SUMMARY

In hydra, nerve cells are a differentiation product of the interstitial cell, a multipotent stem cell. Nerve cell commitment was examined during head regeneration in *Hydra attenuata*. Within 3 h of head removal there is a 10- to 20-fold increase in nerve cell commitment in the tissue which subsequently forms the new head. Nerve cell commitment is unaltered in the remainder of the gastric region. This local increase in nerve cell commitment is responsible for about one half of the new nerve cells formed during head regeneration, while one half differentiate from interstitial cells that migrate into the regenerating tip.

INTRODUCTION

The interstitial cell (i-cell) of hydra is a multipotent stem cell which produces nerve cells (e.g. Davis, 1974) and 4 types of nematocytes (Lehn, 1951; Slatterback & Fawcett, 1959) throughout asexual growth of the animal. David & Murphy (1977) have demonstrated the multipotency of individual i-cells by showing that clonal progeny can include both nerve cells and nematocytes.

Studies have suggested that 3 major influences are involved in the regulation of i-cell commitment to nerve cell or nematocyte differentiation in intact hydra. (1) Nerve cells positively influence i-cells to form nerve cells (Yaross & Bode, 1978a). (2) I-cells positively influence their own commitment to nematocyte production. This results in a negative effect on i-cell population size (Yaross & Bode, 1978a). (3) The type of nematocyte formed depends on the axial position of the i-cell (Bode & Smith, 1977). However, nerve cell commitment is correlated with axial position as well as with nerve cell density (Yaross & Bode, 1978a). This correlation suggests that nerve cell density may be the underlying basis of the position-dependence of nerve cell differentiation in normal, steady-state hydra. However, this does not preclude the possibility that situations exist where nerve cell commitment is governed by position effects unrelated to nerve cell density.

Head regeneration in hydra is known to be accompanied by major changes in the apical regenerating surface. In particular, the cellular composition, especially that of...
the i-cells and their differentiated product cells, rapidly changes from that of the gastric region to that found in the head (Bode et al. 1973; Berking, 1974; Schaller, 1976a). By 24 h following head removal, there is a significant increase in the nerve cell number in the tissue which forms the new head (Bode et al. 1973; Berking, 1974; Schaller, 1976a). To examine the basis of this increase, we have investigated the roles of nerve cell density and positional effects in altering the differentiation behaviour of the i-cell population in the regenerating tissue. We have found that position-related effects are largely responsible for the change that occurs in nerve cell commitment after decapitation.

MATERIALS AND METHODS

Culture of animals

*Hydra attenuata* were cultured at 18 ± 1 °C in media consisting of $1 \times 10^{-3} \text{M} \text{MgCl}_2$, $1 \times 10^{-3} \text{M} \text{KCl}$, $1.25 \times 10^{-3} \text{M} \text{Na}_2\text{EDTA}$, $1 \times 10^{-3} \text{M} \text{CaCl}_2$, and $5 \times 10^{-4} \text{M} \text{NaHCO}_3$ in distilled H$_2$O. Stock cultures were fed 6 days per week with *Artemia* nauplii and washed approximately 8 h after feeding. Budding hydra were used for all experiments; animals were not fed during the course of any experiment.

Cellular composition and nomenclature

The cellular composition of whole hydra or of specific regions was analysed using the maceration technique of David (1973). In all experiments, 8–15 hydra or tissue fragments were macerated together. To determine the density of any cell type (i.e. ratio to epithelial cells), a differential cell count of at least 1000 cells of the appropriate types was made per determination.

The nomenclature used for interstitial cells, unless otherwise noted is that described by Bode & David (1978). Interstitial cells will be those big i-cells (David, 1973) occurring singly or as pairs. This class includes the multipotent stem cells and i-cells committed to nerve cell or nematocyte differentiation (David & Gierer, 1974). Nests of 4, 8, and 16 big i-cells or little i-cells (David, 1973) destined to form nematocytes (David & Gierer, 1974) will be referred to as dividing nematoblasts.

