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REGULATION OF A MULTIPOTENT STEM CELL, THE INTERSTITIAL CELL OF HYDRA

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CONTENTS

I. INTRODUCTION

Stem cells in adult organisms appear to retain features of their embryonic origin—the capacity for continual proliferation and differentiation. Since they are often more accessible to the experimenter than embryonic cells, they have been studied not only for their own sake, but as models for processes fundamental to embryonic development. The interstitial cell* in hydra is particularly interesting in this regard since much of its behavior in terms of differentiation is dependent on its position along the body column, which may be related to the morphogenetic fields controlling the organization of the tissue.

*In the literature the term interstitial cell has been used in different ways which has led to some confusion. In a functional sense it refers to the multipotent stem cell. In a morphological sense it has been used to describe a group of cell types. David (1973) separated this group on morphological grounds into big interstitial and small interstitial cells. The latter, which occur in nests or clusters of 8, 16 or 32 cells were known to be intermediates in nematocyte differentiation. The big interstitial cells which occur singly, in pairs or in fours included the multipotent stem cells and early precursors of nerve and nematocyte differentiation. Since then the function of interstitial cells in the various nest sizes has been further clarified (David and Gierer, 1974) and has led to the following definitions which will be used in this article. The term interstitial cell will refer to cells occurring singly or in pairs identified morphologically as big interstitial cells (David, 1973). In a functional sense these include multipotent stem cells and cells committed to either nerve or nematocyte differentiation. The term stem cells refers to the multipotent stem cells. The terms big interstitial and little interstitial cells will not be used. Instead interstitial cells in nests of 4, 8, 11 or 32 cells will be referred to as nematoblasts capable of cell division.

Interstitial cells in hydra include multipotent stem cells which continually give rise to several types of nerve cells and nematocytes in hydra tissue, and to gametes if the animal enters a sexual phase. Together with differentiating intermediate and product cells, the interstitial cell system comprises about 75% of all the cells in rapidly growing asexually reproducing animals. Stem cells alone constitute about 4% of the total hydra cells. This high density of stem cells in hydra tissue, as well as recently developed techniques for manipulating and assaying hydra cells, has permitted current research to focus on the stem cell itself.

In this review we will first describe the interstitial cell system and its dynamics, and then concentrate on the control mechanisms regulating the stem cells. Two questions are of primary importance: (1) What controls a stem cell's decision to proliferate or differentiate? and (2) What controls the specific type of cell differentiated? Recent work has begun to provide answers to both these questions and to suggest the outline of a model of stem cell control in hydra.

II. THE INTERSTITIAL CELL SYSTEM

1. *Dynamics and Composition of Hydra Tissue*

Hydra is a small (5–10 mm) freshwater coelenterate containing about 100,000 cells distributed among fifteen cell types. Its body is a tube consisting of two epithelial cell layers, ectoderm and endoderm, surrounding a gastric cavity. The mesoglea, a non-cellular basal lamina-like layer, between the cell layers, provides support. The tube is modified at the anterior end to form a head consisting of a mouth region or hypostome surrounded by 5–7 tentacles and a holdfast, termed foot or basal disk, at the other (Fig. 1). The body column is divided into a large gastric region with the budding region at its lower end, and a smaller stalk, or peduncle.

A prominent feature is that tissue growth occurs continuously and throughout the body column (Campbell, 1965, 1967b; David and Campbell, 1972; Campbell and David, 1974). In well-fed cultures the tissue mass doubles in 3–4 days. The animals increase

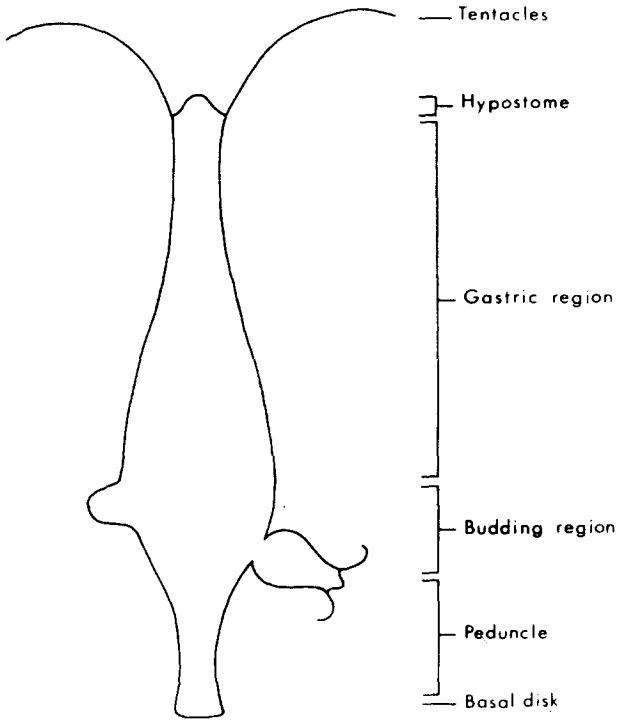

FIG. 1. Diagram of a hydra. The head region consists of the hypostome and tentacles, and the foot region is the basal disk.

in size up to a point and then the excess tissue is removed by budding off young animals which repeat the cycle. About 15% of the tissue is lost by sloughing at the extremities, the tentacle tips and basal disk. The constant proliferation of cells causes a continual displacement of the two tissue layers along the body column into buds and towards the two extremities where they differentiate 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 the epithelio-muscular cells make up the tissue layer. Lodged among these epithelial cells are the 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 the infrequent sexual phases,

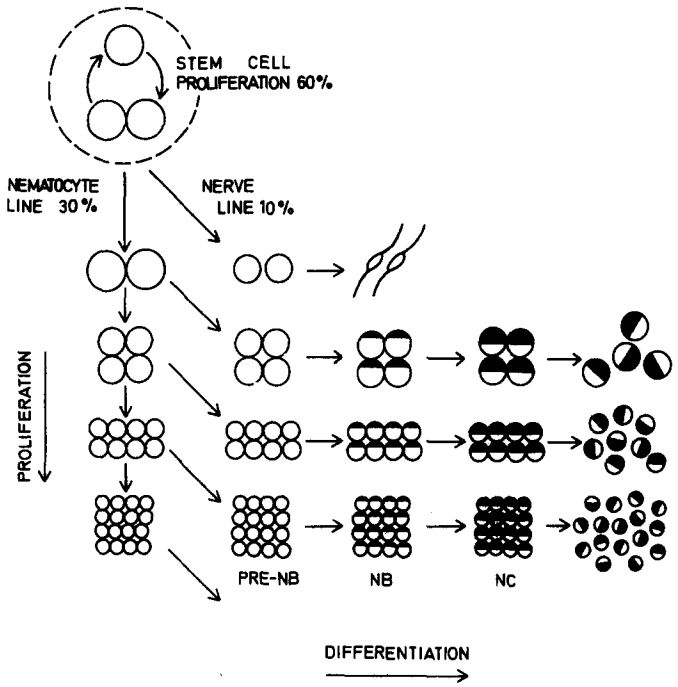

FIG. 2. Cell flow model of the proliferation and differentiation of interstitial cells in hydra. Stem cells, occurring as single interstitial cells or nests of two, proliferate continuously. In each cell generation about 60% remain stem cells, about 30% initiate proliferation of a nest of nematocyte precursors and about 10% initiate nerve differentiation. Nerve differentiation occurs directly from the stem cell pool. In the nematocyte line, after one or more cell divisions, proliferation ceases and a nest commences differentiation of nematocyst capsules. Details of the nematocyte differentiation pathway are shown in Fig. 3. For clarity, nests of 32 differentiating nematocytes have been left off the diagram. (From David and Gierer, 1974).

