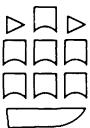


Stem Cells

THEIR IDENTIFICATION AND CHARACTERISATION

EDITED BY

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Stem cell proliferation and differentiation in hydra

INTRODUCTION

The mechanism by which cells decide to proliferate or to differentiate is a central feature of the development of multicellular organisms. Stem cells, either in embryos or adult organisms, retain this decision-making ability and have come under investigation as model systems for the manner in which cells make decisions. Interstitial stem cells in hydra have been intensively investigated in several laboratories in the past decade.

Interstitial cells in hydra are a population of multipotent uncommitted stem cells which continuously give rise to two major classes of differentiated cells: nerves and nematocytes (for a comprehensive review, see Bode & David 1978). In sexual animals, stem cells also give rise to egg and sperm. The interstitial cell system in hydra has a number of advantages for the investigation of stem cell behaviour:

1. Stem cells constitute 4% of hydra cells and thus hydra tissue is a rich source of material,
2. Hydra has a very simple tissue structure and the stem cell population is directly accessible,
3. Analytical techniques have been developed which permit easy scoring of both stem cells and their differentiated derivatives,
4. A culture system has been developed which permits manipulation of the stem cell environment,
5. Several factors have been purified and characterised which affect stem cell differentiation and the prospects are good that other factors controlling stem cell behaviour can also be isolated.
6. Finally, and of particular interest to developmental biologists, is the fact that differentiation to nerve cells or nematocytes depends on stem cell position along the body column and appears to be controlled by the same morphogenetic signals which control other features of hydra development. Thus, an understanding of stem cell behaviour in hydra may also provide insight into the general problem of how, during embryogenesis, patterns of differentiated cells arise under the influence of morphogenetic gradients.

The principal aim of this contribution is to focus attention on the mechanism by which hydra stem cells make decisions. I will summarise the properties of stem cells and the factors which control the fate of stem cells. In particular I will discuss recent work on the control of self-renewal and commitment to nerve and nema-

toocyte differentiation. This work has permitted determination of the time of commitment in the cell cycle and, in the case of nerve differentiation, the identification of molecules which control nerve commitment. Although just beginning, this work has already begun to outline the decision process and thus appears suitable for a book devoted to stem cells.

INTERSTITIAL STEM CELL SYSTEM

a. Dynamics and composition of hydra tissue

Hydra is a small freshwater polyp containing about 100 000 cells distributed among 15 cell types. Its body is a tube consisting of two epithelial layers, ectoderm and endoderm, surrounding a gastric cavity. The body column is modified at the anterior end to form a head consisting of a mouth surrounded by five to seven tentacles, and at the posterior end to form a foot or basal disc. Most of the body column consists of a gastric region with a budding region at its lower end.

Tissue growth in hydra occurs continuously and throughout the body column (Campbell 1967a, David & Campbell 1972, Campbell & David 1974). In well-fed cultures the tissue mass doubles in three to four days. The animals increase in size up to a point and then the excess tissue is removed by budding off young animals which repeat the cycle. Some tissue is also lost by sloughing at the tentacle tips and basal disc. The constant proliferation of cells causes a continual displacement of the tissue from the body column into buds and towards the two ends where it differentiates into head and foot structures. This results in a steady state in which cell loss is balanced by cell production.

The endoderm of the body column is made up primarily of gastrodermal digestive cells with a smaller number of gland and mucous cells interspersed among them. In the ectoderm epithelio-muscular cells make up the tissue layer. Lodged among these epithelial cells are interstitial cells, their differentiated derivatives, nerves and nematocytes, and differentiation intermediates. Together these cells make up the interstitial cell system of an asexually-reproducing animal. During sexual phases of the life cycle gamete differentiation is part of this system.

b. Stem cell proliferation and differentiation

Figure 2.1 outlines schematically cell proliferation and differentiation in the interstitial stem cell system. Analysis of this system of cells has been greatly facilitated by a maceration technique (David 1973, Bode et al 1973) for fixing and dissociating hydra tissue into single cells and cell clusters in the case of interstitial cells connected by cytoplasmic bridges. Cells retain their *in vivo* morphology in macerations permitting unambiguous identification and accurate counting of all cell types. Macerations are also suitable for autoradiography. Thus, in conjunction with appropriate ^3H -thymidine labelling protocols, it has been possible to analyse completely the proliferation and differentiation kinetics of stem cells and differentiating nerves and nematocytes *in vivo*. (Campbell & David 1974, David & Gierer 1974).

Stem cells

Stem cells occur as large single interstitial cells or in pairs (Fig. 2.1). They have

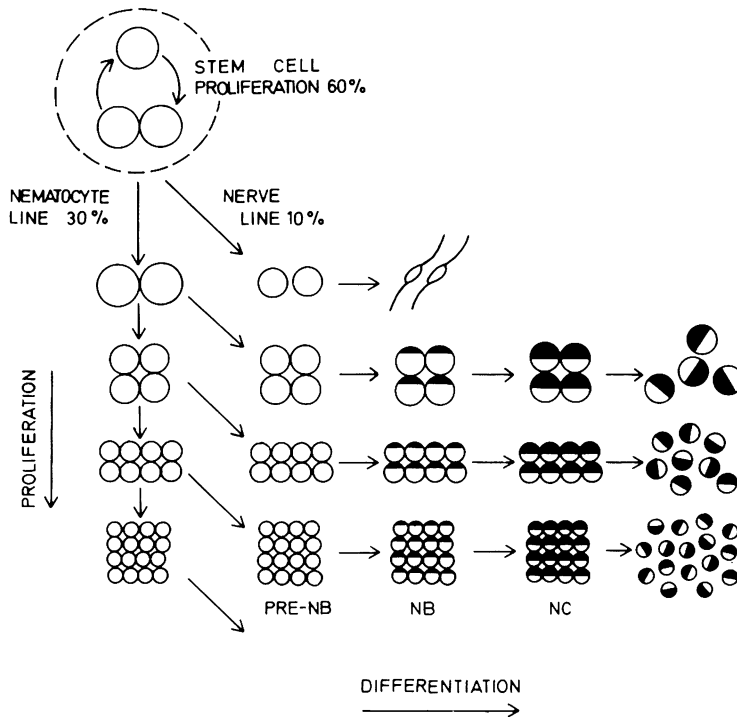


Fig. 2.1 Schematic representation of stem cell proliferation and differentiation in hydra. The cells shown in clusters are held together by cytoplasmic bridges. Nests of proliferating cells are designated by cluster size: 1s, 2s, 4s, etc. Pre-NB, NB and NC represent stages in differentiation of nematocytes. Stem cells and early committed cells in the nematocyte and nerve pathways are morphologically indistinguishable and constitute a class of large undifferentiated interstitial cells. This class, referred to as 1s + 2s, is a convenient measure of stem cells since about 50% of these cells are uncommitted stem cells. (Based on the results of David & Gierer 1974).

