J. Cell Sci. **38**, 155–169 (1979)
Printed in Great Britain © Company of Biologists Limited 1979

STEM CELL GROWTH AND DIFFERENTIATION IN *HYDRA ATTENUATA*

I. REGULATION OF THE SELF-RENEWAL PROBABILITY IN MULTICLONE AGGREGATES

FREDERICK SPROULL* AND CHARLES N. DAVID†
Department of Molecular Biology, Albert Einstein College of Medicine, Bronx, New York 10461, *U.S.A.*

SUMMARY

Interstitial stem cells in *Hydra* are rapidly proliferating multipotent stem cells which continuously give rise to precursors for nerve and nematocyte differentiation. Growth of the stem cell population is controlled by the cell cycle time of the stem cells and the self-renewal probability, P_s (the fraction of stem cells in each generation which divide to yield more stem cells). In normal *Hydra* the stem cell generation time is 24 h and $P_s = 0{\cdot}6$; under these conditions the stem cell population doubles in 3·5 days.

In the present experiments we have systematically investigated the dependence of P_s on stem cell density. We culture stem cells in a feeder layer system consisting of aggregates of nitrogen-mustard (NM)-inactivated *Hydra* cells. In this system stem cell density can be varied over a wide range by changing the number of clone-forming units (CFU) added to the aggregates. We have measured the growth rate of the stem cell population and the cell cycle of stem cells in NM aggregates after 4–7 days of culture. From these data we calculate the value of P_s. The results indicate that the growth rate decreases 4-fold as the number of CFU seeded per aggregate increases from 10 to 400. Under these same conditions the cell cycle remains constant. The values of P_s calculated from these results indicate the P_s decreases from 0·75 in aggregates seeded with 10–30 CFU to 0·55 in aggregates seeded with 200–400 CFU. These results support a model in which P_s is controlled by negative feedback from neighbouring stem cells.

In addition, our experiments indicate that P_s decreases during the growth of stem cell clones. When only a few stem cells are seeded in aggregates, they give rise to isolated clones distributed throughout the aggregate. P_s decreases markedly within such clones as they grow in size presumably due to increasing stem cell content of the clones. Since P_s in such isolated clones declines with growth, we infer that the *local* stem cell concentration is what controls P_s and that the spatial range of the negative feedback signal is short compared to the dimensions of NM aggregates.

INTRODUCTION

In *Hydra* a population of multipotent stem cells (interstitial cells) continuously supplies precursors for 2 major lines of cell differentiation: nerve cells and nematocytes (for review see Bode & David, 1978). In each cell generation, 60% of stem cells divide to yield more stem cells while 40% differentiate (David & Gierer, 1974).

* Present address: Department of Radiology, University of Pittsburgh Medical School, Pittsburgh, Pa. 15261, U.S.A.

† Address for correspondence: Dr Charles N. David, Department of Molecular Biology, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, N.Y. 10461, U.S.A.

Under these conditions the stem cell population increases 20% per cell generation which is just sufficient to match the growth rate of *Hydra* tissue (David & Campbell, 1972). The mechanism by which individual stem cells decide to self-renew or differentiate is unknown. It is, however, clear from 2 recent reports that the probability of self-renewal (P_s) is not a fixed parameter of stem cell physiology but can vary in response to environmental conditions: (1) Bode, Flick & Smith (1976) demonstrated that P_s increases from a value of 0·6 characteristic of normal asexual growth to 0·7–0·8 following depletion of the stem cell population by hydroxyurea treatment. The increase in P_s leads to faster growth of the stem cell population and its recovery to normal levels. (2) David & MacWilliams (1978), using a technique for cloning interstitial stem cells, demonstrated that P_s increases to ~0·8 in young isolated clones and then decreases to ~0·6 by 10–12 generations of clone growth.

Both these results suggest that P_s is controlled by the density of stem cells in *Hydra* tissue. Low densities of stem cells cause higher values of P_s; high densities of stem cells cause lower values of P_s. A model in which P_s is controlled by negative feedback from neighbouring stem cells is qualitatively consistent with the above observations (David & MacWilliams, 1978). To test the negative feedback hypothesis directly, we initiated the present experiments to measure quantitatively the dependence of P_s on stem cell density.

To culture stem cells we have used a recently developed feeder layer technique (David & Murphy, 1977). Aggregates of *Hydra* cells inactivated by nitrogen mustard (NM) treatment are used as host tissue. Varying numbers of stem cells are added to the aggregates and the growth rate of stem population is determined between 4 and 7 days later. Our results show that P_s decreases when increasing numbers of stem cells are added to NM aggregates. The results also show that P_s decreases as the number of stem cells increases during the growth of isolated clones. Both results support the negative feedback model for the control of P_s and, in addition, suggest that the spatial range of the feedback signal is short compared to the dimensions of our NM aggregates.

METHODS

Interstitial cell system in Hydra – tissue maceration and terminology

The maceration technique (David, 1973) permits identification and quantitative scoring of stem cells, differentiating intermediates and differentiated nerves and nematocytes. Maceration dissociates *Hydra* tissue to single cells or small clusters in the case of cells held together by cytoplasmic bridges; cells retain their *in vivo* morphology in macerations enabling easy identification by phase-contrast microscopy. Stem cells and early committed precursors occur in macerations as large single cells and in pairs. We refer to these cells as 1s and 2s and use their numbers as a measure of the stem cell population. Proliferating nematocytes occur in clusters of 4, 8 and 16 cells (referred to as 4s, 8s, and 16s); when differentiation starts, each cell in these clusters develops a nematocyst capsule in its cytoplasm. We refer to these cells as differentiating nematocytes. Terminally differentiated nerve cells and nematocytes are also clearly recognizable in macerations.

