described above.

RESULTS AND DISCUSSION

Evidence for homeostasis

Effect of hydroxyurea on hydra. If the 1:1 i-cell to epithelial cell ratio is maintained by a homeostatic mechanism, then by altering the ratio from 1:1 to 1:100, one would expect the ratio to return to 1:1 in time. Such an alteration can be produced by treating hydra with hydroxyurea, which is known to kill cells in *S*-phase of the cell cycle, and to block the transition of cells from G_1 to *S* (Sinclair, 1965). Since 50% of the i-cell cycle is *S*-phase (Campbell & David, 1974) compared to 20% of the epithelial cell cycle (David & Campbell, 1972), an effect selective for i-cells should be possible. Theoretically, by exposing the animals to a regime in which 24 h of hydroxyurea and 12 h of culture medium are alternated for 3 cycles, all of the i-cells and 70% of the epithelial cells should be killed.

To determine whether or not hydroxyurea affected hydra as it does other systems, the following experiments were carried out. The effect of the drug on DNA synthesis was examined by placing hydra in 10^{-2} M HU and periodically pulse-labelling groups of animals with [^{3}H]thymidine. Thereafter, the labelled animals were macerated and the labelling index determined with autoradiography. As shown in Fig. 1, the label-

ling indices for epithelial cells, big i-cells and little i-cells* decline rapidly indicating that hydroxyurea blocks DNA synthesis almost immediately in these cell types. All cells labelled at 4 and 8 h were labelled one-fifth to one-tenth as heavily as those at time 0. Lower concentrations were less effective for big i-cells and epithelial cells, although DNA synthesis was inhibited in little i-cells at 3×10^{-3} M HU.

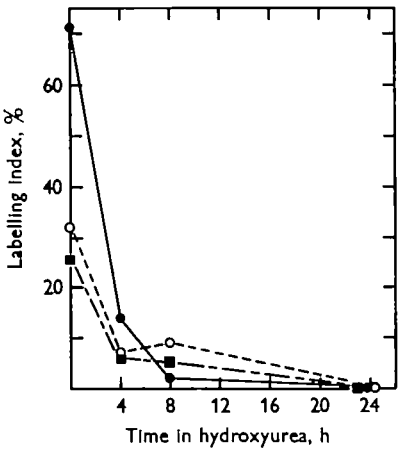

Fig. 1

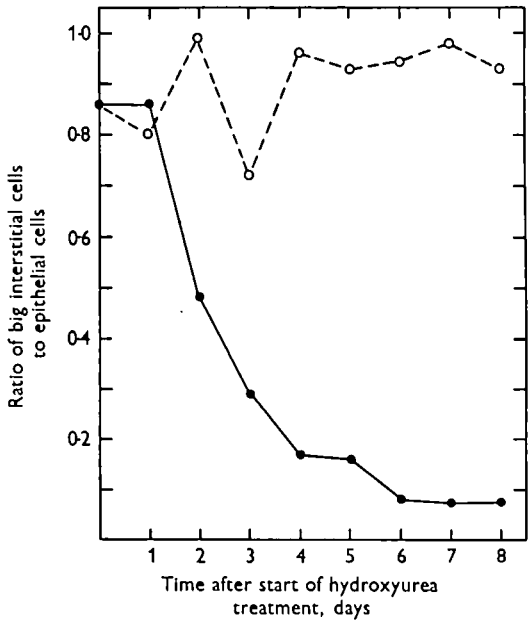

Fig. 2

Fig. 1. Effect of 10^{-2} M hydroxyurea on DNA synthesis (measured as labelling index with [^{3}H]thymidine) of big interstitial cells (●——●), little interstitial cells (○----○), and epithelial cells (■——■).

Fig. 2. Effect of 10^{-2} M hydroxyurea on the Bi:Epi cell ratio. Animals were subjected to 10^{-2} M HU for 4 days and then returned to culture medium. HU-treated animals (●——●) and controls (○---○).

The ability of HU to kill cells was examined by daily measuring the cell composition of animals exposed to 10^{-2} M HU for 4 days and subsequently returned to culture medium. The cell composition was measured with the maceration technique. As shown in Fig. 2, the ratio of big i-cells to epithelial cells drops from 1:1 to 1:12 in 6 days indicating that, as expected, more i-cells than epithelial cells were killed.

To study the long-term (several generations) effect of the drug, hydra were exposed to several different regimes. Animals were exposed to 10^{-2} M HU for 2 days, 4 days,

* The term i-cell will be used to denote the multipotent stem cell. The term big i-cell, as defined by David (1973), describes a cell type identified in maceration preparations. This population includes the multipotent stem cells which are found as single cells or in pairs, and clusters of 4 cells, or occasionally more, which are committed to nematocyte differentiation (David & Challoner, 1974). The term little i-cell refers to cells found in macerates that are morphologically quite different from big i-cells and are intermediates of nematocyte differentiation capable of cell division (see David, 1973).

or for 3 cycles of the alternating regime, 24 h HU followed by 12 h in culture medium. Thereafter, all treated animals were maintained for several weeks in culture medium. As a general indicator of health, the number of buds produced by 10 animals over a period of 3–4 weeks was measured. Fig. 3 shows the cumulative number of buds produced by animals exposed to HU for 2 days and the alternating regime is not much different from the control. In both cases there is a period after the treatment where the rate of bud production slows down, but then approaches a rate similar to