an approximately 24-h cell cycle consisting of S phase 12 h, G_2 12 h, mitosis 1 h and $G_1 < 1$ h (Campbell & David 1974). In exponentially growing Hydra, the stem cell population increases in size 20% per day and thus 60% of stem cell daughters must remain stem cells in each cell generation. Under these conditions the stem cell population doubles in size in about 3·5 days at the same rate as tissue growth. The size of the stem cell population can be estimated from the flow of stem cells into the nerve and nematocyte differentiation pathways. Per day about 1750 stem cells enter the nematocyte pathway and 550 enter the nerve pathway (David & Gierer 1974). Since these committed cells account for about 40% of stem cell daughters per cell generation, the size of the uncommitted stem cell population is about 3500 cells.

Nematocyte differentiation

Nematocytes are stinger cells used for the capture of prey. They are mounted on the external surface of specialised ectodermal cells (battery cells) in the tentacles of hydra. Depending on the size of the animal, individual hydra contain from 5000 to 30 000 nematocytes distributed among four morphologically distinct types: des-

monemes, stenoteles, atrichous isorhizas and holotrichous isorhizas (Bode & Flick 1976). Although most mature nematocytes are located in battery cells in tentacles, their differentiation occurs in the body column.

Stem cells entering the nematocyte pathway undergo three to four rounds of cell division (Fig. 2.1). Each cell cycle lasts about 18 h (Campbell & David 1974) and, since cytokinesis is incomplete, daughter cells form clusters in which cells are connected to one another by cytoplasmic bridges (Slautterback & Fawcett 1959). Subsequently all cells in a cluster undergo synchronous differentiation into one type of nematocyte (Lehn 1951, Rich & Tardent 1969, David & Challoner 1974). Differentiation of the nematocyst capsule starts with the appearance of a small vacuole in the cytoplasm of each cell about 6 h after the last mitosis (David & Gierer 1974). Depending on the type of nematocyte, the vacuole grows in size for 35–50 h. Thereafter, in a rapid series of events, a collagenous shell is laid down in the vacuole and filled with refractile material. After differentiation is complete, the clusters break up into individual cells which migrate through the ectoderm into the tentacles where they are mounted in battery cells.

Nerve differentiation

Differentiation of nerve cells occurs directly from the stem cell compartment (Fig. 2.1). Committed precursors complete the cell cycle, divide and give rise to two nerve cells; there is no evidence for the involvement of clusters (David & Challoner 1974, Davis 1974). Following a ^3H -thymidine pulse, labelled nerve cells begin to appear at 18 h (David & Gierer 1974). Since nerve cells, like nematocytes, have a $2n$ postmitotic DNA content, this 18 h period includes G_2 of the precursor (probably 12 h in normal intact animals but shorter during regeneration; see below) as well as a period of postmitotic differentiation which may be as short as 6 h.

c. Multipotency of stem cells

The cell flow diagram in Figure 2.1 characterises the behaviour of the stem cell population as a whole. It does not, however, provide any information on the differentiated fate of individual stem cells. For example, the stem cell population could consist of several independent populations of stem cells each programmed for a specific differentiation pathway. Alternatively, it could consist of a single homogeneous population of multipotent cells, the fate of individual stem cells being determined by a stochastic process or by environmental signals inducing nerve or nematocyte differentiation. To clarify the situation procedures for cloning hydra stem cells were developed (David & Murphy 1977).

The procedure for cloning hydra stem cells is based on the method of cloning haemopoietic stem cells in lethally irradiated mice (Till & McCulloch 1961). Hydra are treated with nitrogen mustard (NM) which inactivates rapidly proliferating cells of the interstitial cell system and causes their elimination from the tissue (Diehl & Burnett 1964). The remaining epithelial tissue is a suitable host for culturing added interstitial cells. Interstitial cells to be cultured are introduced into host tissue using a technique for dissociating and reaggregating hydra cells (Gierer et al 1972). Reaggregates of NM-treated cells regenerate and interstitial cells added to such aggregates continue to proliferate and differentiate. Stem cells give rise to

clones of stem cells and differentiated products; committed interstitial cells complete differentiation to nerves or nematocytes.

About 1.5% of hydra cells give rise to stem cell clones in NM aggregates (David & Murphy 1977). This estimate is similar to estimates of 3.5–4% stem cells based on the cell flow analysis *in vivo* (David & Gierer 1974) and indicates that the cloning efficiency of stem cells in NM aggregates is high. Thus the cloning procedure is not scoring a selected subpopulation of stem cells. When individual clones were examined for their content of differentiated cells, all were found to contain both nerves and nematocytes in significant numbers (David & Murphy 1977). No clones were found which contained only one differentiated cell type. Although only nine clones were examined by the technique it seems reasonable to conclude that the stem cell population is homogeneous and consists of multipotent cells capable of giving rise to both differentiated cell types.

CONTROL OF STEM CELL PROLIFERATION

There are two basically different ways to control the growth rate of stem cell populations: (1) vary the duration of the cell cycle, and (2) vary the fraction of stem cell daughters which remain stem cells. This is referred to as the self-renewal probability or P_s . Several experiments have now made it clear that in hydra variation in P_s is the principal mechanism by which the growth rate of the stem cell population is controlled (Bode et al 1976, David & MacWilliams 1978, Sproull & David 1979). There is in addition some evidence that cell cycle duration can vary but to date these variations have been associated with differentiation pathways rather than self-renewal (Schaller 1976a,b, Berking 1980, Venugopal & David 1981c).