Growth of interstitial stem cells in nitrogen mustard (NM) aggregates

Interstitial stem cells were cultured in host aggregates of NM-treated *Hydra* tissue as previously described (David & Murphy, 1977). In brief, NM host tissue and live tissue were each dissociated in cell culture medium. Aliquots of the live cell suspension containing from 10 to 400 clone-forming units were mixed with NM cell suspension and the mixture centrifuged. The cells pellets were removed from the centrifuge tubes and incubated as previously described. The growth and differentiation of interstitial cells was analysed between 4 and 7 days. In addition to changing the medium daily we now routinely wash out the aggregates on days 2, 3, and 4 by injecting them with a micropipette and flushing them with a gentle stream of fresh medium. We have found that this procedure enhances the reproducibility of the plating efficiency and the growth rate of stem cells from experiment to experiment. Aggregates prepared in this way are uniform in size and morphology; they contain 20900 ± 2700 total epithelial cells.

Determination of the number of clone forming units (CFU) in a live cell suspension

The number of CFU in a given live cell suspension was calculated using a ratio of 0·048 CFU per 1s+2s added to an aggregate (David & Murphy, 1977). To determine the number of 1s+2s in a live cell suspension, a parallel sample of live tissue was macerated and the number of 1s+2s counted.

Quantitation of stem cells and differentiating cells in NM aggregates

The maceration technique (David, 1973) has been used to identify and count interstitial cells, nematoblasts and nerve cells in NM aggregates. Because of the low cell numbers in many of the experiments reported here, it has not been possible to use a haemocytometer for cell counting as in previous work (Bode *et al.* 1973). We have, therefore, developed the following procedure:

Groups of 4–6 aggregates were macerated in a small volume of maceration solution. After addition of fixative the total volume (~0·2 ml) was determined by weighing the solution. Using a disposable micropipette with the tip cut off to enlarge the opening, 50 μl of solution were transferred to a microscope slide, spread over an area of about 1 cm^2 and allowed to dry. After drying, a drop of 10% glycerol and a coverslip were added and the preparation scored in a phase-contrast microscope with either 25 or 40× objective lenses.

To count cells, a slide was placed on a microscope stage and the position of the right and left edges of the spread determined with the stage micrometer. Then several complete passes across the preparation parallel to these edges were scored for a particular cell type. The passes were spaced uniformly between the right and left edges in order to sample all areas of the cell preparation. The total number of passes in the preparation is equal to the width (from right to left edges) in mm divided by 0·7 mm/pass for 25× objective or 0·45 mm/pass for the 40× objective. The total number of cells in a preparation was calculated from the number of cells counted and the fraction of the spread which was examined. The total number of cells per aggregate was then calculated from the total cells per preparation using the number of aggregates in the sample and the volume spread on the slide.

The above procedure permits accurate cell counting at concentrations as low as 20–50 cells/aggregate. Cell titres calculated from independent spreads of the same cell suspension are reproducible to ±10%. Comparison of the technique with haemocytometer counts of the same cell suspension yielded identical results.

Cell generation times

The generation time of interstitial cells was estimated from the [^{3}H]thymidine labelling kinetics of populations 'continuously' exposed to [^{3}H]thymidine as previously described (Campbell & David, 1974).

Computer simulations of stem cell growth and differentiation

A computer was programmed to simulate growth and differentiation of interstitial stem cells based on the cell flow model of David & Gierer (1974). The program starts with a large number of stem cells and values for the self-renewal probability (P_s), the probability of nerve commitment (P_{nv}) and the probability of nematocyte commitment (P_{nc}). $P_s+P_{nv}+P_{nc}=1{\cdot}0$. To create the next generation the number of stem cells is multiplied by P_s, P_{nv}, and P_{nc} in turn. Each result is then multiplied by 2 since cell division creates 2 stem cells, 2 nerve cells, and 2 nematocyte precursors respectively. After each generation, the number of stem cells and the number of each type of differentiated cell are recorded. Corrections are applied to these numbers for the differences in cell cycle time between stem cells and differentiating cells. Finally the total numbers of 1s+2s, 4s and nerves in each generation are recorded.

By repeating the simulations using different values of P_s between 0·5 and 1, we have generated standard curves relating P_s to the growth rate of 1s+2s (expressed in terms of the number of stem cell generations required to double the population size) and to the ratio of $(1s+2s)_{day\ 4}$ to $(\text{stem cells})_{day\ 0}$. The standard curves are shown in Fig. 3, p. 162, 163.

The simulation for the growth rate of the stem population agrees, as expected, with the analytical equation for growth of a stem population:

$$S/S_0 = (2P_s)^n,$$

where S is population size after n generations, S_0 the initial population size, P_s the self-renewal probability, and n the number of generations of growth. For $S/S_0 = 2$, $n = n_d$ the number of stem cell generations required to double the population size.

RESULTS

Growth of stem cells in nitrogen mustard aggregates

Stem cells can be cultured in a feeder layer system consisting of NM-inactivated *Hydra* tissue (David & Murphy, 1977). To prepare cultures, NM-inactivated tissue and live tissue are dissociated and aliquots of the cell suspensions are mixed and centrifuged to produce a cell pellet (aggregate). Such aggregates regenerate normal *Hydra* structures in 3–4 days. Added live cells proliferate and differentiate normally, thereby replacing the NM-treated cells which are unable to proliferate. Since NM host tissue contains no interstitial cells (Diehl & Burnett, 1966), the proliferation and differentiation of added live stem cells can be followed directly by cell counting using the maceration technique or toluidine blue staining which is specific for interstitial cells and differentiating nematoblasts.

The live cell suspension added to NM host tissue contains all cell types in *Hydra*. No cells types are inactivated by the dissociation and reaggregation procedures, although clusters of proliferating and differentiating nematoblasts are split to single cells by the dissociation procedure. These cells, however, complete their differentiation programme normally in about 4 days and migrate into tentacles of the aggregate (Gierer *et al.* 1972; David, unpublished). Nerve precursors also complete normal differentiation in aggregates in about 2 days (Gierer *et al.* 1972). Thus after 4 days, the only interstitial cells in aggregates are in clones derived from stem cells in the added live cell suspension (David & Murphy, 1977). We have, therefore, chosen the time from 4 to 7 days to measure the growth rate of the stem cell population. We have used the number 1s+2s scored by the maceration technique as an indicator of the stem cell population. Under the conditions of our experiments, between 40 and 70 %

of all 1s+2s are uncommitted stem cells; the exact percentage depends on the self-renewal probability.