gamete differentiation would be part of the system. As sexual animals have not yet been analyzed quantitatively they have been excluded from the present discussion. Characterization of this system of cells has been advanced recently with a maceration technique for fixing and dissociating hydra tissue into single cells (David, 1973). Cells retain their *in vivo* morphology in macerated preparations permitting unambiguous identification of all cell types including several classes of differentiating intermediates. The power of the technique is its simplicity and the ability to precisely measure the numbers of the several cell types in a whole or a particular region of an animal. Based on morphological criteria as well as a variety of pulse-chase experiments with ^{3}H-thymidine that were analyzed with autoradiography, it has been possible to order all classes of cells

into the scheme of differentiation pathways summarized in Fig. 2. Some aspects of the system will now be considered in more detail.

2. *Nematocyte and Nerve Cell Differentiation*

A nematocyte is identified by a large complex organelle, the nematocyst, which is unique to and characteristic of the coelenterates (for reviews, see Picken and Skaer, 1967; Mariscal, 1974). Hydra typically have four types of morphologically very distinct nematocytes: stenoteles, desmonemes, atrichous isorhizas and holotrichous isorhizas (Kanaev, 1952). The great majority (95%; Bode and Flick, 1976) are mounted in the ectoderm of the tentacles, while the remainder are mounted on the surface of the body column. The stenoteles and desmonemes are used to capture prey, while the atrichous isorhizas attach the tentacles to the substrate during the animal's summersaulting form of locomotion (Ewer, 1947). Under the appropriate mechanical and chemical stimulus, the nematocyst explosively discharges everting the contents of the capsule, typically a thread. For example, discharge of a desmoneme results in the eversion of a thread that wraps itself around any nearby hair or spine protruding from the unwary prey that provided the stimulus and thereby caused its own capture.

Although the mature nematocytes are mostly in the tentacles, their differentiation occurs exclusively in the body column. The differentiation pathway from stem cell to nematocyte, which requires 5–7 days is shown in Fig. 3. A stem cell committed to this pathway first undergoes a number of cell divisions, typically 3–4 (Lehn, 1951; Rich and Tardent, 1969). Each round of cell division requires 16–18 hr consisting of a 12 hr S-phase followed by a 3–4 hr G2 (G1 is $\leq$ 1 hr in all hydra cell types, Campbell and David, 1974). The cells remain together in a cluster or nest and are connected to one another by cytoplasmic bridges to form a syncytium (Slautterback and Fawcett, 1959). Subsequently, all the cells of a nest undergo synchronous differentiation into one type of nematocyte (Lehn, 1951; Rich and Tardent, 1969). Differentiation of the nematocyst capsule starts with the appearance of a small vacuole in the cytoplasm of each cell in a nest about 6 hr after the last mitosis (10–12 hr after the end of the last S-phase). Depending on nematocyte type, the vacuole grows in size for 36–50 hr (David and Gierer, 1974). Thereafter, in a rapid series of events, a stiff collagenous shell is laid down in the vacuole (Lenhoff *et al.*, 1957), the shell is filled, and the contents develop into recognizable structures. The nematocyst capsule, now filling most of the cell, is cytologically complete (see Slautterback and Fawcett, 1959, for details of the pathway). Thereafter the nematocytes of the nest disperse and most migrate through the ectodermal tissue into the tentacles (Campbell, 1967c) to be mounted in the battery cells, while the remainder are mounted among the epithelial cells of the ectoderm.

Nematocytes represent 20–25% of the total cells of a hydra (Bode *et al.*, 1973). Since approximately a quarter of them are sloughed daily at the tentacle tips and are replaced

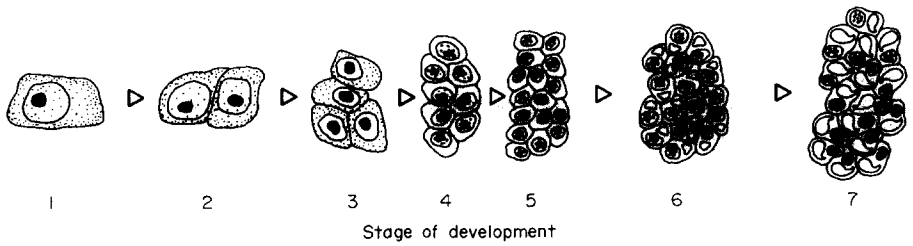

FIG. 3. Nematocyte differentiation pathway. A stem cell (stage 1 or 2) committed to nematocyte differentiation undergoes a number of cell divisions (stages 3–5). Then the cells of the nest undergo synchronous differentiation elaborating a vacuole (stage 6) which develops into a mature nematocyst (stage 7). (Modified from David and Gierer, 1974.)

(David and Gierer, 1974; Bode and Flick, 1976), the production of the four types of nematocytes represents a major synthetic activity of the animal. This is reflected in the large fraction, 50%, of the total cells of the tissue in the body column that are nests of dividing or differentiating nematocyte intermediates (Bode *et al.*, 1973).

In contrast to nematocyte differentiation, nerve cell formation is a simpler and much shorter process. Differentiation of nerve cells probably occurs directly from the stem cell compartment (Fig. 2), as there is no evidence for the involvement of nests. On the contrary, regions of almost exclusive nerve cell differentiation such as the hypostome and the basal disk, contain primarily single interstitial cells (David and Challoner, 1974; Davis, 1974). Following a ^{3}H-thymidine pulse, labelled nerve cells begin to appear at 18 hr (Fig. 2; David and Gierer, 1974). Since nerve cells, like nematocytes have a $2n$ postmitotic DNA content, this 18 hr period includes G2 of the precursor stem cell (probably 12 hr) as well as a period of postmitotic differentiation which may be as short as 6 hr.

In contrast to nematocyte production, nerve cell formation is on a smaller scale. Nerves represent only 4–5% of all cells and turnover less rapidly (David and Gierer, 1974).