In the following sections, the evidence for control of P_s is reviewed. These experiments indicate that P_s is controlled by the concentration of stem cells in tissue: high concentrations lower P_s ; low concentrations raise P_s . The form of this control is that of a negative feedback loop: stem cell concentration controls stem cell proliferation. Since values of P_s less than 0.5 decrease the stem cell population and values of P_s greater than 0.5 increase the stem cell population, the population under steady state will seek a concentration at which $P_s \approx 0.5$.

a. Stem cell homeostasis

Over many generations of asexual reproduction, the ratio of stem cells to epithelial cells in hydra remains constant (Bode et al 1977). Furthermore, if the ratio is experimentally altered, it recovers to its normal level. For example, treatment of hydra with hydroxyurea causes extensive inactivation of cycling cells and depletes the stem cell population dramatically. If treatment is discontinued and such hydra are fed, the stem cell population recovers to its normal level (Bode et al 1976). During the recovery process the stem cell cycle does not change and thus Bode et al concluded that the enhanced growth rate of the stem cell population relative to the epithelial population was due to an increase in P_s to 0.7 from its normal level in a growing hydra of 0.6. Such an increase doubles the growth rate of the stem cell population. Thus recovery of the stem cell population following depletion to 1% of normal level requires 6–7 population doublings (2^{6-7}), or about 25 days as observed.

b. Dependence of P_s on stem cell concentration?

The observations above suggest that P_s depends on stem cell concentration in hydra; low stem cell concentrations increase P_s ; high stem cell concentrations decrease P_s . Direct support for this hypothesis has been obtained by varying the concentration of stem cells in the NM aggregate culture system (see above). Stem cell populations seeded at low concentrations in NM aggregates grow more rapidly than populations seeded at high concentrations (Sproull & David 1979). Under these conditions the cell cycle time of stem cells is constant and thus the variation in growth rate is due to changes in P_s . Figure 2.2 shows the dependence of P_s on stem cell concentration in NM aggregates. At low concentrations $P_s \approx 0.75$ and the stem population doubles in size in 1.7 cell generations. At high concentrations $P_s \approx 0.53$ and stem population doubles in >10 stem cell generations.

Epithelial cells in NM aggregates do not proliferate as a result of the prior treatment with nitrogen mustard. In such aggregates the stem cell population increases in size until a stem cell concentration is attained at which $P_s \approx 0.5$. At this point stem cells continue to cycle but the population stays roughly constant in size. By comparison, in well-fed hydra the epithelium is constantly expanding in size due

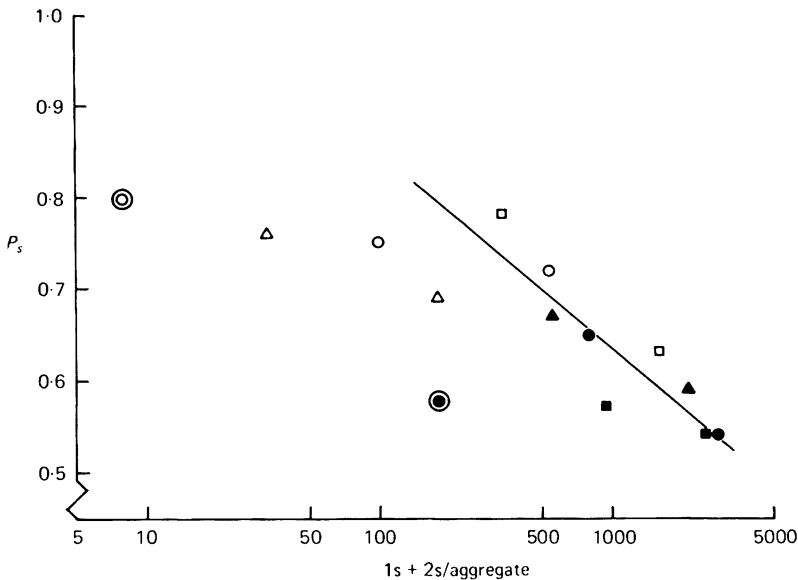


Fig. 2.2 Dependence of P_s on stem cell concentration in NM aggregates. Stem cell concentration is given in terms of $1s + 2s/\text{aggregate}$; about 50% of $1s + 2s$ are uncommitted stem cells. Different symbols indicate NM aggregates seeded with 10 (Δ), 30 (\circ), 100 (\square), 200 (\blacktriangle), 300 (\bullet), and 400 (\blacksquare) stem cells; for each set of aggregates P_s was estimated at two time points, after 2 days and after 5.5 days of growth. Estimates of P_s in single clones in NM aggregates on days 0–5 (\odot) and on days 13–14 (\bullet) are also shown.

In aggregates seeded with 30 or more stem cells growing clones merge with each other and stem cells appear to be homogeneously distributed; a linear regression line has therefore been drawn through these values to indicate the decrease in P_s with increasing stem cell concentration. In aggregates seeded with fewer than 30 stem cells, stem cells occur in isolated clones or small groups of clones. In such aggregates P_s also decreases with increasing stem cell concentration but at lower total concentrations of $1s + 2s/\text{aggregate}$ indicating that the stem cell concentration *within* clones is the dominant factor controlling P_s . (Based on the results of David & MacWilliams 1978, Sproull & David 1979).

to epithelial cell proliferation. Since the epithelium is expanding, the stem cell population never achieves a maximum concentration and P_s remains about 0.6. Under these conditions the stem cell population grows at the same rate as the epithelium in which it is embedded and the ratio of stem cells to epithelial cells remains constant (Bode et al 1977).

c. Changes in P_s during growth of stem cell clones

When very low numbers of stem cells are seeded in NM aggregates, individual stem cells give rise to isolated clones (David & Murphy 1977). Clones start from single stem cells but rapidly increase in size (number of stem cells) and also begin to differentiate nerves and nematocytes. These changes reflect changes in P_s which are due to the dramatic change in stem cell concentration during clone growth.