NM aggregates were prepared containing varying numbers of CFU from 10 to 400 per aggregate. On days 4, 5, 6 and 7 groups of aggregates were macerated and the number of 1s+2s per aggregate determined by counting. In each experiment, control aggregates containing no added live cells were macerated and scored to be certain that all interstitial cells had been eliminated from the host tissue by NM treatment. The results of a number of experiments are presented in Fig. 1; the line through the experimental points is the least squares regression line. In all cases the population of 1s+2s increased exponentially. There was, however, a marked decrease in the rate of growth in aggregates seeded with large numbers of CFU.

In addition to the 4–7-day growth rate we have used the absolute number of 1s+2s on day 4 as a measure of stem cell growth. The results show a change in stem cell growth from a 12-fold increase in aggregates seeded with 10 CFU (Fig. 1A) to 5-fold increase in aggregates seeded with 400 CFU (Fig. 1F). There is some scatter in the absolute numbers of 1s+2s on day 4 due to changes in plating efficiency from experiment to experiment (David & Murphy, 1977). As expected, high values on day 4 are associated with generally higher values on day 5–7 as well. For example, in 10 experiments in which aggregates were nominally seeded with 30 CFU (Fig. 1B) 2 experiments have consistently almost 2-fold more 1s+2s per aggregate between days 4–7 than 8 other experiments. We have not attempted to correct for variations in plating efficiency in individual experiments. Instead we have averaged the results from all the experiments in order to minimize errors.

Stem cell cycle

The duration of the stem cell cycle is required in order to estimate P_s from the growth rate of the stem population. We have used a simple [^{3}H]thymidine continuous labelling technique (Campbell & David, 1974) to estimate the cell cycle of 1s+2s. The results in Fig. 2 indicate that the [^{3}H]thymidine labelling kinetics of 1s+2s on day 5 is similar in aggregates seeded with 30 and 400 stem cells. Therefore, the decrease in growth rate (Fig. 1) is not due to changes in cell cycle duration or the fraction of cycling stem cells. The duration of the cell cycle can be estimated from continuous labelling results as the time required to reach 100% labelling plus 12 h for *S*-phase (Campbell & David, 1974). For the cell populations in Fig. 2, this is 24–28 h which is very similar to estimates of stem cell cycle in normal animals.

Methods for measuring P_s

In the present experiments we have used 2 measures of stem cell growth to estimate P_s: (1) the increase in 1s+2s between 4 and 7 days of growth, and (2) the total number of 1s+2s on day 4 normalized to the number of stem cells seeded on day 0. We have used computer simulation of the growth and differentiation of *Hydra* stem cells (see Methods) to prepare standard curves relating each of the growth measures to P_s. The standard curves are shown in Fig. 3A, B; P_s for a given experimental value of the growth rate was read from the standard curve.