$[^3]H$thymidine administration and autoradiography

Hydra were radioactively labelled by injection of $[^3]H$thymidine (New England Nuclear, 6 Ci/mM) into the gastric cavity using a polyethylene needle (David & Campbell, 1972). For pulse labelling, 50 μCi/ml $[^3]H$thymidine were injected a single time; for 'continuous' labelling experiments, animals were injected with 25 μCi/ml $[^3]H$thymidine every 8 h. As the S-phase of hydra cells is 12 h (David & Campbell, 1972; Campbell & David, 1974), the latter regime is effectively equivalent to continuous labelling as all cells which enter S-phase become labelled.

Autoradiography was performed as follows: slides were washed in distilled H$_2$O, dipped in 1 : 1 distilled H$_2$O : Kodak NTB$_2$ Nuclear Track Emulsion, and dried. After 7 days exposure at 4 °C, autoradiographs were developed for 7 min with Kodak D-19, rinsed in distilled H$_2$O or Kodak Stop bath, fixed for 5 min with Kodak Rapid-Fix, and washed thoroughly with distilled H$_2$O.

Assay for nerve cell commitment

Nerve cell commitment was assayed as described previously (Yaross & Bode, 1978a). Hydra were pulse-labelled with $[^3]H$thymidine to label the i-cell population. After sufficient time to allow detection of newly committed nerve cells (26 h), 8–15 animals or fragments were macerated (David, 1973). The resulting cell suspensions were spread on slides and processed autoradiographically.
Position-dependent nerve differentiation

The fraction of the pulse-labelled stem cells committed to nerve cell differentiation was determined from the distribution of the radioactively labelled cells derived from the pulse-labelled i-cells 26 h after the [3H]thymidine pulse. At this time, i-cells in nests of 1 and 2 cells, dividing nematoblasts in nests of 4 cells, and nerve cells would all be derived from the cohort of labelled i-cells (cf. Yaross & Bode, 1978a). The nerve cell commitment fraction was therefore defined as follows:

\[
\text{Nerve cell commitment fraction} = \frac{\text{Labelled nerve cells}}{\text{Total cells (1's, 2's, 4's, & nerve cells)} \text{ at 26 h.}} \text{ derived from pulse labelled i-cells}
\]

From 300 to 1000 cells of the appropriate categories were counted per determination.

The validity to this assay, developed for steady-state conditions (Yaross & Bode, 1978a), depends on all pulse-labelled i-cells and their progeny remaining in the cohort of labelled cells at the time of analysis, 26 h after labelling. During head regeneration, some dividing nematoblasts in nests of 4 cells are lost, presumably due to cell death (Yaross & Bode, 1978b). However, one can calculate that the loss which occurs would affect the assay by at most a factor of 1.5-2 during the time period examined.

Transplantation techniques

Head regeneration was initiated in all cases by removal of the H1 region (head plus the apical quarter of the gastric region; see Wolpert, 1969, for nomenclature). The apical tip of regenerating animals was defined as the apical one-eighth to one-twelfth of the gastric region remaining after H1 removal. Hydra were grafted by threading appropriate fragments, as described in Results, on nylon fishline (Herlands & Bode, 1974). Three- to five-millimetre pieces of polyethylene tubing just large enough to fit snugly on the fishline were used to keep the cut edges in contact for 1-2 h to permit healing prior to removal from the fishline. In some experiments hydra were vitally marked by injecting India ink (Pelikan) into the epidermis using a glass micropipette (Campbell, 1973). This permitted accurate identification of the graft junction at any later time. As the cellular composition of carbon-marked tissue is unaltered (Yaross, unpublished observations), it is unlikely that any cell type is adversely affected by the carbon injection.