By analysing quantitatively several properties of growing clones, it has been possible to estimate P_s at various stages of clone growth (David & MacWilliams 1978). For example, the size variability between clones is generated primarily when clones are small and subject to statistical fluctuations in the number of stem cells which self-renew. By comparison, the ratio of differentiated cells to stem cells in older clones is a measure of P_s in late clone growth at the time the differentiated cells left the stem cell pool. Using this approach P_s was shown to decrease from 0.8 during the first five generations of clone growth to 0.6 by 10 generations of growth.

d. A model for the control of P_s

An interesting feature of the growth studies in NM aggregates is that P_s is lower in a single large clone than when the same number of stem cells are spread out randomly in a NM aggregate (Fig. 2.2). This result suggests that the *local* stem cell concentration is what controls P_s and that the spatial range of the interaction between stem cells is short compared to the dimensions of NM aggregates (Sproull & David 1979). The interaction does not appear to be mediated by cell contact since individual stem cells do not appear to touch each other in clones or in whole animals. Rather the results support the idea that stem cell interactions which affect P_s are mediated by a short-range diffusible molecule.

The results above can be summarised in the form of a model in which P_s is controlled by negative feedback from neighbouring stem cells (David & MacWilliams 1978, Sproull & David 1979). The model is based on three postulates: stem cells secrete a diffusible factor, stem cells sense the ambient concentration of the factor, and factor concentration controls the value of P_s . In the model the value of P_s in any given stem cell is set by the ambient concentration of the factor which, in turn, depends on the number and proximity of neighbouring stem cells. In the case of isolated stem cells with few or no stem cell neighbours, the factor concentration would be low and P_s would be high. Conversely, for a cell surrounded by numerous neighbouring stem cells, the factor concentration would be high and P_s correspondingly lower.

e. Other factors affecting P_s

The negative feedback model specifically predicts that the concentration of stem cells should be uniform in hydra tissue and that all available ectodermal space

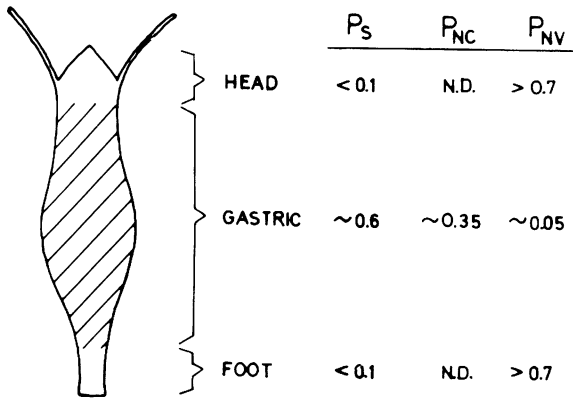


Fig 2.3 Localization of self-renewal, nerve and nematocyte commitment in different regions of hydra. The results are expressed as the fraction of stem cell daughters per cell generation which enter the self-renewal pathway (P_s), the nematocyte pathway (P_{nc}) and the nerve pathway (P_{nv}). The shaded area in the hydra represents the region of high stem cell concentration in the body column; the head and foot regions contain lower concentrations of stem cells. There is quite a sharp boundary between the regions of low and high stem cell concentration. (Summarised from the results of Bode et al 1973, David & Gierer 1974, Yaross & Bode 1978a, David & Plotnick 1980, Venugopal & David 1981a).

should be filled with stem cells. Irregularities in stem cell concentration or empty areas would tend to be evened out by local changes in P_s . Stem cells do appear to be homogeneously distributed throughout most of the hydra body column in agreement with the model's prediction. However, the concentration of stem cells in head and foot tissue is 20-fold lower than in gastric tissue (David & Plotnick 1980). This stem cell depletion can not be explained by the negative feedback model and thus it is necessary to postulate that a second factor (or factors) localised in head and foot tissue causes a decrease in P_s in these regions. As discussed below, factors which control nerve differentiation are localised in head and foot tissue and nerve commitment is very high in these regions compared to gastric tissue (Fig. 2.3; Yaross & Bode 1978a, Venugopal & David 1981a). Thus, the simplest hypothesis is to assume that nerve commitment competes with self-renewal for the same stem cell population and that, in head and foot tissue, this competition effectively removes stem cells from the self-renewal pathway. The persistence of low levels of stem cells in head and foot tissue is due to continuous slow movement of tissue containing stem cells from the gastric region into these regions (Campbell 1967b).

CONTROL OF DIFFERENTIATION

a. Localisation of nerve and nematocyte differentiation in the body column

A striking feature of nerve and nematocyte differentiation in hydra is its localisation in different regions of the body column. This localisation is shown schematically in Figure 2.3. The localisation of nematocyte differentiation can be directly observed from the distribution of intermediates in the nematocyte pathway identified either in macerations of different body regions (Bode et al 1973) or by staining with thiolacetic acid and lead nitrate (David & Challoner 1974). The latter technique is particularly useful since it stains differentiating nests (Fig. 2.1) during

a brief period just prior to their dissolution into single mature nematocytes. Since no other tissue in hydra stains with the reagent, whole mounts can be examined and the *in vivo* distribution of nests directly visualised. Differentiating nests are distributed homogeneously throughout the gastric region but are absent from head and foot regions. Thus, there appear to be two environments in hydra: head and foot tissue which do not support nematocyte differentiation and gastric tissue which supports extensive nematocyte differentiation.

In contrast to nematocyte differentiation, nerve differentiation occurs throughout the body column (David & Gierer 1974, Yaross & Bode 1978a, Venugopal & David 1981a). In particular there is extensive nerve differentiation in head and foot regions despite the low concentration of stem cell in these regions (David & Plotnick 1980). Such a distribution could be due to migration of committed nerve precursors from the stem cell population in the gastric region or to a higher proportion of stem cells entering the nerve pathway (P_{nv}) in head and foot regions compared to the gastric region. These possibilities were distinguished by comparing the distribution of ^3H -thymidine labelled nerve precursors with the distribution of newly differentiated nerves in whole hydra and in hydra which had been cut into head, gastric, and foot sections immediately after labelling (Venugopal & David 1981a). Since the distribution of labelled nerves was the same in both whole hydra and sectioned hydra there can be no redistribution of nerve precursors between S phase and terminal differentiation. As discussed below nerve commitment occurs in S phase, and thus it is clear that the high level of nerve differentiation in head and foot tissue is due to the high proportion of stem cells undergoing nerve commitment in these regions. From the ratio of labelled nerves to stem cells one can estimate that $P_{nv} > 0.7$ in head and foot tissue and $P_{nv} < 0.05$ in gastric region (Fig. 2.3).