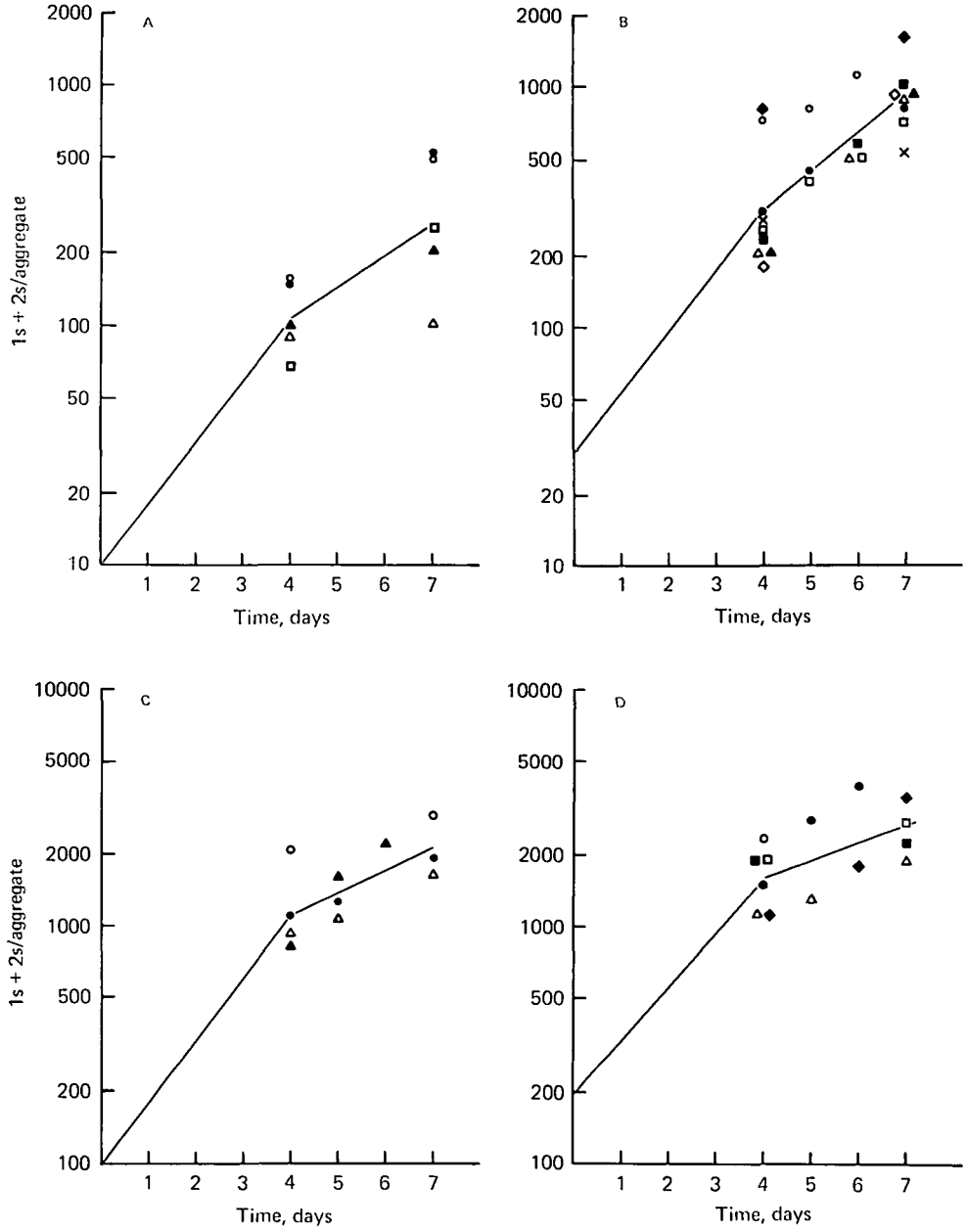

Fig. 1. Growth rate of 1s+2s in NM aggregates. NM aggregates seeded with varying numbers of CFU were macerated on days 4 through 7. 4–5 aggregates were used for each sample and the total number of 1s+2s per aggregate determined. Different symbols indicate independent experiments. Lines drawn through the experimental points were calculated from a least squares regression analysis. Aggregates seeded with 10 (A), 30 (B), 100 (C), 200 (D), 300 (E), and 400 CFU (F).

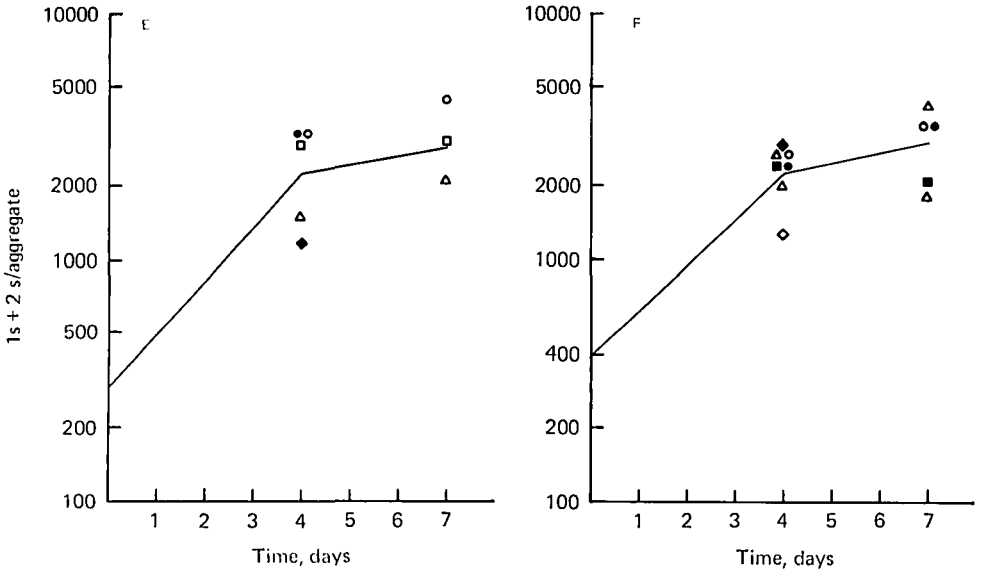

Fig. 1E–F. For legend see opposite.

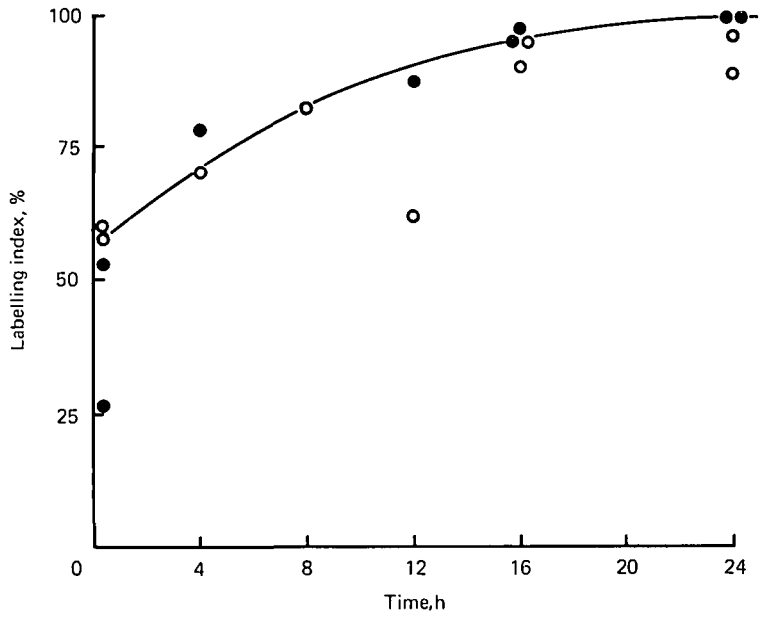

Fig. 2. 'Continuous' [^{3}H]thymidine labelling kinetics of the population of 1s+2s in NM aggregates. NM aggregates were seeded with 30 (●) or 400 CFU (○). Starting on day 5, aggregates were labelled by injecting [^{3}H]thymidine (100 μCi/ml; 0·1 μl) into the central cavity at t_0 and at 1 h prior to sampling. All aggregates were injected at least once every 12 h. This labelling schedule catches all cells once in their cell cycle since the *S*-phase of interstitial cells is about 12 h (Campbell & David, 1974). Five aggregates were macerated for each time point and the labelling index of the 1s+2s determined following autoradiography.