3. *Dynamics of the Interstitial Cell System*

Differentiation of nerve cells and nematocytes occurs continuously in hydra from the population of interstitial cells. This population in turn maintains itself by proliferation. To measure the cell flow in the interstitial cell system, David and Gierer (1974) measured the rates of nerve cell and nematocyte differentiation by labelling hydra with ^{3}H-thymidine and following the kinetics of appearance of label in these populations. The observed rates, 900 nerve cells per day and 9000 nematocytes per day, were converted to cells leaving the stem cell pool by correcting for the number of differentiated cells produced per stem cell initiating differentiation (Fig. 2) and for growth of the system during the time course of differentiation, which in the case of nematocyte differentiation is almost 6 days or two tissue doublings. After making these corrections, the flow of stem cells to the nematocyte line was observed to be 1750 cells/day and to the nerve line 550 cells/day.

Interstitial cells have a cell cycle of 24–27 hr (Campbell and David, 1974). Since only a fraction of the daughter cells remain stem cells, the doubling time of the stem cell population is longer than the stem cell cycle. For rapidly growing hydra, the doubling time of the interstitial cell population is 3 days (David and Campbell, 1972) which leads to an estimate of the self-renewal probability (Ps) of 0.6 under these conditions, i.e. 60% of the stem cells will divide in each cell cycle.

The minimum number of stem cells in the interstitial cell system can be calculated from the self-renewal probability and the measured number of stem cells initiating differentiation. Under conditions where 40% of stem cells in each generation initiate differentiation ($Ps = 0.6$), a stem cell population of 3500 cells is required to support the commitment of 2300 stem cells to differentiation per day. This minimum estimate is roughly comparable to the size of the stem cell population *in vivo* (David and Gierer, 1974).

It should be emphasized here that the cell flow analysis presented above characterizes the dynamics of the *total* interstitial cell population. As we will discuss in detail below, there are marked deviations from this average behavior in individual regions of the body column.

4. *Multipotency of the Stem Cell*

Until recently evidence for the multipotency of the stem cell was indirect. It was based, primarily, on histological observation (Brien, 1953) and on experiments in which the differentiation of the interstitial cell population was altered from primarily one pathway to another (Brien, 1961; Burnett and Diehl, 1964). In an effort to obtain direct

evidence on this question, and in general, to examine stem cell behavior more directly, David and Murphy (1977) have developed a procedure for cloning interstitial cells.

The technique is analogous to the method of cloning hemopoietic stem cells in lethally irradiated mice (Till and McCulloch, 1961). Host tissue for cloning is prepared by treating hydra with nitrogen mustard (NM) to inactivate proliferating cells. Although such animals die after 3–4 weeks, they have proved to be suitable hosts for cloning added live cells during this period. Furthermore, since NM-treatment causes the rapid elimination of interstitial cells from host tissue, clones of interstitial cells derived from added live cells are easily visualized.

Live cells for cloning were introduced into NM-treated host tissue using a technique for reaggregating dissociated hydra cells (Gierer *et al.*, 1972). Reaggregates prepared with normal hydra cells regenerate normal hydra; reaggregates of NM-treated cells also regenerate morphologically normal hydra structures which, like NM treated hydra, die after 3–4 weeks. Interstitial cell clones were prepared by coaggregating 100–200 live cells with about 10^5 NM cells. After 1–2 weeks of growth, clones of interstitial cells could be easily scored in host tissue stained with toluidine blue. About 1–2% of the input live cells are capable of forming interstitial cell clones. This compares with *in vivo* estimates of 3.5–4.0% stem cells ($3500/10^5$ cells in a standard animal) and suggests that the cloning efficiency is quite high.

Evidence that stem cells are indeed "multipotent" has been obtained by scoring the occurrence of differentiated cells in clones derived from single stem cells (David and Murphy, 1977). To distinguish clone-derived cells from residual host cells, which are incapable of DNA synthesis, clones were labelled with ^{3}H-thymidine. Nine clones were examined by the procedure and all were found to contain both differentiated nerve cells and nematocytes, thus clearly demonstrating that stem cells are multipotent. In this experiment there was no evidence for sub-populations of determined stem cells capable of extensive proliferation but giving rise to only one type of differentiated product.

5. *Regulation of Stem Cell Behavior*

The cell flow model presented above provides a quantitative description of the daily behavior of the interstitial cell population in animals reproducing asexually: 60% divide and the remaining 40% differentiate. Of the latter, 10% are committed to nerve cell formation and 30% to nematocytes, which in turn are divided among the four types in fixed ratios (David and Gierer, 1974; Bode and Flick, 1976). Although this summarizes the behavior of the interstitial cell system as a whole, it masks the complexity of the system. It does not consider major differences in stem cell proliferation and differentiation associated with specific regions of hydra, nor does it consider differential loss of cell types associated with tissue movements (e.g. budding). To achieve this complex behavior it is likely that the stem cell population is subject to a network of controls based on cell–cell interactions.

In the following we will discuss a variety of experiments which outline some of the elements of control. These have been ordered to focus on two questions: what influences commitment of a stem cell to division instead of differentiation? and what affects commitment to a particular differentiation? The dominant themes underlying this discussion are regional or positional effects, which are particularly important for the type of differentiation, and the homeostasis of the stem cell population, which strongly affects commitment to division.

III. CONTROL OF STEM CELL DIVISION

1. *Homeostasis of the Stem Cell Population*

A consideration of the tissue dynamics suggests that the several cell populations might be subject to homeostatic controls. The two tissue layers, or phrased differently, the

cell populations of the body column, are continually expanding. Over hundreds of asexual generations the cell composition of the animal has been observed to remain constant. Variations in the relative proportion of each cell type between individuals maintained under the same conditions is less than $\pm 30\%$ (Bode *et al.*, 1977). Further, the composition remains constant in animals differing several-fold in size such as freshly detached buds and adults or in animals maintained at 25°C compared to 15°C. All of these results suggest a set of homeostastic controls for maintaining the population sizes relative to one another.

When experiments were performed to determine the extent of these controls it was found that they were less restrictive than originally expected. Conditions were found whereby some populations changed in size and others did not. Animals maintained on different feeding regimes ranging from 1 to 20 shrimp larvae per day reached different steady state sets of ratios of population sizes (Bode *et al.*, 1977). The epithelial cell populations increased in size with each increase in level of feeding. However, the gland, mucous and nerve cell populations remained about the same size. Hence, their ratios with respect to the epithelial cell population decreased with each higher level of food intake. The nematocyte to epithelial cell ratio went up at each level from 1 to 6 shrimp larvae per day, but then declined as the feeding level increased further. Thus, there are a number of steady states of cell population sizes or ratios in which hydra can grow and reproduce normally. These results suggest that any controls that may be governing the size of some cell populations are not so stringent as to rigidly fix them to one set of values. Instead the population sizes or ratios of population sizes can vary over a limited range.