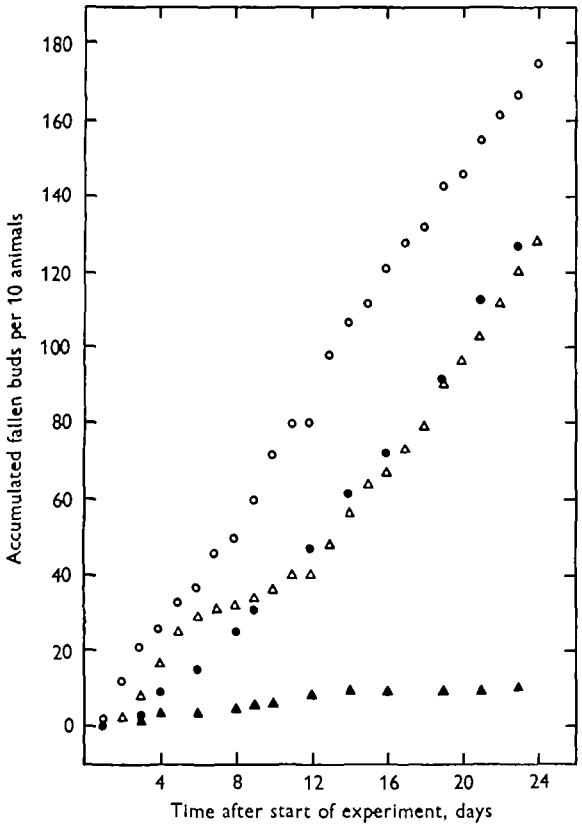

Fig. 3. Effect of 10^{-2} M hydroxyurea on growth rate as measured by the rate of accumulation of detached buds. Controls (○); 2-day-treated animals (●); 4-day-treated animals (▲); animals treated with the alternating regime (see text) (△).

the control rate. These animals appeared normal, fed normally, though those on the alternating regime ingested fewer shrimp larvae for a 1–2 week period, 2 weeks after beginning of treatment. Those exposed to HU for 4 days, ceased budding and feeding soon after the end of the treatment. They became transparent and gradually reduced in size over 35 days.

Macerates of animals during or 1 day after treatment showed large numbers of swollen big i-cells and epithelial cells. The cells were 2-3 times as large as normal. Three days after the treatment ended all cells were of a normal size. To measure the

number of cells capable of DNA synthesis after treatment with hydroxyurea, animals were pulse-labelled with [^{3}H]thymidine 1 day following the various HU treatments. The labelling index for big i-cells and epithelial cells, as shown in Table 1, is similar to or greater than the controls in all cases, indicating that the effect of the drug on the

Table 1. *Ability of cells to incorporate [^{3}H]thymidine 1 day after hydroxyurea treatment*

HU treatment	Fraction of cell type labelled 1 day after treatment, %	
	Big i-cells	Epithelial cells
Control	44	18
1 day HU	67	10
2 days HU	69	35
3 days HU	30	18
Alternating regime*	42	26

* Alternating regime: 3 cycles of 24 h in hydroxyurea followed by 12 h in culture medium.

Table 2. *Changes in cell numbers with time after hydroxyurea treatment; fraction (%) and number (#) of each cell type*

Day*		Epi	Bi	Li	Nb	Nc	Ner	GL	Σ
0	%	20	28	21	24	3	2	3	
	#	19100	28800	20100	22900	3200	1500	2900	95600
3	%	66	9	—	1	8	6	10	
	#	21500	2900	—	390	2600	1800	3300	32600
7	%	83	1	—	2	3	3	7	
	#	25800	400	—	600	1100	900	2200	31100
14	%	84	5	1	—	1	3	6	
	#	20250	1100	270	—	290	800	1300	24100
21	%	66	12	5	9	1	4	3	
	#	22300	4100	1700	3000	340	1500	1000	33800
28	%	50	21	10	10	4	3	3	
	#	14000	5900	2700	2700	980	800	950	28000
35	%	36	27	10	16	4	3	4	
	#	15700	11700	4400	7100	1600	1500	1500	43500
41	%	32	28	13	17	4	3	3	
	#	23500	20900	9900	12800	2700	2000	2500	74200
Control†	%	25	31	14	18	6	2	4	
	#	23200	28600	12800	16300	5400	1800	3500	91600
		±1990	±3950	±2120	±2360	±1170	±370	±560	±6100

* Time after beginning of treatment.

† Animals maintained in culture medium. Values are mean values ± S.D. of control measurements made on same days as hydroxyurea-treated animals.

Epi, epithelial cells; Bi, interstitial cells; Li, little i-cells; Nb, nematoblasts; Nc, nematocytes; Ner, nerve cells; GL, gland cells.

Numbers are for the whole animal minus the head and buds which were removed.

surviving cells was reversible in 1 day. If a large fraction of the cells were not in the mitotic cycle, the fraction of labelled cells in the HU-treated animals would be lower than controls. Since this did not occur, the observed populations of big i-cells and epithelial cells are apparently cycling normally, and the killed cells have disappeared.