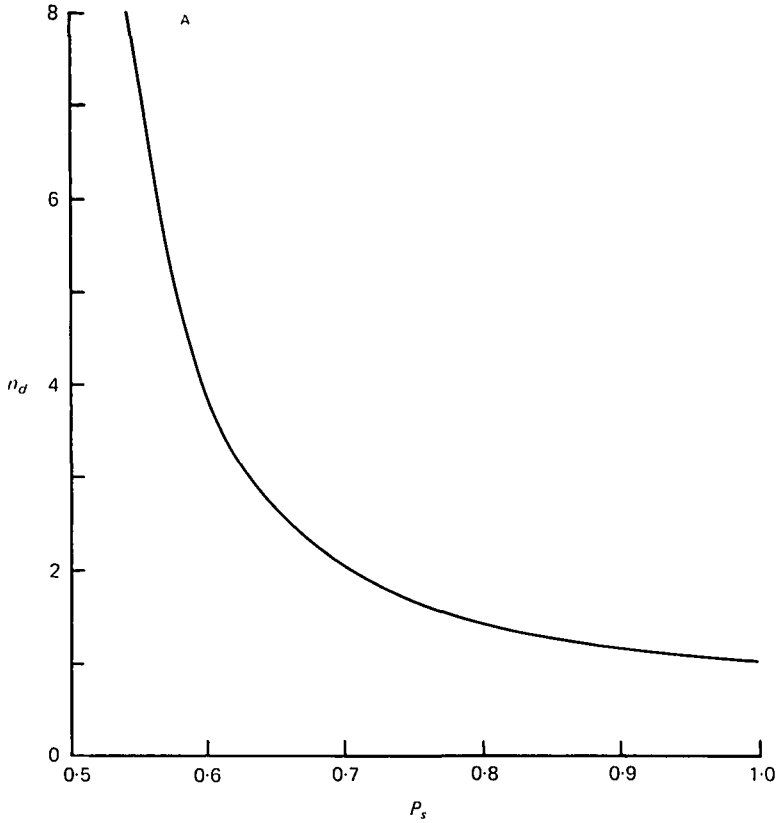

Fig. 3. Standard curves relating P_s to n_d (A) and to $(1s+2s)_{day\ 4}/(stems)_{day\ 0}$ (B). A computer was programmed to simulate the growth of the stem cell population (see Methods). Stem cells were assumed to have a 24-h cell cycle. P_s was varied and the total number of 1s+2s computed for 4 to 7 generations of growth. The growth rate of the population of 1s+2s was calculated from these results and plotted in (A) as the number of stem cell generations required to double the stem cell population (n_d). The ratio of $(1s+2s)_{day\ 4}/(stems)_{day\ 0}$ was also calculated from the simulations and is shown in (B).

Fig. 3A shows the dependence of doubling time (n_d) of a stem cell population on P_s. Above $P_s=0{\cdot}75$, n_d is relatively insensitive to P_s. However, below $P_s=0{\cdot}75$, n_d increases rapidly with decreasing P_s. In this range n_d is a useful measure of P_s and we have used the results in Fig. 3A in conjunction with measurements of growth rate (Fig. 1) to estimate P_s. We refer to this estimate as P_s on day 5·5 since the increase in 1s+2s/aggregate between days 4 and 7 was used to estimate growth rate (n_d).

In addition to the 4–7 day growth rate we have used the total number of 1s+2s on day 4 produced per stem cell plated on day 0 as a measure of growth. In Fig. 3B, the ratio of 1s+2s on day 4 to stem cells on day 0 is given as a function of P_s. In order to use this standard curve, it is necessary to correct the experimental results, which are given in terms of 1s+2s per CFU, to 1s+2s per stem cell. The number of stem cells is greater than the number of CFU if P_s is less than 1·0 because some input stem cells differentiate instead of self-renewing, thus 'extinguishing' a potential clone.

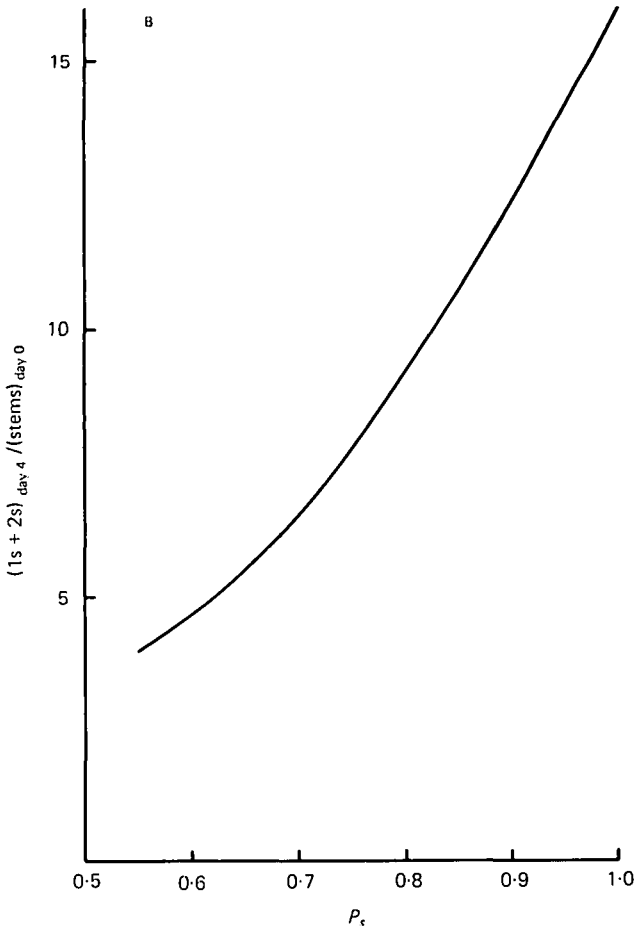

Fig. 3B. For legend see opposite.

For $P_s = 0{\cdot}8$, which is characteristic of young stem cell clones (David & MacWilliams, 1978), the probability that a clone will extinguish is 25 % (Vogel, Niewisch & Matioli, 1969). After correcting the experimental values of 1s+2s/CFU for clone extinction (Table 1) we have used Fig. 3B to determine P_s. We refer to this result as P_s on day 2, since it is an average of the behaviour during the first 4 days of clone growth.

Calculation of P_s in multiclone aggregates

Using the experimental results in Fig. 1 and the standard curves in Fig. 3, P_s has been calculated for aggregates containing increasing numbers of CFU. Table 1 shows the calculations in detail for P_s estimated on day 2 and day 5·5, Fig. 4 shows the change in P_s as a function of increasing clone density in NM aggregates.