b. The pattern of nerve commitment is controlled by morphogenetic signals

Morphogenesis in hydra is controlled by signals which are distributed in a pattern along the body column. The same or very similar signals also appear to be involved in controlling head and foot regeneration and budding. Much has been learned about these morphogenetic signals from studies involving regeneration of head and foot tissue and transplantation of small pieces of tissue from one position to another in the body column. This work has been extensively reviewed elsewhere (MacWilliams et al 1970, Wolpert et al 1971, Gierer 1977, Webster 1971, MacWilliams 1982a,b) and only one aspect of these studies will be discussed here, namely the ability of small pieces of tissue to induce head or foot structures when transplanted to the gastric region of a host animal. We refer to this property as 'head (or foot) activation': high levels of activation are associated with transplants which can induce; low levels with transplants which cannot induce. Some high levels of activation are closely correlated with high levels of nerve commitment it seems likely that signals controlling 'activation' also control nerve commitment.

Head and foot tissue express high levels of activation; gastric tissue expresses a low level of activation. As discussed above this pattern is very similar to the pattern of nerve commitment along the body column (Yaross & Bode 1978a, Venugopal & David 1981a). When a head (or foot) is cut off, gastric tissue at the cut surface develops a high level of head (or foot) activation within a few hours after

cutting; tissue elsewhere in the regenerate does not change its state of activation. Coincident with the increase in activation at the cut surface, there is an increase in nerve commitment which is, like the change in activation, confined to the cut surface (Yaross & Bode 1978b, Venugopal & David 1981b, Yaross et al 1982).

During bud formation there is also a strong correlation between nerve commitment and morphogenesis. Buds form by local evagination of tissue on one side of the lower gastric region. Prior to budding the tissue of the prospective bud is the same as tissue elsewhere in the gastric region. However, at the time of bud formation this tissue becomes 'activated' so that, when transplanted, it will induce bud formation (Li & Yao 1945, Berking 1979a). Coincident with this activation there is a wave of nerve commitment in stem cells localised at the site of the prospective bud (Bode et al 1973, Berking 1980).

c. Timing of commitment in the nerve and nematocyte pathways

Nerve commitment

Differentiation of nerve cells is preceded by a decision to enter the nerve pathway which is the result of an interaction between uncommitted stem cells and morphogenetic signals. We refer to this decision as nerve commitment. Recently, three groups have investigated the timing of this decision process. Although each group used different techniques, all the results indicate that commitment occurs in S phase of the stem cell precursor.

Venugopal & David (1981b,c) and Yaross et al (1982) used head regeneration as a stimulus to induce extensive nerve commitment. Committed nerve precursors were assayed by their ability to continue nerve differentiation when transplanted to NM aggregates or to continue differentiation when a small piece of tissue from the regenerating tip was isolated. In both cases cells were 'transplanted' by these procedures into an environment which consisted primarily of gastric tissue and which was shown to contain low levels of signals stimulating nerve commitment. Cells which continued nerve differentiation under such conditions were defined as 'committed' since their differentiation had become independent of environmental signals.

When these tests were applied to tissue regenerating a head, committed nerve precursors began to appear within 2–3 h after the onset of regeneration (Fig. 2.4; Yaross et al 1982, Venugopal & David 1981b). Newly differentiated nerves began to appear in the same tissue after about 14 h and thus nerve commitment precedes nerve differentiation by about 12 h. Using standard cell cycle techniques, the G1/S and S/G2 boundaries of the precursor population undergoing nerve commitment were determined (Venugopal & David 1981c). These boundaries occur 18 and 9 h, respectively, before terminal nerve differentiation and thus nerve commitment, which occurs 12 h prior to nerve differentiation, must occur in the S phase (Fig. 2.4).

Berking (1979b) used a quite different approach to localise nerve commitment. He started from the observation that feeding causes a brief burst of nerve differentiation throughout hydra tissue. Using ^3H -thymidine labelling he showed that the precursor population responding to this feeding stimulus was in mid-S phase. Since he was able to argue that the stimulus causing nerve differentiation occurred

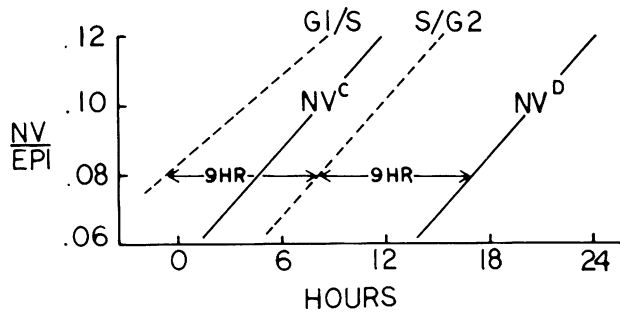


Fig. 2.4 Kinetics of appearance of committed nerve cells (NV^c) and differentiated nerve cells (NV^d) at the site of head regeneration. The number of nerve cells is expressed as the ratio of nerve cells to epithelial cells (NV/EPI) in regenerating tissue. Dotted lines show the G1/S and S/G2 boundaries of the precursor population which is committed to nerve cell differentiation. (Based on the results of Venugopal & David 1981c).

close to the time of feeding, he concluded that commitment occurred in mid-S phase. In additional experiments, Berking showed that an inhibitor of head morphogenesis, which has been partially purified from hydra tissue (Berking 1977), blocked nerve differentiation when added 30 min after feeding but not when added 3 h later. The simplest interpretation of this result is that the inhibitor blocks the process of nerve commitment but that, once committed, nerve precursors become independent of exogenous signals such as the inhibitor. Since Berking's results indicated that such cells were just past the middle of S phase, his assignment of commitment to mid-S phase is in good agreement with the results above, which were based on the ability of committed nerve precursors to continue differentiation in the absence of signals causing commitment.