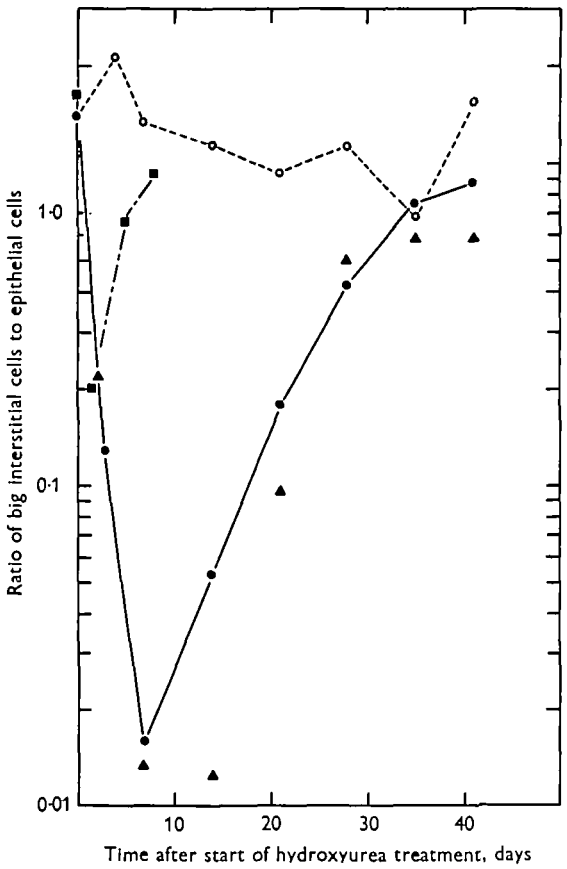

Fig. 4. Recovery of the Bi:Epi cell ratio after hydroxyurea treatment. Hydroxyurea-treated animals (●——●) and their buds (▲); controls (○---○); animals exposed to hydroxyurea for 1 day (■).

Recovery of the i-cell population after HU treatment. To examine the effect of the hydroxyurea treatment on the populations of the various cell types and their ratios to one another, a group of animals was exposed to the alternating regime of 24 h in 10^{-2} M HU followed by 12 h in culture medium for 3 cycles. Periodically 5 animals were macerated and the numbers of each type determined (Table 2). It is evident that over the time course of the experiment the numbers of epithelial cells did not vary substantially from the control values. In contrast, the numbers of big i-cells dropped to about 1 % of the control value by 7 days after start of the treatment and then recovered to control values within the next 35 days. The little i-cells which are more sensitive to the drug and whose *S*-phase is 75 % of the cell cycle (Campbell &

David, 1974) vanish completely, and after 14 days reappear and recover. A similar pattern is shown by nematoblasts which arise from the little i-cells. The other 3 types of cells, nematocytes, nerve cells and gland cells also decline in number and recover, but not as dramatically.

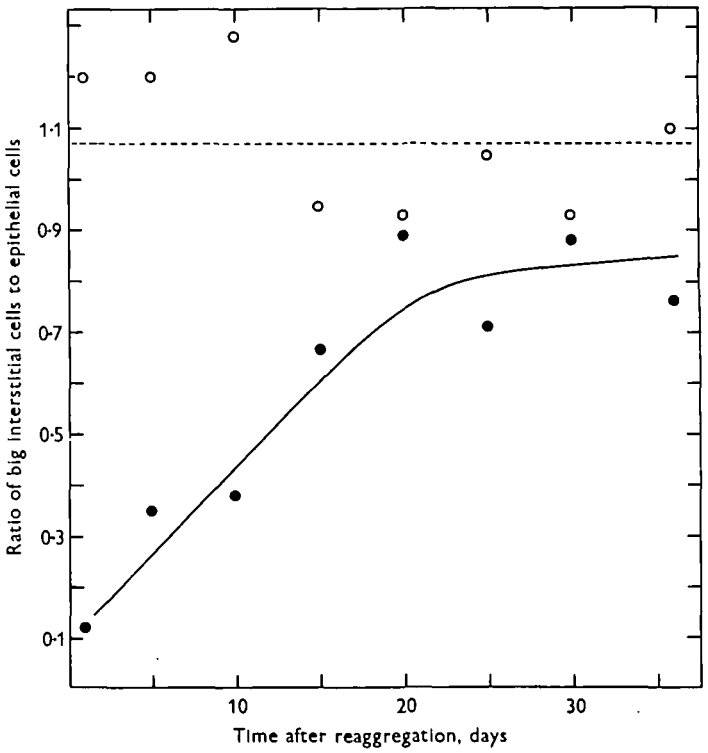

Fig. 5. Recovery of the Bi:Epi cell ratio in mixed reaggregates of colchicine-treated animals and normal animals. Reaggregates of cells of colchicine animals:normal animals in a 10:1 ratio (●——●); reaggregates of cells of only normal animals (O---O).

The data for the epithelial cells and big i-cells are presented graphically in Fig. 4. The ratio of the big i-cells to the epithelial cells in the controls remains around 1:1. The experimental ratio drops to 1:70 and subsequently recovers to 1:1. The buds were analysed separately to determine whether the cell composition differed from the adults, perhaps reflecting a different recovery pattern. No differences were found, as the ratio of Bi:Epi cells was similar in adults and buds throughout the experiment. Fig. 4 also indicates that a 1-day treatment with HU leads to a drop in the Bi:Epi ratio, although a much smaller one, and a recovery to control values.

The experiment described above was repeated with the same results in 5 of 6 attempts. In the sixth experiment, the animals stopped feeding after 20–25 days and became pale. Examination of the cell composition indicated that the Bi-cell population did not recover beyond a 1:40 ratio in 35 days. Similar results were obtained by treating animals for 4 days with hydroxyurea. Thus, one effect of prolonged exposure to

HU is failure of the i-cell population to recover to normal levels. These animals cease feeding because of a lack of nematocytes in the tentacles.