On day 2, P_s is relatively constant at 0·75–0·79 over a 10-fold range in clone density (10–100 clones/aggregates). However, as clone density increases from 100 to 400 clones/aggregate, P_s drops sharply to 0·55. The results on day 5·5 are qualitatively

similar to day 2: at low clone densities, P_s is relatively constant at about 0·7; at high clone densities P_s decreases to values of 0·55. However, the day-5·5 estimates of P_s are uniformly lower than the day-2 estimates at all but the highest densities indicating that P_s decreases during clone growth.

Table 1. *Calculation of P_s from growth rate of 1s+2s*

CFU/ aggregate	1s+2s/aggregate*		P_s estimated on day 5·5		P_s estimated on day 2		
	Day 4	Day 7	n_d†	P_s‡	$(\text{Stems})_{\text{day 0}}$§	$\frac{(1s+2s)_{\text{day 4}}}{(\text{Stems})_{\text{day 0}}}$	P_s‖
10	107	275	2·2	0·68	13·3	8·05	0·76
30	310	920	1·9	0·72	40	7·75	0·75
100	1120	2180	3·1	0·63	133	8·4	0·78
200	1590	2660	4·0	0·59	266	5·9	0·67
300	2260	2900	8·3	0·54	400	5·6	0·65
400	2260	3000	8·9	0·54	532	4·3	0·58

* Results from linear regression line in Fig. 1.

† Calculated from equation for exponential growth:

$$n_d = \frac{[t/T_s]\,0{\cdot}69}{\ln (S_7/S_4)},$$

where n_d = number of stem cell generations required to double stem cell population; T_s = stem cell generation time (24 h in these calculations); t = time (3 days in these calculations); S_4, S_7 are stem cell numbers (expressed as 1s+2s) on day 4 and 7.

‡ Determined from standard curve in Fig. 3A.

§ Stem cells/aggregate on day 0 was calculated by correcting CFU/aggregate for clone extinction. $P_s = 0{\cdot}8$ during the first day of clone growth (David & MacWilliams, 1978). For $P_s = 0{\cdot}8$ the probability that a clone will extinguish due to differentiation of all stem cells is 0·25 (Vogel *et al.* 1969). Therefore, stem cells/aggregate = CFU/aggregate × 1·33.

‖ Determined from standard curve in Fig. 3B.

Distribution of stem cells in NM aggregates

When single stem cells are added to NM aggregates they give rise to clones which occur as discrete isolated patches of cells in the ectodermal epithelium of the aggregates (David & Murphy, 1977). These patches are easily distinguished in whole mounts of aggregates stained with toluidine blue (Diehl & Burnett, 1964) which stains the stem cells and differentiating nematoblasts of the clones more darkly than the surrounding host tissue. To determine the distribution of clones in the present experiments, aggregates were stained with toluidine blue on days 4 and 7. Aggregates seeded with 10 and 30 CFU showed distinct clones on day 4, although in the 30 CFU aggregates growth has increased the size of clones to the point where some of the clones have begun to merge with their neighbours. In aggregates seeded with 100 or more CFU, individual clones could no longer be discerned on day 4; all the interstitial cells appeared in very large patches. By day 7 individual clones could only be discerned in aggregates seeded with 10 CFU; in all other aggregates the interstitial cells occurred

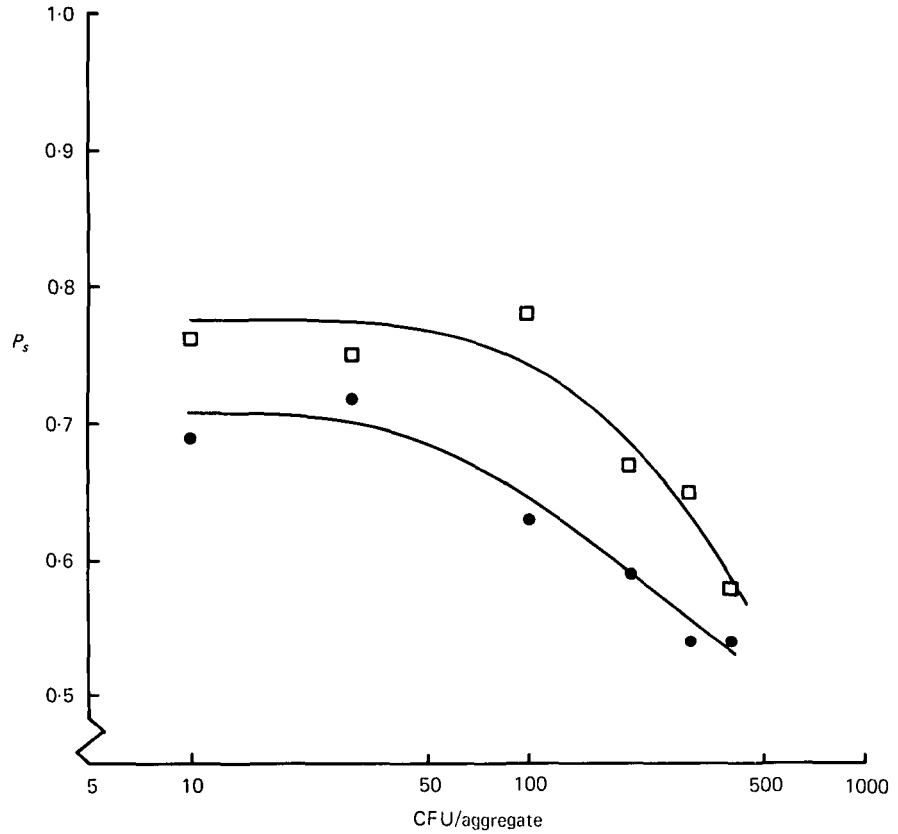

Fig. 4. The change in the self-renewal probability (P_s) with increasing numbers of CFU per aggregate. P_s was calculated from the growth rate of the population of 1s + 2s. Details of the calculations are given in Table 1. The results in the figure are for P_s estimated on day 2 (□) and on day 5·5 (●).

in large patches or in the case of 300 and 400 CFU aggregates, the aggregates were essentially completely filled with interstitial cells.