One exception to this is the relationship between the interstitial and epithelial cells, which is significant since they are the primary proliferating cell types and the source of most of the differentiated cells. In the above experiments the ratio of the interstitial to epithelio-muscular cells remained constant despite a several fold increase in the epithelio-muscular cell population size over the range of feeding regimes. They also remain tightly coupled under a variety of other conditions which affect epithelial cell population size. This suggests a homeostatic mechanism which keeps these two cell types in register. Alternatively, both cell populations could be responding to the altered environmental conditions in the same way. However, there is evidence indicating that the interstitial cells, but not the epithelial cells, are subject to homeostatic control.

2. *Mechanism of Stem Cell Homeostasis*

In principle the epithelial cells and interstitial cells could be kept in register by exerting control on the cell cycle time of one or both cell types and/or on the fraction of stem cells undergoing self-renewal. Two lines of evidence indicate that the second mechanism is responsible for regulating the interstitial cell growth rate. Bode *et al.* (1976) reduced the interstitial cell population to 1% of normal with hydroxyurea and found that it recovered to control levels in 35 days (Fig. 4). The epithelial cell population was unaffected. They observed no changes in the cell cycle times of either the interstitial cells or the epithelial cells during the recovery process. Instead these authors found that the fraction of stem cells committed to nematocyte production was considerably reduced during the early part of the recovery period. The results suggest that the recovery of the interstitial cell population was due to an increase in the fraction committed to division at the expense of differentiation. A shift in the fraction of stem cells committed to division from 60% to 70%, or, expressed in terms of a self-renewal probability, from 0.6 to 0.7, would be sufficient to obtain the observed recovery (Bode *et al.*, 1976).

Similar increases in the self-renewal probability were observed early in the growth of clones of interstitial cells. Single clones were grown in NM host aggregates (Section II.4). During the first 3–4 generations of clone growth the self-renewal probability was found to be ~ 0.8. After 10–12 generations of clone growth the clones contained several

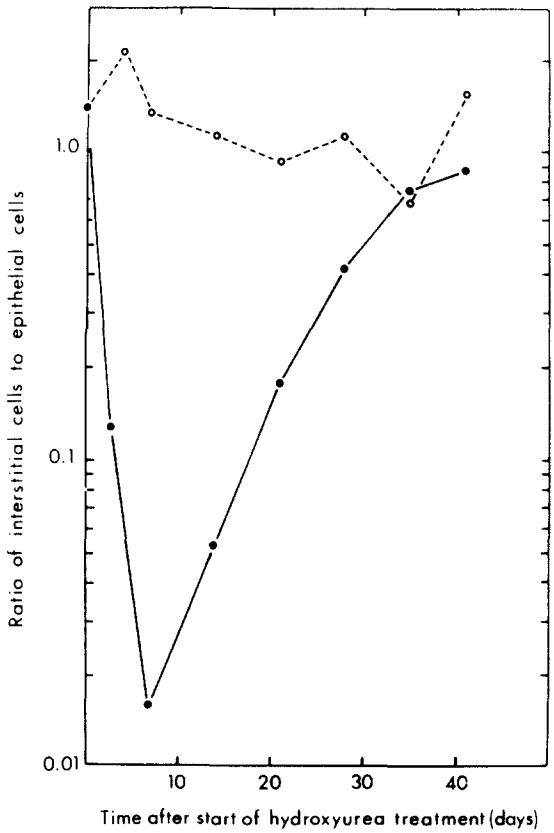

FIG. 4. Recovery of the interstitial cell: epithelial cell ratio after hydroxyurea treatment. Hydroxyurea-treated animals (●---●) and controls (○---○). (Modified from Bode *et al.*, 1976.)

hundred cells including numerous stem cells, and the self-renewal probability had dropped to ~0.6 (David and MacWilliams, unpublished results).

Decreases in the self-renewal probability have also been observed. Animals maintained on one shrimp larva/day no longer bud nor do they change appreciably in size. Cell division continued, but the cell populations remain roughly constant in size as production just balanced turnover. The epithelial cell cycle increased from 3 to 13 days, although the interstitial cell cycle time changed much less (Otto and Campbell, personal communication). Instead the self-renewal probability of the stem cells dropped from 0.6, typical of growing populations, to 0.5, which was expected for steady state populations (David, unpublished results).

All of these results are consistent with a mechanism in which the self-renewal probability varies in response to the concentration of stem cells in hydra tissue. Assuming that the mechanism is based on an interaction of the stem cells among themselves, there are two simple possibilities. Either there is a global control acting over the entire animal in which a stem cell "measures" the total stem cell population size, or the interaction occurs at a local level and the stem cell is "measuring" the density of stem cells among its immediate neighbors.

If the mechanism were a global one, then nematocyte differentiation would not be expected until the stem cell population recovered close to control levels. However, nematocyte differentiation was observed before the interstitial cell population had recovered to 10% of normal in the hydroxyurea-treated animals (Bode *et al.*, 1976). A similar result was observed in stem cell clones. For the first few days the clones contained only interstitial cells, the clone size increasing daily. Thereafter, nematocyte precursors appeared (nerves were not detectable with the method) although the overall epithelial

cell:interstitial cell ratio, or the interstitial cell population size was far below normal. The local interstitial cell density in the tissue, however, was comparable to that of normal tissue. Thus, the evidence is consistent with a short range cell–cell interaction among the stem cells which affects the self-renewal probability.

However, stem cell density cannot be the only influence on the self-renewal probability. The 1:1 interstitial cell:epithelial cell ratio is true for the body column but not for the head and foot where the ratios are about 0.09:1 (Bode *et al.*, 1973; David and Challoner, 1974). Since the cell cycle times of interstitial cells and epithelial cells in the extremities are the same as in the body column (David and Campbell, 1972; Campbell and David, 1974), there must be regional influences on the self-renewal probability in addition to the effects of stem cell density. These positional influences on stem cell behavior are explored in the following section.

IV. CONTROL OF STEM CELL DIFFERENTIATION

1. *Position-dependent Differentiation*

The most obvious feature of interstitial cell differentiation is that the types of product cells made varies from region to region along the axis of the body column. Nematocyte differentiation is easily visualized in whole mounts of hydra since the nematocyst capsules of nematoblasts in nests at a late stage of development stain specifically with lead nitrate–thioacetic acid (David and Challoner, 1974). Nests of stained nematoblasts are abundant throughout most of the body column, but rare in either the head or foot or the one tenth of the body column adjacent to these extremities. There are also differences among the four nematocyte types in terms of their axial pattern of differentiation. For example, in the gastric region nests of desmonemes occur five times as frequently as stenoteles (Fig. 5), whereas the reverse is true in the peduncle (Bode and Smith, 1977). Also, for one type of nematocyte, the stenotele, there is a gradient in nematocyst size along the body column with the high point at the upper end near the head (Rich and Tardent, 1969; Bode and Smith, 1977).