Nematocyte commitment

The nematocyte differentiation pathway is longer and more complex than the nerve pathway: stem cells first proliferate nematoblast nests and then all cells in each nest differentiate into one type of nematocyte (Fig. 2.1). In view of this complexity it seems likely that there are stages to nematocyte commitment, e.g. commitment to proliferation of nests and then commitment to a specific type of nematocyte differentiation. Fujisawa & David (1981, 1982) have recently investigated stenotele commitment using a transplantation approach analogous to that described above for analysing nerve commitment (Venugopal & David 1981b). They took advantage of the fact that stenotele differentiation occurs primarily in proximal regions of the body column and less frequently in distal regions (Bode & Smith 1977). When distal portions of the body column are isolated, they regenerate normal hydra and, as a result, stenotele differentiation is induced in tissue which previously supported very little stenotele differentiation. Comparison of the kinetics of stenotele induction during regeneration with the known differentiation pathway (Fig. 2.1; David & Gierer 1974) indicated that the cells which responded to the regeneration stimulus were in the final cell cycle preceding overt cytodifferentiation. Thus proliferating nests, although committed to the nematocyte pathway, are not committed to a specific type of nematocyte differentiation. Nematocyte commitment, therefore, must consist of at least two steps, one of

which occurs long after precursors have left the stem cell pool. The nature of the signals controlling these commitment events is still unknown.

d. Molecular signals which affect nerve commitment

Several factors affecting morphogenesis have been identified in recent years. The best characterised of these factors is a peptide hormone referred to as the head activator (Schaller 1973, Schaller & Gierer 1973) which stimulates head regeneration and budding and thus appears to be involved in morphogenesis and differentiation of head structures (Schaller 1975). The head activator is an undecapeptide whose structure has recently been determined (Schaller & Bodenmüller 1981). An inhibitor of head and bud morphogenesis has also been identified and partially purified (Berking 1977, Schaller et al 1979). It has a molecular weight of ~ 500 and is not a peptide. A foot activator and foot inhibitor have also been identified but not yet extensively characterised (Schmidt & Schaller 1976, Grimelikhuijzen & Schaller 1977).

Several laboratories have investigated the effects of these factors on nerve commitment (Schaller 1976b, Berking 1979b, Venugopal & David, in preparation). Figure 2.5 shows the results of one such experiment in which hydra were treated with a crude extract of hydra tissue or with purified head activator. The results indicate that treatment with either crude extract or head activator causes commitment of large numbers of nerve precursors which then differentiate in the period following treatment. Untreated hydra exhibit a low rate of nerve commitment typical of gastric tissue (David & Gierer 1974). From the rate of increase in nerve cells in treated hydra it is possible to calculate that most stem cells which

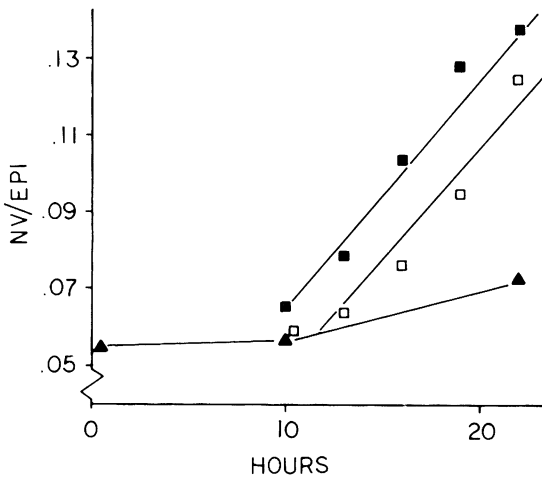


Fig. 2.5 Nerve cell differentiation induced by treatment of whole hydra with a crude extract of hydra tissue (■) or with purified head activator (□). Hydra were treated for 10 h and then sections of the body column were excised and incubated in fresh medium to allow differentiation of committed nerve precursors. At the times shown, samples were macerated and the number of nerves determined. The number of nerve cells is expressed as a ratio of nerve cells to epithelial cells (NV/EPI) in each sample. Crude extract was prepared by sonicating hydra and diluting the extract to 0.25 O.D.₂₈₀; head activator was used at 15 BU/10 ml (Schaller 1973). Control animals (▲) were incubated in culture medium from 0–10 h and then processed in parallel with experimental animals. (Venugopal & David, unpublished results).

pass through S phase during the treatment are committed to nerve cell differentiation. Thus, stem cells which normally enter the self-renewal and nematocyte pathways are committed to the nerve pathway by the treatment.

The most striking feature of the results in Figure 2.5 is that crude extract and head activator cause similar increases in nerve cells but with slightly different kinetics. From the timing of the increase it is possible to conclude that cells in mid-S phase respond to crude extract whereas cells entering S phase respond to head activator. This has also been confirmed by independent ^3H -thymidine labelling experiments: when hydra were labelled 1 h before treatment with crude extract or head activator, the former caused the appearance of labelled nerve cells, the latter did not (Venugopal & David, in preparation).

The results in Figure 2.5 indicate that stem cells in early and mid-S phase differ in their sensitivity to factors causing nerve commitment. Cells in mid-S undergo commitment when stimulated by crude extract but not when stimulated by head activator; cells in early S respond to head activator. The simplest interpretation of these observations is that a second factor in crude extract modifies the sensitivity of cells in the first half of S phase such that they can respond to head activator. This has been directly demonstrated by treating hydra first with a crude extract lacking head activator and then with purified head activator. Under these conditions stem cells in the first half of S phase undergo nerve commitment in response to head activator (Venugopal & David, in preparation).

Factors which inhibit nerve commitment have also been identified. Berking (1979b) has shown that a partially purified factor (or factors), which inhibit(s) head regeneration and budding in hydra (Berking 1977), also inhibit(s) nerve cell commitment when applied at any time during the first half of S phase of the stem cell precursor. Inhibitor applied after the middle of S phase has no effect; committed nerves continue differentiation.

The results above on the stimulation and inhibition of nerve commitment begin to outline several features of commitment. First, commitment is a process which extends over a finite period of time, in this case the first half of S phase. During this period stem cells appear to interact with competing signals in the environment before becoming committed to a particular pathway. Second, the commitment process appears to involve a progressive decrease in sensitivity to exogenous signals. For example, stem cells in early S phase respond to head activator; stem cells in mid-S phase do not (Fig. 2.5). Third, the commitment process, once started, is not irreversible since inhibitory factors can still block commitment as late as mid-S phase. Such factors even appear to restore the original naive state characteristic of cells at the beginning of S phase.