DISCUSSION

Dependence of P_s on stem cell density

The results in Fig. 1 demonstrate that increasing the number of CFU in NM aggregates decreases the growth rate of the stem cell population. In addition we have shown that the stem cell generation time is essentially constant in aggregates seeded with 30 and 400 CFU (Fig. 2) under conditions where the 4–7-day growth rate differs by 4-fold ($n_d = 2{\cdot}0$ against $n_d = 8{\cdot}0$; Table 1). To achieve such a difference in growth rate by changing the cell cycle would require an increase in cell cycle time from 24 to 96 h which is clearly inconsistent with the results in Fig. 2. Thus we conclude that the change in growth rate of the stem cell population is due to a change in the fraction of stem cells undergoing self-renewal and that we can use the observed growth rate to estimate P_s from the standard curves in Fig. 3.

Fig. 4 shows the change in P_s as the number of CFU added to aggregates increases. The 2 curves, based on P_s estimates on days 2 and 5·5, show qualitatively the same result – namely that P_s decreases with increasing clone density. In addition, the estimates of P_s on day 5·5, when clones are larger, are uniformly lower than the day 2 estimates for each clone density. Both results suggest that increasing numbers of stem cells – due either to clone growth or to increasing input of CFU – cause a decrease in P_s.

The present results are in good agreement with the results of other investigators. David & MacWilliams (1978) measured P_s in single stem cell clones in NM aggregates. They estimated that $P_s \cong 0{\cdot}8$ during the first generations of clone growth and, in addition, they showed that P_s decreased during the growth of single clones to $\cong 0{\cdot}6$ after 10–12 generations. Both results are in agreement with the results in Fig. 4 and suggest that 10 clones per aggregate behave very much as 1 clone per aggregate. Furthermore, Bode *et al.* (1976) have shown that depletion of stem cell density in normal *Hydra* by hydroxyurea treatment increases P_s to 0·7–0·8. The stem cell density in their depleted tissue was similar to our NM aggregates seeded with 30–100 CFU and thus Bode *et al.*'s observations are similar to ours. Bode *et al.* also observed that P_s decreased to 0·6 as the stem cell density recovered to normal levels again in agreement with the results in Fig. 4.

A model for the control of P_s

The present results suggest that P_s in *Hydra* stem cells is controlled by negative feedback from neighbouring stem cells. We have previously suggested a simple model to explain this behaviour (David & MacWilliams, 1978). Stem cells are postulated to secrete a diffusible factor into their environment; in addition, they can sense the concentration of factor in their environment. It is then postulated that the ambient factor concentration at a stem cell regulates the value of P_s within that cell: high concentrations of factor decrease P_s; low concentrations of factor increase P_s.

An important parameter of the negative feedback model is the spatial range over which stem cells can 'sense' one another. If the range is similar to the dimensions of the aggregate, then all stem cells in the aggregate interact with each other and P_s is determined only by the total number of stem cells per aggregate; the spatial distribution of stem cells in the aggregate is unimportant. If, however, the spatial range of the feedback signal is much smaller than the aggregate, the distribution of stem cells in the aggregate will profoundly affect the value of P_s; stem cells clustered together will have lower P_s than the same number of stem cells spread throughout the aggregate.

In the present experiments we have gathered information about the distribution of stem cells in aggregates and about the value of P_s. We are, therefore, in a position to ask whether the value of P_s is dependent only on the total number of stem cells per aggregate or whether the distribution of stem cells within the aggregate also influences the value of P_s. To determine the relationship between P_s and the total number of stem cells per aggregate we have replotted the estimates of P_s in Fig. 4 versus the number of 1s + 2s per aggregate at the time P_s was determined (Fig. 5). In addition

we have added results from single clones grown in NM aggregates (David & MacWilliams, 1978). The points show a considerable degree of scatter in this plot. Consequently, it is not possible to draw a smooth curve through all the points without accepting large deviations for some values. Thus our data do not appear to support the hypothesis of a single relationship between P_s and the total number of stem cells per aggregate. Our data, therefore, also do not support the idea that the feedback signal has a very long spatial range.

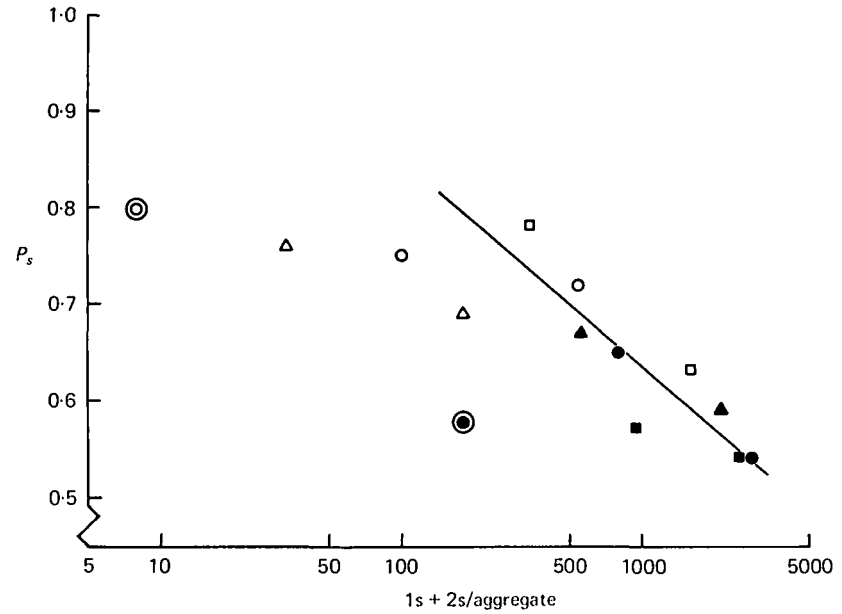

Fig. 5. Change in the self-renewal probability (P_s) with the concentration of 1s + 2s per aggregate. The values of P_s in Fig. 4 are plotted versus the number of 1s + 2s per aggregate at the time P_s was determined. The number of 1s + 2s was determined from Fig. 1 on day 2 and 5·5. Aggregates seeded with 10 (△), 30 (○), 100 (□), 200 (▲), 300 (●), and 400 CFU (■). Estimates of P_s in single clones in NM aggregates from the results of David & MacWilliams (1978), have been added to the figure. These results are for P_s averaged over days 0–5 (◎) and on days 13–14 (⊙).