Nerve cell differentiation shows a regional distribution that is roughly the inverse of the nematocytes. Nerves are found in high densities in both head and foot, but in a much lower density in the body column (Bode *et al.*, 1973). Use of ^{3}H-thymidine labelling coupled with autoradiography indicates the nerve cell differentiation pattern parallels their distribution in the animal (Yaross, personal communication; Fig. 5). Stem cells in the two extremities differentiate almost exclusively into nerves, whereas only 10% do so in the body column (David and Gierer, 1974).

Gamete differentiation has not been as thoroughly characterized, but it is clear that differentiation is localized along the body column. When a hydra enters the sexual phase, stem cells are committed to the production of one or other type of gamete (or both in hermaphroditic species). In males, a cone-shaped testis forms in the ectoderm near the head. Histological sections indicate the testis contains large numbers of sperm, differentiation intermediates, and interstitial cells (Brien, 1961). In females, one or a number of prominent eggs form in the ectoderm near the budding zone. Each arise from the fusion of a number of interstitial cells (Brien, 1961).

Implicit in the foregoing is that the regional pattern of differentiation is the result of regional influences on the stem cells residing there and not the result of a massive influx of interstitial cells already committed someplace else. This point has yet to be rigorously proven in experiments which specifically assay stem cell commitment. Nevertheless, in at least one instance—regeneration—the changes in stem cell differentiation appear to be too large to explain with cell migration. Bisection of a hydra perpendicular to the body axis instantly changes the position of stem cells near the cut surface from a mid-gastric environment to that of an extremity, either head or foot. The apical end of the lower half will regenerate a head while the basal end of the upper half will regenerate a foot. The interstitial cells in both regenerating tips undergo rapid

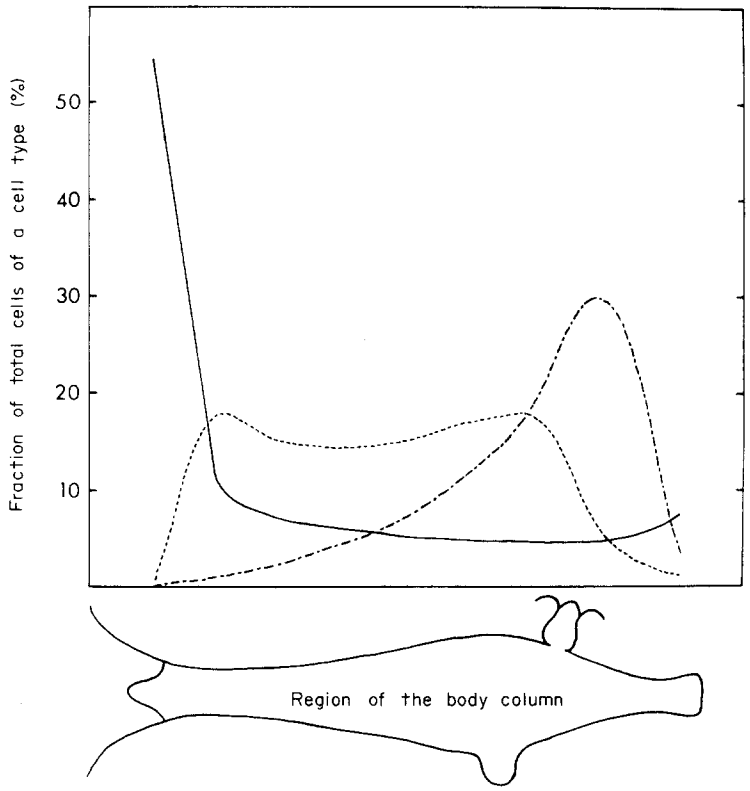

FIG. 5. Axial distribution of the differentiating cells of three cell types: differentiating nerve cells (——), desmoneme nematoblasts (-----), and stenotele nematoblasts (—·—).

changes in differentiation. For example, there is a marked increase in the number of nerves due to new differentiation by 24 hr (Bode *et al.*, 1973). Further, Yaross and Bode (unpublished results) pulse-labelled hydra with ^{3}H-thymidine 3 hr after bisection and subsequently examined the differentiation fate of the labelled interstitial cells in the regenerating apical tip. The number of interstitial cells becoming nerves increased twentyfold, while nematocyte differentiation decreased sixfold compared to the same region in control animals. No changes in interstitial cell behavior occurred in the region below the regenerating tip. Apparently the multipotent stem cell reacts to signals in the immediate environment, and is able to rapidly adjust its response to new signals as its regional position changes.

In the particular case of regeneration it is unlikely that the changes are due to the selective migration into the region of interstitial cells committed to nerve cells. Only a small fraction of the interstitial cells migrate (Herlands and Bode, 1974) and their number would be insufficient to account for the observed change. In other instances of localized differentiation, e.g. nematocyte differentiation along the body column, the role of interstitial cell migration in redistributing committed cells remains unclear. Although small numbers of interstitial cells are known to migrate along the body column (Tardent and Morgenthaler, 1966; Campbell, 1967c), the numbers are probably too small to account for all nematocyte differentiation. Moreover, migration is primarily in an apical direction (Herlands and Bode, 1974).

That the type of differentiation a cell will undergo is affected by position is common in embryology. The unusual aspect of regional differentiation in hydra is that the tissue layers are in a dynamic steady state in contrast to the relatively static state of most tissues. Assuming localized stem cell differentiation does indeed reflect localized stem cell commitment every stem cell and its progeny are continually changing their probabili-

ties for division or a particular differentiation because they are constantly changing their axial position. All interstitial cells are subject to the slow but constant tissue displacements up or down the column. In addition, the small number of interstitial cells that migrate experience the changes much more rapidly since they can traverse half the length of the animal in several hours (Tardent and Morgenthaler, 1966). By grafting the lower half of an animal labelled with ^{3}H-thymidine to an unlabelled upper half, Herlands and Bode (1974) demonstrated this point clearly. They found that labelled interstitial cells migrating into the upper gastric region formed nematocyte precursors, while those that moved all the way into the hypostome differentiated into nerves.

What is the basis or nature of the control of regional stem cell commitment? Although little is known, there are a few experiments which bear on the question, and some interesting correlations between the results of these experiments and the processes underlying pattern regulation in hydra. In order to discuss these correlations, a brief description of the major findings about pattern regulation is presented.

2. *Pattern Regulation in Hydra*

A large body of regeneration and transplantation experiments have led to a consistent model for pattern regulation in hydra. For detailed reviews of this work, Webster (1971), Wolpert *et al.* (1971), Bode (1973) and Campbell (1974) should be consulted.

Removal of head and foot from the body column of hydra result in the regeneration of each at the appropriate end. This phenomenon is termed the polarity of regeneration. With respect to the head, this polarity can be explained formally in terms of two gradients. One of them is related to the property that any part of the body column is capable of forming a head. This quality, which is a property of the tissue, is distributed in an axial gradient being maximal in the head. The other, which is not a property of the tissue, is the quality of inhibition of head formation. It originates in the head and decreases monotically in intensity down the body column (Webster and Wolpert, 1966; Webster, 1966a, b; Wilby and Webster, 1970a, b; Wolpert *et al.*, 1971).