Recovery of the i-cell population after colchicine treatment. As an alternate method for reducing the i-cell population, a method described by Campbell (1974) was used. He has shown that following treatment of hydra with 0·4% colchicine for 8 h, the numbers of i-cells are sharply reduced to around 1% of the control values and do not recover readily. In contrast, the epithelial cell population is unaffected in size and ability to undergo cell division. Using the reaggregation technique (Gierer *et al.* 1972; Bode, 1974), low numbers of normal i-cells can be added to the epithelial cells of colchicine-treated animals, thus creating animals with artificial Bi:Epi ratios. Normal animals and colchicine-treated animals were dissociated separately and mixed in a ratio of 1:10, reaggregated and allowed to develop. Periodically, 4–6 reaggregates were macerated and the cell composition determined. Fig. 5 indicates that 1 day after reaggregation the ratio was around 1:10 and recovered to approximately 1:1 in 20 days.

The experiments with HU-treated animals and with colchicine-treated animals showed that if the i-cell population was reduced, thereby lowering the Bi:Epi ratio significantly below 1:1, the i-cell population recovered. This result indicates that there is a homeostatic mechanism for maintaining the Bi:Epi ratio at 1:1. If, however, the animals are exposed to severe treatments, such as prolonged treatment with HU, homeostasis is no longer apparent.

Mechanism for homeostasis

Change in cell-cycle times. The next question that arises is the basis of the homeostatic mechanism. Two straightforward hypotheses exist. The recovery of the i-cell population could be due to a shift in the division:differentiation ratio of 60:40 to something higher such as 70:30 until recovery is complete, and, subsequently, drop back to 60:40. Alternatively, this ratio may remain unchanged but the cell cycle of the epithelial cell might lengthen, or that of the i-cell shorten, or both, until recovery is complete. Both of these hypotheses were tested.

If the mechanism were based on a change in the cell cycle, one can calculate how large a change in the cell cycle must take place in order that the recovery occur in roughly 35 days. By assuming that the i-cells maintain a 1-day cell cycle time and a 60:40 ratio (see legend of Fig. 6) and by varying the epithelial cell generation times, recovery curves of Bi:Epi from 1:100 to 1:1 can be calculated. Several are shown in Fig. 6. It is clear that the mean epithelial cell cycle must be lengthened from the 3 days observed in normal animals (David & Campbell, 1972) to more than 6 days to explain the recovery. Conversely, the same could be achieved by shortening the mean i-cell cycle from 1 to less than 0·5 day, while maintaining the epithelial cell cycle at 3 days.

The cell cycle times of the big i-cells and epithelial cells of HU-treated and control animals were measured to determine whether changes of this magnitude had taken place. The length of the cell cycle can be measured by continuously labelling animals with [^{3}H]thymidine. The time required for 100% of all cells to become labelled is a measure of cell cycle length minus *S*-phase (see Cleaver, 1967). The theoretical

continuous labelling curves can be calculated (assuming cell-cycle time of either 3 or 6 days for the epithelial cells) and are presented in Fig. 7A. The important point is that the curves are distinctly separate and that 100% labelling is reached at significantly different times.

The experiment was carried out by injecting animals at 8-h intervals for the first day (to ensure that all of the i-cells were labelled) and at 12-h intervals for the next 6 days to label all the epithelial cells if, in fact, the epithelial cell cycle were extended to 6 days. The cells were analysed autoradiographically and the results are shown in