Formation of aggregates

Aggregates of dissociated cells were prepared as described by Gierer et al. (1972). The dissociation medium used consisted of 5 x 10^{-3} M CaCl_2, 1 x 10^{-3} M MgSO_4, 2.8 x 10^{-3} M KCl, 1.1 x 10^{-3} M TES, 3.3 x 10^{-4} M Na_2HPO_4, 6.5 x 10^{-4} M KH_2PO_4, 5 x 10^{-3} M Na-pyruvate, 5 x 10^{-4} M Na_3 citrate, and 50 mg/l rifampicin; the pH was adjusted to 6.9.

Aggregates were allowed to develop for at least 24 h before assaying either cellular composition or nerve cell commitment. For nerve cell commitment studies aggregates were labelled by inserting a polyethylene needle directly through the epithelia to the centre of the hollow aggregates. All other procedures were as described for intact hydra.

RESULTS

Nerve cell differentiation in the regenerating tip

One of the early events that accompany head regeneration is an increase in the nerve cell number, resulting in an increase in nerve cell density (i.e. nerve cells per epithelial cell) in the head regenerating tissue (Bode et al. 1973; Berking, 1974; Schaller, 1976a). To determine whether this increase in nerve cell density results from a local increase in i-cell commitment to nerve cell differentiation, nerve cell commitment was assayed in regenerating animals and intact control hydra. Head regeneration was initiated by removing and discarding the H1 region (head plus apical quarter of the gastric region; Wolpert, 1969) from groups of hydra. Animals
were injected with \[^3\text{H}\text{H}^\text{thymidine} to pulse-label the stem cell population at times following H\text{I} removal. Twenty-six hours after labelling, the apical tip of the regenerating animals, the remainder of the gastric region, and comparable regions of the control animals (see Fig. 1A) were excised and the nerve cell commitment fraction determined as described in Materials and methods. As seen in Fig. 1B, 3 h after H\text{I} removal, the nerve cell commitment fraction rose from a control level of 0.005 ± 0.001 (± standard error) to 0.115 ± 0.021 in the apical tip of the regenerating animals. This represents a 10- to 20-fold increase. Nerve cell commitment remained at this elevated level until at least 48 h. That the increased nerve cell commitment is restricted to the regenerating tissue is seen in Fig. 1C. Nerve cell commitment in the remainder of the gastric region of the regenerating animals did not increase significantly above control.

Fig. 1. Nerve cell commitment during head regeneration. Regions indicated in A were excised from 10 hydra for each measurement. B, nerve cell commitment in the regenerating tip (1: •) and the comparable control fragment (3: ---- O----). C, nerve cell commitment in the remaining gastric region of regenerating (2: ---- x ----) and control (4: ----  □ ----) hydra.
levels. The results therefore suggest that there was a local increase in the nerve cell commitment in the regenerating tip.

Table 1. *Nerve cell commitment in aggregates of dissociated cells*

<table>
<thead>
<tr>
<th>Time after reaggregation, h</th>
<th>Nerve cell commitment fraction</th>
<th>Nerve cells/ epithelial cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 Aggregates</td>
<td>0.093 ± 0.019</td>
<td>0.137 ± 0.030</td>
</tr>
<tr>
<td></td>
<td>Intact</td>
<td>0.037 ± 0.008</td>
</tr>
<tr>
<td>48 Aggregates</td>
<td>0.065 ± 0.024</td>
<td>0.230 ± 0.015</td>
</tr>
<tr>
<td></td>
<td>Intact</td>
<td>0.032 ± 0.008</td>
</tr>
<tr>
<td>72 Aggregates</td>
<td>0.080 ± 0.031</td>
<td>0.284 ± 0.055</td>
</tr>
<tr>
<td></td>
<td>Intact</td>
<td>0.024 ± 0.009</td>
</tr>
</tbody>
</table>

Each value represents the mean ± standard error of 2–4 independent experiments. 5–15 aggregates or 4–10 intact hydra were analysed together per determination. Neither aggregates nor intact animals were fed during the course of the experiment.