Not only is the head the origin of the gradient of inhibition, but it controls the gradient of head formation. By removing the gastric region from an animal and grafting a head to its basal end, Wilby and Webster (1970) were able to reverse the polarity of head regeneration of the gastric region. Thus, the head influenced the capacity for head formation in the tissue such that the capacity is highest at the end of the body column nearest the head. The head, therefore, plays a crucial role in the establishment of both gradients and with it the control of the regeneration polarity.

However, the head region is not the only organizing region in the animal. Formation of the foot has been shown to be under the control of a pair of gradients originating in the foot that have similar properties to those of the head (MacWilliams and Kafatos, 1968; MacWilliams *et al.*, 1970; Hicklin and Wolpert, 1973).

At present there is no direct knowledge what the basis of these gradients might be. There are transplantation experiments which indicate that the transfer of head inhibition (Wolpert, 1972) or foot inhibition (MacWilliams, 1974) through the body column is consistent with a substance emanating from the appropriate organizing center and diffusing through the tissue. Further, Gierer and Meinhardt (1972, 1974) have proposed a model in which both gradients governing head formation are based on diffusible substances. The model explains a large number of regeneration and transplantation results.

A number of attempts have been made to isolate substances affecting pattern regulation in hydra. Two activities, one stimulatory and one inhibitory that affect head regeneration have been described. Lentz (1965) and Lesh and Burnett (1964, 1966) reported that a fraction of hydra extract obtained by centrifugation caused the formation of supernumerary head structures during head regeneration. Extending these initial studies, Schaller (1973) has purified an activity about 10^6-fold that has a molecular weight of approximately 1000 daltons and is sensitive to proteolytic enzymes. The material is stored in granules primarily in the nerve cells, and is distributed in a gradient along

the body column with a maximum in the head region (Schaller and Gierer, 1973). The substance, termed head activator, is active at concentrations of less than 10^{-10} M. At these concentrations it increases the rate of head regeneration and the number of tentacles regenerated in decapitated animals as well as increasing the rate of bud formation in intact animals (Schaller, 1973). It has no effect on foot regeneration.

Perhaps most striking is the ability of the head activator to confer properties of the head on aggregates of cells from the gastric region. Because of its importance, the experiment will be described in detail. Aggregates of hydra cells obtained by centrifuging dissociated cells into clumps will develop into normal hydra, the number depending on aggregate size (Gierer *et al.*, 1972). Further, aggregates made of gastric cells will also develop head structures, although fewer and at a slower rate than those made of only head cells. When aggregates of cells from these two regions are combined shortly after formation into triplet aggregates of composition HGH or GHG (H = aggregate of head cells; G = aggregate of gastric region cells), head structures are formed only where the head aggregates are (Gierer *et al.*, 1972; Schaller, 1975). Head structures are apparently suppressed in the gastric region aggregates. Schaller (1975) obtained this same result by treating gastric regions with head activator (G+) and making the triplet aggregates G+GG+, GG+G, or GGG. In the first two, head structures formed only where G+ aggregates were, while in the controls head structures formed all over. Hence, the head activator can substitute for head cells in organizing these aggregates. Although there is no direct evidence, these results suggest that the substance is involved in the stable head-forming property of the tissue demonstrated in the transplantation experiments.

The second activity is in many ways the antithesis of the head activator. In a number of reports an inhibitory activity of head regeneration in homogenates of hydra (Lenique and Lundblad, 1966; Davis, 1967) or in the culture medium (Loomis and Lenhoff, 1957; Davis, 1966) has been described. More recently, Berking (1977) has characterized an activity which depending on concentration retards or inhibits the development of buds from bud anlagen, but does not affect the number of anlagen. It will also inhibit both head and foot regeneration and, thereby, is not specific for head formation as is the head activator. The activity which has been enriched at least 1500-fold has a molecular weight of 300–1000 daltons (Berking, 1977). It is distributed in a gradient along the body axis with a maximum in the head region, and is stored in either nerve or epithelial cells or both. The chemical nature of the activity remains unknown, though it is stable to boiling in strong acid or base and to degradative enzymes for a number of macromolecules. Though suggestive, it is open question if this inhibitory activity is related to the quality of inhibition found in transplantation experiments.

3. *Control of Stem Cell Differentiation and Pattern Regulation*

Since little is known about the control of the regional pattern of stem cell differentiation, the possibilities are many. An interesting one is that the gradients underlying pattern regulation may be responsible. In the intact animal these gradients may have the role of maintaining the pattern in the face of continuous growth and tissue displacement. Body column tissue is continuously converted into head or foot tissue. As an example at a cellular level, the proliferating epithelio-muscular cells of the ectoderm on the body column are converted into non-dividing battery cells as they are displaced from the body column onto the tentacles. One of the gradients, or both, could provide a means for the epithelial cells to continuously monitor their position and alter their cellular activity in response to changes in position. Similarly, stem cells could "measure" their position using one or both gradients and differentiate accordingly.

There is some evidence correlating the processes of pattern regulation and regional interstitial cell commitment. Some of it concerns the influence of the head, which plays a central role in both gradients. It is also known to directly affect two interstitial cell differentiation pathways and modify the behavior of another.

Stagni (1961) (see also Vannini, 1974) carried out grafting experiments which clearly indicate that stem cell-to-gamete transitions occur only at specific distances from the head. If the gastric region of the hermaphroditic species, *Chlorohydra viridissima*, bearing both a developing testis near the apical end and a developing egg near the basal end is removed and grafted back in an inverted position (see Fig. 6a), the gonads undergo the following changes. The existing gonads regress and disappear. Shortly thereafter, a new testis will form near the head, at the original basal end of the gastric region.

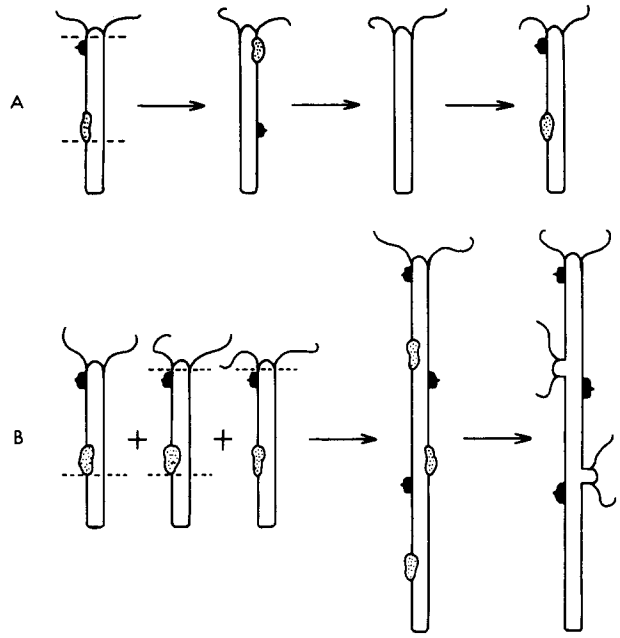

FIG. 6. Effect of the alternation of axial position on gonad development. (A) Inversion of the body column, (B) Three body columns grafted together with normal polarity. Testes are black and developing ovaries are dotted. Results are discussed in the text. (Modified from Stagni, 1961.)