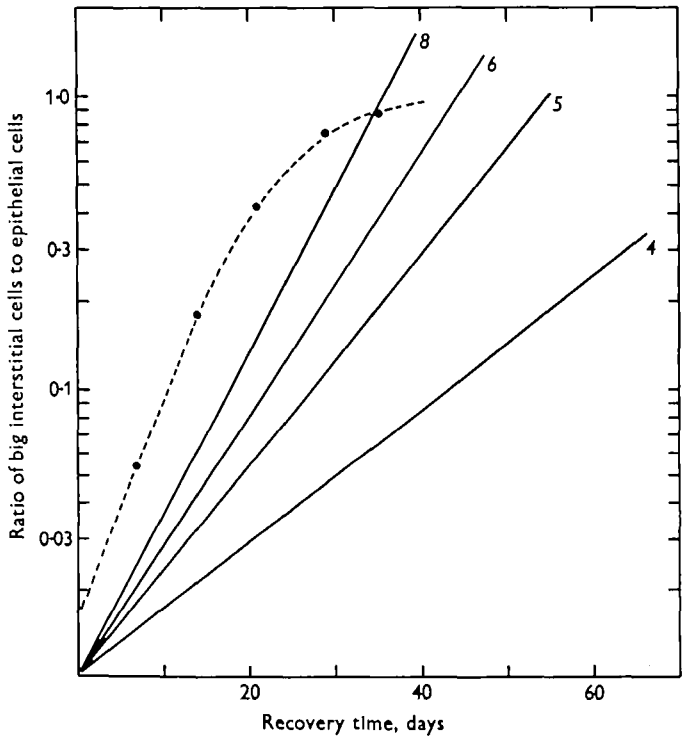

Fig. 6. Recovery rate of the i-cell population as a function of the epithelial cell cycle time. The epithelial cell cycle time is assumed to be the value (in days) indicated for each curve (solid line). These curves were calculated as follows. The i-cell cycle time was fixed at 1 day and the division:differentiation ratio at 61·5:38·5. This ratio maintains a 1:1 i-cell:epithelial cell ratio assuming a 1-day cell cycle for the i-cells and a 3-day cycle for the epithelial cells. Then, knowing the size of the i-cell population on day t, the population on day $t+1$ was calculated from the relationship, $i(t+1) = i(t) \times 1{\cdot}23$. Similarly, the population size of the epithelial cells on day $t+N$, where N was the assumed cell cycle time was $\mathrm{Epi}(t+N) = \mathrm{Epi}(t) \times 1{\cdot}85$. This expression instead of $\mathrm{Epi}(t) \times 2$, as one would expect for a simple doubling of the population, was used because allowance was made for sloughing of epithelial cells at the extremities (15%, Campbell, 1967). Also, the entire population of epithelial cells was in the mitotic cycle (David & Campbell, 1972), so that a correction for a growth fraction was unnecessary. Starting with an initial ratio of i-cells:epithelial cells of 1:100 and using the above procedures for increasing the 2 populations, the ratios of the 2 population sizes were calculated periodically. These ratios were used in generating the recovery curves. The dashed line is the experimental data of Fig. 4.

Fig. 7B. The data points for both the HU-treated and control animals lie so close together that the same curve can be fitted for both sets of points. They do not show the separation expected if the cell cycle times were significantly different. Therefore, we conclude that the recovery is not due to a shift in the epithelial cell cycle time. The difference in the shapes of the theoretical and experimental curves is due in large part to a variable G_2 phase known to exist (David & Campbell, 1972) which was not taken into account in the theoretical curve.

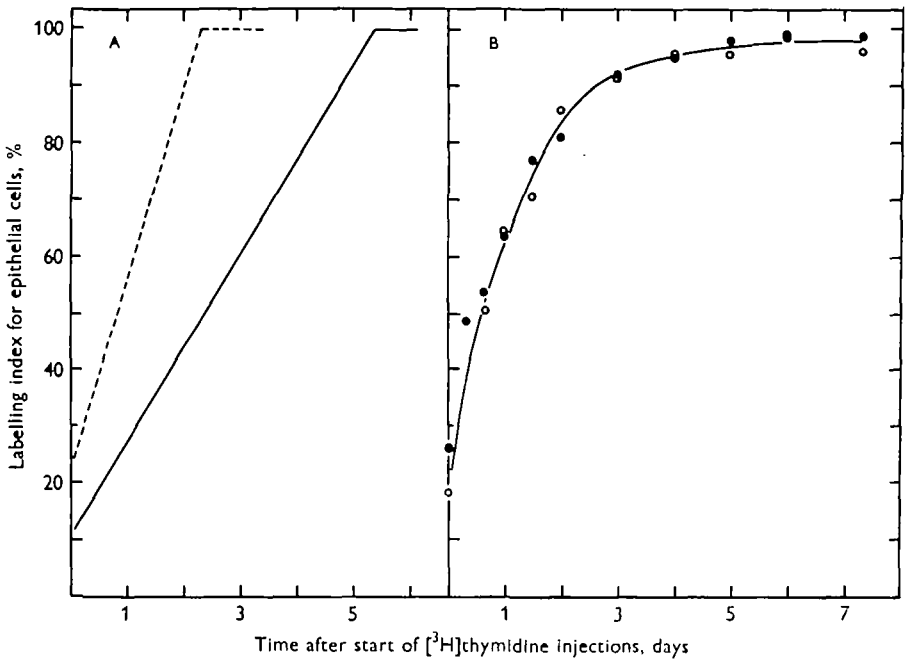

Fig. 7. Theoretical (A) and experimental (B) continuous labelling patterns of epithelial cells. The curves in A represent the calculated continuous labelling pattern for cells with a 3-day cell cycle time (– – –) and a 6-day cell cycle time (——). In B, the continuous labelling pattern for the epithelial cells of hydroxyurea-treated animals is represented by (●) and control animals (○). The same curve is drawn for both sets of data points.

Similar arguments can be made for the i-cells. The theoretical curve for a one-half day generation time and the predicted curve for a 1-day generation time based on known data (Campbell & David, 1974) are presented in Fig. 8A. Since the S-phase is reasonably constant and the G_2 phase quite variable under normal conditions (Campbell & David, 1974), the shortening of the cell cycle is assumed to occur at the expense of the G_2 phase. Again, the chief feature is the separateness of the curves, and in this case, the fraction labelled at $t = 0$ is much higher in the one-half day cell cycle time compared to the 1-day cell cycle time. The experimental data were obtained in the same experiment as for the epithelial cells and are shown in Fig. 8B. Again it is clear that the 2 curves are very similar, indicating that the HU animals do not have a shorter i-cell cycle time. The fact that the fraction of Bi-cells labelled at $t = 0$ for the HU

animals is the same as for the controls suggests the mean length of G_2 did not change in HU animals, and underscores the preceding conclusion. Thus, a shortening of the i-cell cycle does not play a major role in the recovery of the i-cell population. A repeat of this experiment yielded the same results. These experiments indicate that the recovery of the Bi:Epi ratio to 1:1 is not due to changes in the mean length of the cell cycle of either cell type.