CONTROL OF THE DIFFERENTIATION OF COMMITTED NERVE AND NEMATOCYTE PRECURSORS

In addition to controlling the rate of commitment, there is evidence that hydra control the differentiation of both committed nerves and nematocytes. In the nerve pathway there appears to be a differentiation 'block' in late G_2 of the precursor (Schaller 1976a,b). In normal intact hydra committed precursors pause at the 'block' for a period of hours before completing nerve differentiation. However,

during regeneration or simply in isolated pieces of tissue, the 'block' is removed such that the differentiation time following ^3H -thymidine labelling is only 9–12 h (Yaross et al 1982, Venugopal & David 1981c) compared to 18 h in intact animals (David & Gierer 1974, Berking 1979b). It seems likely that the 'block' is localised in late G_2 since conditions, such as treatment with head activator, which accelerate cells into mitosis and thus shorten G_2 (Schaller 1976a) also accelerate the appearance of labelled nerves following ^3H -thymidine labelling (Schaller 1976b). In addition to regeneration and treatment with head activator, there is also evidence that the budding process can regulate the timing of differentiation since terminal nerve differentiation occurs at a precise time during bud morphogenesis rather than a fixed time after commitment (Berking 1980).

In the nematocyte pathway, there is evidence that the survival of differentiating stenoteles and desmonemes is regulated. In well-fed intact hydra essentially all committed stenoteles and desmonemes appear to complete differentiation (David & Gierer 1974). However, during regeneration many of these cells are killed (Yaross & Bode 1978c) and thus the flow of differentiated nematocytes is sharply reduced. The inactivation appears to be selective (Fujisawa & David, unpublished results); committed and differentiating cells are killed whereas nests of proliferating interstitial cells, which are not yet committed to a specific nematocyte pathway (Fujisawa & David 1981, 1982), are not affected. At present it is not clear whether the effect is due to absence of a hormone required for stenotele and desmoneme differentiation or due to release of a poison by regenerating tissue which selectively inactivates differentiating cells.

SUMMARY AND CONCLUSIONS

Hydra contain a population of multipotent stem cells which continuously give rise to differentiated nerve cells and nematocytes. Large well-fed animals contain about 3500 stem cells with a cell cycle time of 24 h. In each stem cell generation about 60% of daughter cells remain stem cells, 30% initiate nematocyte differentiation and 10% initiate nerve differentiation. Under these conditions the stem cell population increases in size about 20% per day at the same rate as tissue growth.

The growth rate of the stem cell population is regulated by changes in the self-renewal probability (P_s) rather than changes in the stem cell generation time. Several experiments now indicate that the value of P_s is controlled by the concentration of stem cells in tissue: high stem cell concentrations decrease P_s ; low concentrations increase P_s . This negative feedback control loop gives rise to a uniform distribution of stem cells throughout the body column and leads to homeostatic maintenance of the stem cell population at a particular concentration.

Stem cell differentiation to nerve cells and nematocytes is localised in different regions of the body column and appears to be under the control of morphogenetic signals which regulate other features of hydra morphogenesis. One such signal, a neuropeptide which is involved in head morphogenesis, has been shown to cause extensive nerve cell commitment. Only stem cells at the beginning of S phase respond to the factor; stem cells later in the cell cycle are committed to self-renewal or a differentiation pathway and are unable to respond. There is, however, evidence that the sensitivity of stem cells to the peptide can be altered during the

first half of the S phase by a second factor present in hydra extracts. Thus it seems likely that the process by which stem cells decide their fate requires a period of a few hours during which stem cells sample their environment for the presence of factors which regulate stem cell behaviour.

In addition to factors controlling stem cell commitment to the nerve and nematocyte pathways, there is evidence that the organism regulates the rate of nerve and nematocyte differentiation by controlling the time required to differentiate and the survival of differentiation intermediates.

REFERENCES

- Berking S 1977 Bud formation in hydra: inhibition by an endogeneous morphogen. *Wilhelm Roux's Archives* 181: 215.
- Berking S 1979a Analysis of head and foot formation in hydra by means of an endogenous inhibitor. *Wilhelm Roux's Archives* 186: 189
- Berking S 1979b Control of nerve cell formation from multipotent stem cells in hydra. *Journal of Cell Science* 40: 193
- Berking S 1980 Commitment of stem cells to nerve cells and migration of nerve cell precursors in preparatory bud development in hydra. *Journal of Embryology and Experimental Morphology* 60: 373
- Bode H R, David C N 1978 Regulation of a multipotent stem cell, the interstitial cell of hydra. *Progress in Biophysics and Molecular Biology* 33: 189
- Bode H R, Flick K M 1976 Distribution and dynamics of nematocyte populations in *Hydra attenuata*. *Journal of Cell Science* 21: 15
- Bode H R, Smith G S 1977 Regulation of interstitial cell differentiation in *Hydra attenuata*. II. Correlation of the axial position of the interstitial cell with nematocyte differentiation. *Wilhelm Roux's Archives* 181: 203
- Bode H R, Flick K M, Bode P M 1977 Constraints on the relative sizes of the cell populations in *Hydra attenuata*. *Journal of Cell Science* 24: 31
- Bode H R, Flick K M, Smith G S 1976 Regulation of interstitial cell differentiation in *Hydra attenuata*. I. Homeostatic control of interstitial cell population size. *Journal of Cell Science* 20: 29
- Bode H R, 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's Archives* 171: 269
- Campbell R D 1967a Tissue dynamics of steady-state growth in *Hydra littoralis*. I. Patterns of cell division. *Developmental Biology* 15: 487
- Campbell R D 1967b Tissue dynamics of steady-state growth in *Hydra littoralis*. II. Patterns of tissue movement. *Journal of Morphology* 121: 19
- Campbell R D, David C N 1974 Cell cycle kinetics and development in *Hydra attenuata*. II. Interstitial cells. *Journal of Cell Science* 16: 344
- David C N 1973 A quantitative method for maceration of hydra tissue. *Wilhelm Roux's Archives* 171: 259
- David C N, Campbell R 1972 Cell cycle kinetics and development of *Hydra attenuata*. I. Epithelial cells. *Journal of Cell Science* 11: 557
- David C N, Challoner D 1974 Distribution of interstitial cells and differentiating nematocytes in nests in *Hydra attenuata*. *American Zoologist* 14: 537
- David C N, Gierer A 1974 Cell cycle kinetics and development of *Hydra attenuata*. III. Nerve and nematocyte differentiation. *Journal of Cell Science* 16: 359
- David C N, MacWilliams H K 1978 Regulation of the self-renewal probability in hydra stem cell clones. *Proceedings of the National Academy of Sciences* 75: 886
- David C N, Murphy S 1977 Characterization of interstitial stem cells in hydra by cloning. *Developmental Biology* 58: 372
- David C N, Plotnick I 1980 Distribution of interstitial stem cells in hydra. *Developmental Biology* 76: 175
- Davis L E 1974 Ultrastructure studies of the development of nerves in hydra. *American Zoologist* 14: 551
- 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. *Journal of Experimental Zoology* 155: 253
- Fujisawa T, David C N 1981 Commitment during nematocyte differentiation in hydra. *Journal of Cell Science* 48: 207