Conversely a developing egg appears near the budding zone which is the original apical end of the gastric region. In a second experiment three body columns, each bearing a testis and an egg were grafted end to end with parallel polarity (Fig. 6B). As has been demonstrated repeatedly (e.g. Wolpert *et al.*, 1971), secondary heads will form at the graft junctions thereby increasing the number of heads in such graft combinations. The effect on the gonads is that all the testes remain while the developing eggs regress. These results indicate that stem cell–sperm differentiation require a close proximity to the head, whereas stem cell–egg transitions require the stem cell to be far from the head.

The second example concerns the gradient of size in the stenotele nematocyst, which is maximal in the upper gastric region near the head. Tardent *et al.* (1971) have shown that the formation of large nematocysts depends on the presence of the head. By decapitating a hydra and removing the regenerating apical tip on the two following days, these authors showed that stem cells committed to stenotele differentiation during that period of time all produced small nematocysts. In another experiment they removed head and foot and grafted them to the opposite ends of the body column. Interstitial cells in the peduncle committed to stenotele formation shortly after grafting of the head to the basal end formed large stenotele nematocysts where formerly they produced

small ones. Conversely, interstitial cells in the upper gastric region close to a foot and far from a head formed small nematocysts instead of the previous large ones. The gradient in nematocyst size suggests that the influence of the head declines monotonically with distance.

However, proximity to the head is not the only factor in these experiments. In an analogous experiment to the first one, Tardent *et al.* (1971) removed the foot and the subsequent regenerating basal tips for two days. They found that no small nematocysts were made at the lower end by interstitial cells committed to stenotele formation during this period. Earlier, Lehn (1951) found a similar effect. He bisected an animal in the mid-gastric region and examined stenotele nematocyst size at the basal end of the upper gastric region during the subsequent regeneration of a foot. He found that interstitial cells forming stenoteles had small nematocysts even though the distance from the head had changed very little.

Changes occurring in the regenerating apical tip of an animal decapitated directly below the tentacles provides another line of evidence. One characteristic of hypostome tissue is its ability to form a head and a secondary axis when transplanted into the body column near the host head (Browne, 1909; Webster and Wolpert, 1966). No other tissue in the animal has this ability (Browne, 1909). If a regenerating tip is transplanted 0–2 hr after decapitation, it is absorbed into the body column. If, however, it is transplanted 4–6 hr after decapitation, the tip will form a head and a secondary axis in 50% of the cases (Webster and Wolpert, 1966). The regenerating tip has acquired a property of the hypostome. Within the same period, sharp changes in the interstitial cell differentiation pattern have occurred. Before decapitation interstitial cells differentiated predominantly into nematocytes, whereas by 3 hr after decapitation, interstitial cells in the tip formed primarily nerve cells which is typical of the hypostome (see Section IV.1). Thus, the pattern-forming characteristics and interstitial cell behavior of the regenerating tip changed in parallel.

Another type of evidence concerns the substances that affect head regeneration. Schaller has shown that the head activator has effects on interstitial cell behavior. Not only does the head activator increase the rate of head regeneration, it has a mitogenic effect by shortening the cell cycle of the interstitial cell and the other proliferating cell types in hydra (Schaller, 1976a). It also causes an increase in the number of interstitial cells differentiating into nerves and a decrease in nematocyte formation (Schaller, 1976b). This, too, has a correlate in regeneration. Shortly after bisection large amounts of head activator are released at and near the cut surface (Schaller, 1973, 1976c). At the same time, as just mentioned above, nerve cell differentiation increases and nematocyte formation decreases in this area.

Berking has characterized the effects of the inhibitor activity on interstitial cell behavior as well as on regeneration. At concentrations where it blocks head regeneration it also blocks both interstitial cell division and interstitial cell differentiation into nerves. Removal of the inhibitor results in a burst of mitotic activity in the interstitial cells and a sudden increase in the number of nerve cells formed as well as resumed head regeneration (Berking, 1974).

Thus, there are parallel influences of the head and two substances on regeneration, or pattern regulation, and on interstitial cell behavior. Also, there are correlated changes in the head-forming ability and interstitial cell behavior in a regenerating apical tip. All these results suggest that the mechanisms underlying pattern regulation may also be responsible for the regional control of stem cell differentiation. In part, this hypothesis may be attractive because ignorance hides other possibilities. Pattern regulation and interstitial cell behavior have been studied more extensively than other aspects of development in hydra so that it may be easy to draw parallels between the two. Some recent results obtained by Campbell (1976) urge caution in so doing. He has been able to produce nerve-free hydra, using colchicine. These animals undergo head regeneration (Marcum and Campbell, personal communication) as do normal animals suggesting that nerve cells are not necessary for this process. An apparent consequence of

this is that the head activator, which is produced primarily in nerve cells, may not be necessary for head regeneration and may only affect stem cell differentiation. More likely, the results suggest the processes are more complicated than currently envisioned and any hypothesis should be considered as tentative.

4. *Other Influences on Stem Cell Differentiation*

(a) *Feedback from the Product Cells*

In addition to the effects of position, there is some evidence indicating other influences on stem cell differentiation. The continuous production and turnover of nerves and nematocytes suggests that feedback from the product cells to the stem cells might exist to maintain the size of the product cell populations. Zumstein and Tardent (Zumstein and Tardent, 1971; Zumstein, 1973) reported evidence for a negative feedback loop governing stenotele nematocyte production. They reduced the stenotele population by 90% by selectively discharging the stenotele nematocysts by stroking the tentacles with a glass rod. Periodically thereafter they quantitated the nests of nematoblasts by type in the ectoderm. They found an increase, compared to controls, in the number of nests of stenoteles, but no other nematocyte type, several days after treatment. Similarly, Smith, Nadeau and Bode (unpublished results) reduced the stenotele population by 90% by subjecting the animals to pulses of electric current. They found a transient 10% increase in the number of stenotele nests 5–7 days after treatment. The results were the same even when repeated treatments over 28 days maintained the stenotele population at 10% of normal (Smith *et al.*, unpublished results). Since each nest arises from a single stem cell and the differentiation time to the assayed stage is 5–7 days (David and Gierer, 1974), the results suggest that reduction of the stenoteles caused an increase in stem cell commitment to stenotele differentiation.