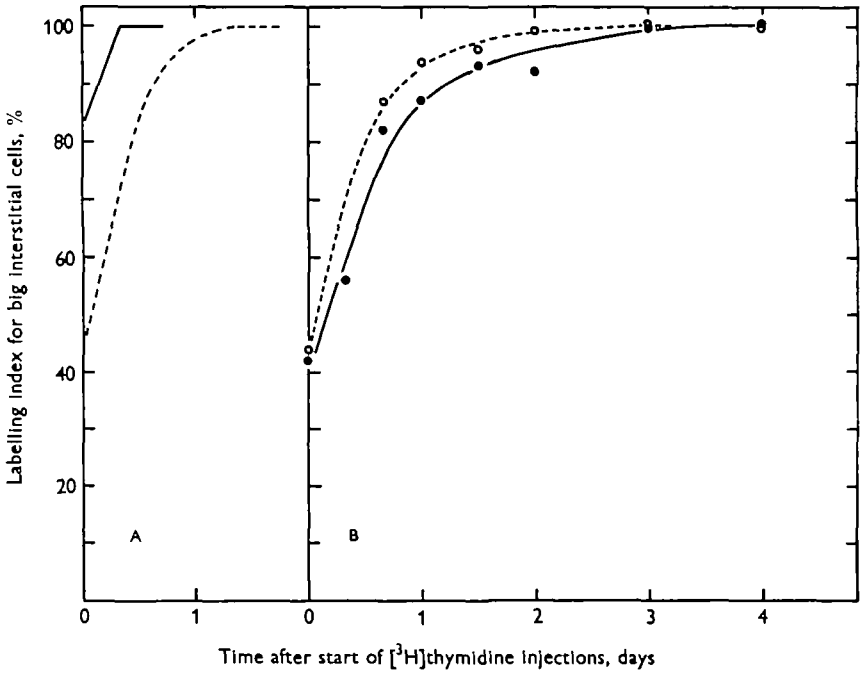

Fig. 8. Theoretical (A) and experimental (B) continuous labelling patterns of i-cells. In part A the curves represent labelling pattern for i-cells with a 1-day cell cycle time (– – –) and for i-cells with a 0·5-day cell cycle time (——). In part B the curves represent the continuous labelling patterns of i-cells of HU-treated animals (●——●) and of control animals (○– – –○).

Change in division:differentiation ratio. The second possible explanation for the recovery of the i-cell population is that the division:differentiation ratio shifted from 60:40 to a value with a higher fraction of i-cells in division. Assuming the cell cycle times of epithelial cell and i-cell to be unchanged, a series of theoretical recovery curves were generated by shifting the ratio. Starting with a Bi:Epi of 1:100, the recovery curves to a 1:1 ratio are shown in Fig. 9. For recovery to occur in the observed 35 days, the 60:40 ratio need shift not more than to 70:30, which is not very large and not easy to measure. However, these curves were calculated assuming recovery to be linear throughout the entire recovery period until the normal 1:1 Bi:Epi ratio is reached. As shown in Fig. 4, this is not the case. The recovery is steeper initially indicating a larger shift than 70:30 at the beginning and a smaller one near the end. In order to maximize the chances of observing a change in this ratio, we examined the 2-week period after the end of treatment.

If there were a substantial shift in the direction of division away from differentiation, we would have expected to observe fewer intermediates of nematocyte differentiation. I-cells committed to nematocyte differentiation undergo 3–5 divisions. These cells, termed little i-cells (David, 1973), remain together in a syncytial nest and proceed to differentiate synchronously into nematoblasts, and later nematocytes (Lehn,

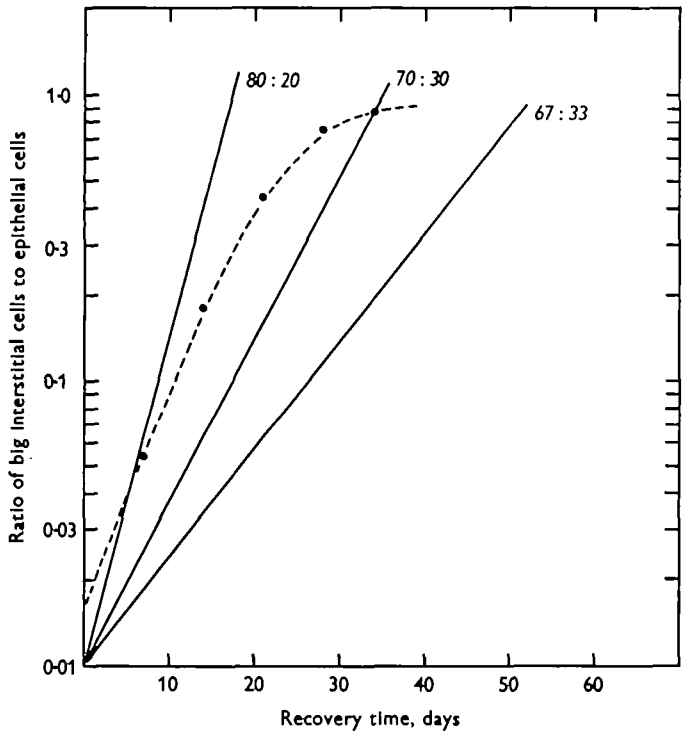

Fig. 9. Calculated rates of recovery of the i-cell population as a function of the division: differentiation ratio of i-cells. The ratio assumed for each curve is indicated near the calculated curve (solid lines). The epithelial cell cycle was assumed to be 3 days and this population grew according to the relationship, $\mathrm{Epi}(t+3) = \mathrm{Epi}(t) \times 1{\cdot}85$. The assumptions concerning growth of the epithelial cell population were the same as described in the legend of Fig. 6. The i-cell population increased according to the relationship $i(t+1) = i(t) \times 2Y$, where Y was the fraction of i-cells assumed to be in division, and the i-cell cycle was assumed to be 1 day. Starting with an initial ratio of i-cells:epithelial cells of 1:100 and using the above procedures for increasing the populations, the ratios of these 2 population sizes were calculated periodically. These ratios were used in generating the recovery curves. The dashed line is the experimental data of Fig. 4.