- Fujisawa T, David C N 1982 Commitment during stenotele differentiation in hydra is localized near the S/G₂ boundary in the terminal cell cycle. *Developmental Biology*, in press
- Gierer A 1977 Biological features and physical concepts of pattern formation exemplified by hydra. *Current Topics in Developmental Biology* 11: 17
- Gierer A, Berking A, Bode H, David C N, Flick H, Hansmann G, Schaller C, Trenkner E 1972 Regeneration of hydra from reaggregated cells. *Nature New Biology* 239: 98
- Grimmelikhuijzen C J P, Schaller H C 1977 Isolation of a substance activating foot formation in hydra. *Cell Differentiation* 6: 297
- Lehn H 1951 Teilungsfolgen und determination von I-Zellen für die Cnidenbildung bei hydra. *Zeitschrift für Naturforschung* 66: 388
- Li H P, Yao T 1945 Studies of the organizer problem in *Pelmatohydra oligactis*. III. Bud induction by developing hypostome. *Journal of Experimental Biology* 21: 155
- MacWilliams H K 1982a Hydra transplantation phenomena and the mechanism of hydra head regeneration. I. Properties of the host. *Developmental Biology*, in press
- MacWilliams H K 1982b Hydra transplantation phenomena and the mechanism of hydra head regeneration. II. Properties of the transplant donor. *Developmental Biology*, in press
- MacWilliams H K, Kafatos F C, Bossert W H 1970 The feedback inhibition of basal disk regeneration in hydra has a continuously variable intensity. *Developmental Biology* 23: 380
- Rich F, Tardent P 1969 Untersuchung zur Nematocyten-Differenzierung bei *Hydra attenuata*. *Revue suisse de Zoologie* 76: 779
- Schaller H C 1973 Isolation and characterization of a low molecular weight substance activating head and bud formation in hydra. *Journal of Embryology and Experimental Morphology* 29: 27
- Schaller H C 1975 Head activator controls head formation in reaggregated cells of hydra. *Cell Differentiation* 4: 265
- Schaller H C 1976a Action of the head activator as a growth hormone in hydra. *Cell Differentiation* 5: 1
- Schaller H C 1976b Action of the head activator on the determination of interstitial cells in hydra. *Cell Differentiation* 5: 13
- Schaller H C, Bodenmüller H 1981 Isolation and amino acid sequence of a morphogenetic peptide from hydra. *Proceedings of the National Academy of Sciences* 78: 7000
- Schaller H C, Gierer A 1973 Distribution of the head activating substance in hydra and its localization in membranous particles in nerve cells. *Journal of Embryology and Experimental Morphology* 29: 39
- Schaller H C, Schmidt T, Grimmelikhuijzen C J P 1979 Separation and specificity of action of four morphogens from hydra. *Wilhelm Roux's Archives* 186: 139
- Schmidt T, Schaller H C 1976 Evidence for a foot-inhibiting substance in hydra. *Cell Differentiation* 5: 151
- Slautterback D B, Fawcett D W 1959 The development of cnidoblasts of hydra. An electron microscope study of cell differentiation. *Journal of Biophysical and Biochemical Cytology* 5: 441
- Sproull F, David C N 1979 Stem cell growth and differentiation in *Hydra attenuata* I. Regulation of the self-renewal probability in multiclone aggregates. *Journal of Cell Science* 38: 155
- Till J E, McCulloch E A 1961 A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. *Radiation Research* 14: 213
- Venugopal G, David C N 1981a Spatial pattern of nerve differentiation in hydra is due to a pattern of nerve commitment. *Developmental Biology* 83: 366
- Venugopal G, David C N 1981b Nerve commitment in hydra. I. Role of morphogenetic signals. *Developmental Biology* 82: 353
- Venugopal G, David C N 1981c Nerve commitment in hydra II. Localization of commitment in S phase. *Developmental Biology* 83: 361
- Webster G 1971 Morphogenesis and pattern formation in hydroids. *Biological Reviews* 46: 1
- Wolpert L, Hicklin J, Hornbruch A 1971 Positional information and pattern formation in regeneration of hydra. In: *Control mechanisms of growth and differentiation. Symposium for the Society of Experimental Biology* 25: 391
- Yaross M S, Bode H R 1978a Regulation of interstitial cell differentiation in *Hydra attenuata*. III. Effects of i-cell and nerve cell densities. *Journal of Cell Science* 34: 1
- Yaross M S, Bode H R 1978b Regulation of interstitial cell differentiation in *Hydra attenuata*. IV. Nerve cell commitment in head regeneration is position-dependent. *Journal of Cell Science* 34: 27
- Yaross M S, Bode H R 1978c Regulation of interstitial cell differentiation in *Hydra attenuata*. V. Inability of regenerating head to support nematocyte differentiation. *Journal of Cell Science* 34: 39
- Yaross M S, Baca B A, Chow M H, Bode H R 1982 Commitment of hydra interstitial cells to nerve cells occurs by late S-phase. *Developmental Biology* 89: 425