It is unlikely that such negative feedback loops play an important role in the control of stem cell behavior. If they did, the nematocyte population sizes would be expected to remain quite constant. They do not. In hydra of constant size (maintained on a constant feeding regime) the stenotele population can undergo four fold variations in the same animal over a period of four months. Also, animals maintained on different feeding regimes differ several-fold in the nematocyte population sizes and, more importantly, in the ratio of the nematocyte: interstitial cell population sizes (Bode *et al.*, 1977). Finally, the large and prolonged reduction of the stenotele population described above did not elicit a large and continued increase in production of stenoteles to make up the deficit. Thus, this negative feedback loop is unlike, for example, the fairly stringent one operating in the mammalian blood tissue to maintain the size of the erythrocyte population. Small changes in the population size result in the appropriate increase or decrease in the number of erythrocyte precursor cells entering the differentiation pathway (e.g. Erslev, 1971; Morley *et al.*, 1970). In contrast, the stenotele feedback loop, at best, is effective only when a large reduction in stenotele numbers has taken place.

(b) *Hormonal Influence on Sex Determination*

Though the type of gamete a stem cell will form is clearly dependent on axial location, there is also evidence for a hormonal influence that is unrelated to position. Though gamete formation is an occasional and transient behavior of the stem cells, one aspect of gamete production is retained throughout the long periods of asexual reproduction, namely the sex of the animal. Some species of hydra are hermaphroditic, some dioecious and some unstably dioecious. An animal of a species of the last category, such as *H. attenuata*, usually maintains its sex, but occasionally undergoes sexual inversion. This can occur in either direction. At a cellular level this means that stem cells producing one type of gamete switch completely and permanently to the production of the other type.

By making reciprocal grafts between male and female animals Tardent (1968) obtained some interesting results bearing on the problem of how a stem cell is influenced to

form one or other type of gamete. He found that all the animals resulting from the grafts were male. Grafts in which only the head or the stalk (peduncle and basal disk) of male animals were grafted to complimentary parts of the female animal yielded the same results. Further, the male half need only be in contact with the female for 72 hr to switch the sex of the female portion. These experiments indicate that either a cell capable of migration or a substance in the male tissue invaded the female tissue and induced the stem cells of the female tissue to form sperm. Because the stalk was as effective as the head this migrating cell type or the cell type releasing the factor must occur throughout the body column.

In another series of experiments Tardent (1968) X-irradiated male animals before making the reciprocal half–half grafts. In this case all animals arising from the grafts were female indicating that the treatment had destroyed the "masculinizing" effect. Because the total dose eventually led to the death of the irradiated animals, it is possible that animals resulting from the graft eventually consisted wholly of female tissue. However, males treated with sub-lethal doses were propagated and found to have undergone male-to-female inversions at a rate much higher than in untreated animals. These results are formally similar to the case in mammals where an animal will develop into a female unless a male hormone is produced very early during development (Whalen, 1968).

Both sets of experiments were carried out on asexual animals so that the "masculinizing" effects or their loss were maintained in the tissue for a number of asexual generations. The grafting experiments could be interpreted in terms of invading "male" interstitial cells which for some reason supplanted the "female" interstitial cells of the female animal. However, the high rate of male-to-female inversions following sub-lethal doses of X-irradiation cannot be explained in this manner. Alternatively, the results can be explained in terms of a substance produced in male tissue that promotes stem cell–sperm transitions. In the graft, transfer of the substance and subsequent production by any one of a number of cell types (epithelial cell, interstitial cell, nerve cell) would maintain the "male" environment in the formerly female tissue. This influence on stem cell behavior is not position-dependent, nor is it homeostatic or environmental, but is one based on cell–cell interactions and clearly of importance when the animal enters a sexual phase.

(c) *Environmental Influences*

All the influences described so far are internal to the animal. In addition, it is quite clear that altering the environment external to the animal affects stem cell differentiation. Most of the work on environmental influences on stem cell behavior concerns gamete formation. This area also remains one of the most elusive for analysis because factors inducing one species to enter the sexual phase have no effect on another species, nor does a given treatment always work with the same species. Among the conditions described to induce egg or sperm gamete formation are sudden increases or decreases in temperature (reviewed by Park *et al.*, 1965), starvation (Gross, 1925), particular feeding regimes (Lenhoff, personal communication; Bode, unpublished observations), changes in day length (Burnett and Diehl, 1964), or changes in the composition of the culture medium. The latter has been achieved by allowing the medium to stagnate, altering the p_{CO_2} or simply changing the chemical composition (Loomis, 1964). A change in the ion composition of the medium has also been reported to affect the pattern of nematocyte differentiation (Macklin and Burnett, 1966).

Whether these influences affect the stem cell directly or indirectly is unknown. In analogy to vertebrates entering a sexual phase due to an environmental change (e.g. length of daylight), the effects of the stimuli on gamete production in hydra could be only indirectly on the interstitial cell. However, considering the position of hydra on the evolutionary scale, the analogy to protozoa which are often directly affected by environmental stimuli to undergo differentiation (e.g. Fulton and Dingle, 1967; Fulton, 1972) may be equally appropriate. In this case, the influence would be directly on the stem cell.

V. SUMMARY

Although the knowledge is still rudimentary, elements of control affecting stem cell behavior are beginning to emerge. The commitment to division or differentiation of the stem cells is affected by their local density. There is a regional pattern of the several differentiations which has been in part correlated with distance from the head. Progress in unraveling these controls, more than likely a network of cell–cell interactions, should proceed more rapidly with methods developed recently. The ability to quantitate cells precisely, the ability to clone a stem cell and follow its behavior from the single cell stage on, and the ability to alter the cell composition of the stem cell environment with transplantation and reaggregation techniques should all prove useful. In addition, the simplicity of an animal with only fifteen cell types suggests that the network of controls might be decipherable.

One aspect of the controls influencing the stem cells that is of particular interest is its positional dependence. Ever since Driesch (1892) first noted the phenomenon of position-dependent differentiation, the question of the basis of this phenomenon in animals has remained unanswered. With the discovery of morphogenetic fields, the problem was phrased in terms of how such fields control the spatial distribution of cell differentiations (e.g. Weiss, 1939, or, more recently, Wolpert, 1969). Examination of a multipotent cell known to undergo different cell differentiations in separate locations affords a good opportunity for investigating this problem. Because few multipotent cells have been studied to any degree, clear cut evidence for positional effects are known for only two other cells in addition to the stem cell of the interstitial cell system. The differentiation of neural crest cells of chick embryos is affected by location along the body axis (e.g. Le Douarin and Teillet, 1974), and Trentin *et al.* (1974) have shown that the hemopoietic stem cell behavior of the adult mouse is affected by the regional location in the spleen, although there is no indication of morphogenetic fields in the latter case. The stem cell of the interstitial cell system of hydra is a particularly good candidate because of the observed parallels between pattern regulation and regional stem cell differentiation. The head region, which is known to be the source of the morphogenetic field affecting pattern formation and regulation also affects the location of at least two interstitial cell differentiation pathways. An understanding of the mechanisms underlying both processes should help elucidate the role of fields in position-dependent differentiation.

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