1951; Slautterback & Fawcett, 1959). If fewer i-cells are committed to differentiation, the ratio of little i-cells:big i-cells should be lower in HU-treated animals than in controls. An even earlier stage of nematocyte differentiation can also be analysed. In macerates, big i-cells are found singly or in groups of 2 or 4. David & Gierer (1974) have presented kinetic data that indicate that the populations of single and pairs of big i-cells contain the stem cells while groups of 4 are already committed to

nematocyte differentiation. Thus, if in HU-treated animals, more i-cells are committed to division, the 4:1+2 ratio should be lower in HU-treated animals than in control animals.

To examine these possibilities a large group of animals was treated with the alternating HU regime. Then, starting 1 day after the end of the treatment, samples of 5 animals were taken periodically, macerated, and the numbers of epithelial cells, big i-cells, and little i-cells determined. The results of one of three experiments are presented in Table 3 and show that the Bi:Epi ratio has dropped well below 1:1.

Table 3. *Effect of hydroxyurea on nematocyte production during the first 2 weeks after treatment*

Time after start of HU treatment, days	Bi/Epi		Li/Bi		4/1+2*	
	Control	HU	Control	HU	Control	HU
6	0·66	0·014	1·39	0·80	0·43	0·0
8	0·94	0·058	1·46	0·28	0·44	0·10
10	1·00	0·007	0·80	0·54	0·28	0·08
12	0·81	0·027	1·01	0·38	0·65	0·32
14	0·63	0·014	0·98	0·34	0·18	0·14
19	0·75	0·022	0·85	0·49	0·40	0·24
21	0·61	0·024	1·03	0·65	0·23	0·23
23	0·56	0·28	1·25	1·39	0·41	0·32
Average	0·74	—	1·10	—	0·38	—

* The fraction is the ratio of nests of 4 Bi cells compared to the sum of nests of 1 and 2 Bi cells.

Also, the ratios of Li:Bi and 4:1+2 are lower in the HU-treated animals than in the controls. Later, when the Bi:Epi ratio was closer to normal (day 23 of this experiment and the later days in the experiment presented in Table 2), the Li:Bi ratio was also closer to normal. These results are consistent with the view that when the Bi:Epi ratio is considerably less than 1:1, a larger fraction of the i-cell population undergoes division instead of differentiating into nematocytes. Because of the variability, no attempt was made to calculate the extent of the shift.

These reduced Li:Bi and 4:1+2 ratios would also have been obtained if a considerable fraction of the i-cells were not active at all but simply quiescent. However, the continuous labelling experiments indicated that the entire i-cell population of the HU-treated animals undergoes DNA synthesis and at the same rate as the control population of i-cells. Also, the pulse-labelling experiment indicates that 1 day after the end of the treatment, the labelling index is the same in both HU-treated and control animals. Thus, it is doubtful that a sizeable fraction of the i-cells is quiescent.

GENERAL DISCUSSION

The experimental results indicate that a homeostatic mechanism exists for maintaining the size of the i-cell population with respect to the epithelial cell population

in *Hydra attenuata*. When the i-cell population was reduced in size by factors of 10–100, it was observed gradually to regain its normal size with time. This recovery was shown not to involve changes in the length of the cell cycle of either the epithelial cell or the i-cell. Instead the results were consistent with the idea that the fraction of i-cells daily committed to division (~ 60% in controls) was increased to some higher value, while the fraction committed to differentiation was lowered. In the hydroxyurea-treated animals an average shift in the division:differentiation ratio from 60:40 to 70:30 was sufficient to account for the observed recovery in 35 days. However, the shift was probably initially higher than 70:30 and then gradually returned to 60:40 as the i-cell population size approached the normal one.

A similar effect was found by Hall, Lajtha & Oliver (1962) in their work with the root tips of *Vicia faba*. There is a growth zone behind the root tip whose population size is reasonably constant. To maintain this constant population size, 50% of the daily dividing cells become committed to differentiation and cease dividing. These cells then elongate and thereby increase the length of the rootlet. By irradiating the roots with X-rays the elongation rate was retarded, but gradually returned to the normal rate in about 40 days. In earlier work, an analysis of the stem cell population indicated that shortly after X-irradiation, only 1% of the cells were capable of DNA synthesis and that the cell-cycle time was unchanged (Hornsey, 1956).

As Hall *et al.* (1962) pointed out, had the cells of the growth zone undergone division exclusively until the population size was normal, there would have been a period of time at the beginning of the recovery in which the root did not elongate at all. This did not occur. Instead the root did elongate, but initially at a very low rate. The low rate was probably due in part to a small population of dividing cells, and in part due to a reduced fraction (less than the normal 50%) of this population committed to differentiation. If 50% of the dividing cells continued to be committed to differentiation after the irradiation treatment, the dividing population would never regain its normal size and the elongation rate would not return to normal as, in fact, it did. Thus, the 50:50 division:differentiation ratio apparently shifted to an increased fraction of cells in division, and then gradually returned to 50:50 as the growth zone population recovered. This shift in the division:differentiation ratio found for the i-cell population in hydra and the root of *Vicia faba* may be a general one in tissues and organs in which a stem cell population has been sharply reduced in size and subsequently recovers.