Assuming, instead, that the range of the feedback signal is short permits a more satisfactory interpretation of the points in Fig. 5. Toluidine blue staining (see Results) shows that, in aggregates with 30 CFU or more, interstitial cells are close to uniformly dispersed, while in aggregates containing 10 CFU or fewer, interstitial cells are clustered in clearly defined clones. Since the spatial distribution of interstitial cells is similar in all aggregates with 30 CFU or more, the relationship between P_s and cell density should be similar in all these aggregates even if spatial distribution is an important variable. We have accordingly fitted a single line in Fig. 5 to all points derived from these aggregates. There is still a certain amount of scatter of points about the line, but the deviations are much less than would be found if the line had been fitted to all the points in Fig. 5. If the spatial range of the feedback signal is short, one expects

that the values of P_s observed in uniformly populated aggregates will also be obtained in aggregates containing isolated clones. In the latter case, however, the total number of interstitial cells/aggregate will be substantially lower. One, therefore, expects curves describing the relationship between P_s and 1s+2s/aggregate for aggregates containing 1 and 10 CFU to lie to the left of the curve for aggregates containing 30 CFU or more. This expectation is fully justified by the results in Fig. 5. Fig. 5, therefore, seems to support the idea that the feedback signal has a short range.

An equivalent way of coming to this conclusion is to observe that stem cells localized in isolated clones have P_s values similar to stem cells located in very large arrays of cells (compare 1 or 10 CFU aggregates to 100–400 CFU aggregates having the same value of P_s in Fig. 5). Thus stem cells in clones do not appear to sense whether the area beyond the perimeter of a clone is empty or filled with other stem cells. The range of the feedback signal must, therefore, be comparatively short.

One interesting implication of the short range of stem-stem feedback is that stem cells in different regions of *Hydra* are independent of each other. Such independence means that the entire stem cell population in a *Hydra* can not behave in unison (see also Sproull & David, 1979.) This could prevent situations in which many or most stem cells in one generation differentiate or self-renew simultaneously. The apparent short range of the feedback signal, thus, effectively inhibits large-scale oscillations in stem cell number, which might adversely affect the physiology of the organism.

The authors thank Dr H. K. MacWilliams for helpful discussions and critical reading of the manuscript. Ms. Ida Plotnick provided expert technical assistance. The research was supported by grants from the NIH (GM 11301), the NSF (77–25426). C.N.D. is recipient of a Career Development Award (FRA–132) from the American Cancer Society.

REFERENCES

BODE, H., BERKING, S., DAVID, C. N., GIERER, A., SCHALLER, H. & TRENKNER, E. (1973). Quantitative analysis of cell types during growth and morphogenesis in *Hydra*. *Wilhelm Roux Arch. EntwMech. Org.* **171**, 269–285.

BODE, H. & DAVID, C. N. (1978). Regulation of a multipotent stem cell, the interstitial cell of *Hydra*. *Prog. Biophys. molec. Biol.* **33**, 189–206.

BODE, H. G., FLICK, K. M. & SMITH, G. S. (1976). Regulation of interstitial cell differentiation in *Hydra attenuata*. 1. Homeostatic control of interstitial cell population size. *J. Cell Sci.* **20**, 29–46.

CAMPBELL, R. D. & DAVID, C. N. (1974). Cell cycle kinetics and development of *Hydra attenuata*. II. Interstitial cells. *J. Cell Sci.* **16**, 349–358.

DAVID, C. N. (1973). A quantitative method for maceration of *Hydra* tissue. *Wilhelm Roux Arch. EntwMech. Org.* **171**, 259–268.

DAVID, C. N. & CAMPBELL, R. (1972). Cell cycle kinetics and development of *Hydra attenuata*. I. Epithelial cells. *J. Cell Sci.* **11**, 557–568.

DAVID, C. N. & GIERER, A. (1974). Cell cycle kinetics and development of *Hydra attenuata*. III. Nerve and nematocyte differentiation. *J. Cell Sci.* **16**, 359–375.

DAVID, C. N. & MACWILLIAMS, H. (1978). Regulation of the self-renewal probability in *Hydra* stem cell clones. *Proc. natn. Acad. Sci. U.S.A.* **75**, 886–890.

DAVID, C. N. & MURPHY, S. (1977). Characterization of interstitial stem cells in *Hydra* by cloning. *Devl Biol.* **58**, 372–383.

DIEHL, F. & BURNETT, A. L. (1964). The role of interstitial cells in the maintenance of *Hydra*. I. Specific destruction of interstitial cells in normal, sexual and non-budding animals. *J. exp. Zool.* **155**, 253–259.

GIERER, A., BERKING, S., BODE, H., DAVID, C. N., FLICK, K., HANSMANN, G., SCHALLER, C. & TRENKNER, E. (1972). Regeneration of *Hydra* from reaggregated cells. *Nature, New Biol.* **239**, 98–101.

SPROULL, F. & DAVID, C. N. (1979). Stem cell growth and differentiation in *Hydra attenuata*. II. Regulation of nerve and nematocyte differentiation in multiclone aggregates. *J. Cell Sci.* **38**, 171–179.

VOGEL, H., NIEWISCH, H. & MATIOLI, G. (1969). Stochastic development of stem cells. *J. theor. Biol.* **22**, 249–270.

(*Received* 22 *September* 1978 –
Revised 15 *January* 1979)