One explanation for this phenomenon in hydra is that the i-cells can 'measure' and regulate their own density within the tissue by some form of feedback mechanism. Since the i-cells are located in the interstices among the epithelial cells of the ectoderm and are for the most part not in physical contact with one another, the feedback information would probably be humoral. In a simple form of the idea, the i-cell would secrete a regulatory substance into the tissue at a constant rate. High concentration in the tissue would cause the i-cell to leave the mitotic cycle and, possibly, enter a differentiation pathway. When the i-cell population is low, the substance would be in low concentration and the i-cells would preferentially divide. Further, the recovery patterns of the i-cell and the differentiation product cell populations would require

that such a substance have a short diffusion range, as suggested to us by C. David. If it diffused rapidly from the i-cell throughout the tissue, the concentration would be low in an animal treated with hydroxyurea and one would expect the i-cell population to divide without undergoing any differentiation until the population size returned to close to normal. This did not occur. In macerated preparations nematocyte precursors appeared at a time when the i-cell population was only 10% of normal.

Similar information was obtained with another method. Hydroxyurea-treated animals were stained as whole mounts with toluidine blue (Diehl & Burnett, 1964) periodically after the end of the treatment. Shortly after the end of the treatment, big i-cells were found as single cells or in pairs scattered throughout an otherwise empty epithelium. Later, groups of cells were found that were probably derived from single or a pair of big i-cells by division and differentiation, and, therefore, could be clones. At early times, the small clones consisted predominantly of nests of 1 and 2 big i-cells. At later times, the clones contained many more cells. Big i-cells occurred singly and in pairs, and in nests of 4, 8 and 16, which are committed to nematocyte differentiation (David & Gierer, 1974). Also, nests of differentiating nematoblasts were found. These developing nematoblasts were found even when large areas of the epithelium were empty and the numbers of big i-cells were well below 20% of the normal population. Thus, localized high concentrations of a short-range substance could prevent i-cell division or induce i-cell differentiation long before the total i-cell population had recovered to normal size.

Thus, an i-cell would effectively measure the number of its neighbours, or the local i-cell density, instead of measuring the size of the entire i-cell population in the animal. In this way i-cells could maintain the observed 1:1, big i-cell:epithelial cell ratio and the 60:40 division:differentiation ratio observed in normal animals. Such a mechanism would also account for the shift in the 60:40 ratio towards a higher fraction in division when the i-cell population is smaller than normal. This hypothesis has dealt with the feedback of i-cells to themselves to regulate division. It can be extended to include some of the factors regulating their differentiation.

Some information has been obtained on 2 other types of influences affecting the division and differentiation behaviour of the i-cell. The particular differentiation undertaken is to some extent influenced by the position of the i-cell along the axial column of the animal. Nerve cells are the only i-cell differentiation product found in the head region (Bode *et al.* 1973; David & Challoner, 1974). I-cells found in the body column below the head region form both nerves and nematocytes, but the type of nematocytes is influenced, in part, by the region (Bode, unpublished observations). I-cell differentiation is also influenced to some extent by the size of the differentiated product cell population. This has been demonstrated for one of the types of nematocytes, the stenoteles. Reduction of the stenotele population by 90% leads to an increase in the number of stenoteles produced (Zumstein & Tardent, 1971; Zumstein, 1973) which is due to an increase in the number of i-cells committed to stenotele differentiation (Smith, Nadeau & Bode, in preparation). Thus, there may also be a feedback signal for each product cell type.

The question arises as to how an individual i-cell deals with the variety of signals.

Since in growing hydra cultures, the i-cell population is expanding exponentially and the i-cell has a 24-h generation time, every i-cell undergoes a commitment process to division or to a particular differentiation once each day. The particular commitment may be the result of simple competition among the several signals with the strongest prevailing. Then, in hydroxyurea-treated animals in which the i-cell population was reduced to 1% of normal, the feedback signal (or lack thereof in case of negative feedback) from the i-cell would be strongest, and more i-cells would divide than differentiate. As the i-cell population recovers and localized high densities of i-cells appear, feedback signals from the nematocyte populations could compete favourably and differentiation would occur. However, there is some evidence which suggests a hierarchy in these controls which would also satisfactorily explain the data. By changing the feeding regime (number of shrimp larvae ingested per day), the ratios of the population sizes of the various nematocytes and nerve cells to the epithelial cells were altered by factors of 2–3. In contrast the ratio of the i-cells to epithelial cells remained essentially unchanged (Bode, unpublished observations). This suggests that the maintenance of i-cell density or population size may be more closely controlled than that of the product cells. There could be a hierarchy of decisions for the i-cell with division taking precedence over differentiation. These possibilities, simple competition of equally strong signals and a hierarchy of decisions, are currently under study.

We thank Patricia Bode for her critical reading of the manuscript, and acknowledge the helpful comments on the manuscript of our colleagues Richard Campbell, Ted Pardy, Marcia Yaross and Howard Lenhoff. This research was supported by a grant from the National Institutes of Health (HD 08086).

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(*Received* 15 *July* 1975)