To study further the effect of regeneration on nerve cell commitment, aggregates of dissociated cells were examined. Reaggregated hydra cells undergo morphogenetic processes very similar to those occurring during head regeneration, forming head structures (hypostomes and tentacles) between 24 and 48 h after aggregate formation (Gierer et al. 1972; Bode, 1974). This is accompanied by a doubling of the nerve cell density (Gierer et al. 1972), paralleling that seen in the apical tip of regenerating animals. The nerve cell commitment fraction and nerve cell density were therefore assayed in aggregates at 24, 48, and 72 h after reaggregation (Table 1). Nerve cell commitment was 2- to 3-fold higher in the aggregates than in intact animals comparable to those dissociated to prepare the aggregates throughout the time period examined. As a result, the nerve cell density at 72 h is higher in the aggregates than in the intact hydra, despite the fact that their initial nerve cell densities were identical. The nerve cell density of the intact controls also increased somewhat between 24 to 72 h, most likely due to starvation (cf. Bode, Flick & Bode, 1977; Yaross & Bode, 1978a). However, the increase in nerve cell density in the aggregates was greater than in the intact control hydra during this period. Thus regeneration in aggregates was also accompanied by an increase in the fraction of the i-cells undergoing nerve cell differentiation.
However, the possibility exists that the i-cells and progeny present in the regenerating tip at the time nerve cell commitment was assayed (26 h after labelling with \(^{3}H\)thymidine) migrated into the apical tip during that time period and were in fact committed elsewhere. To determine whether the results obtained by assaying the apical portion of regenerating animals were characteristic of the behaviour of the i-cells present in the regenerating tip at the time of labelling, the following experiment was performed (see Fig. 2). Hydra were pulse-labelled with \(^{3}H\)thymidine 3 h after H1 removal as previously described. One hour after labelling, the former 2-region (apical one-third of the remaining gastric region) was removed from the labelled animals and grafted to the basal half of an unlabelled hydra immediately after H12 removal. Care was taken to maintain the original polarity of the grafted ring of labelled tissue. At 26 h after labelling, the apical tip of the graft was macerated and the nerve cell commitment fraction determined. As the nerve cell commitment assay analyses only the labelled cells, any cells that migrated into the labelled tissue from below would not be scored. Migrating i-cells therefore could not influence the result in this experiment. The mean nerve cell commitment fraction in 2 such experiments, each of which involved 12 grafts, was 0.065 ± 0.038, clearly well above 0.005 ± 0.001, the mean control level of nerve cell commitment at this time (Fig. 1B). This result indicates that the elevated nerve cell commitment assayed in the regenerating surface does reflect a change in the differentiation behaviour of the i-cell population in that region.

However, apical migration of i-cells does occur in both normal and regenerating hydra (Tardent & Morgenthaler, 1966; Vögel, 1972; Herlands & Bode, 1974). Consequently i-cells migrating into the regenerating tissue and differentiating to nerve cells could additionally contribute to the increase in nerve cell density. To determine the extent to which the nerve cells formed during the initial 48 h of head regeneration are derived from apically migrating cells, hydra were treated as shown in Fig. 3. Animals were injected 3 times at 8-h intervals with \(^{3}H\)thymidine, 25 μCi/ml, to label the i-cell population. They were also vitally marked with carbon particles (Campbell, 1973) in the mid-gastric region. The H12 region of these labelled animals was then discarded, and an unmarked, unlabelled 2-region grafted to the remaining basal half, again taking care to retain the original polarity of the ring of tissue. The grafts were then allowed to undergo regeneration for 48 h, at which time the apical half of the unmarked tissue was removed and macerated, as was the remainder of the
gastric region below the graft junction identified by the carbon particles. After autoradiography, the labelling indices of the i-cells and nerve cells were determined for each of these regions (Table 2). Thirty-one percent of the nerve cells in the upper 2-region were radioactively labelled. Since $\sim 84\%$ of the i-cells in the labelled animals were radioactively labelled, this indicates that $31\% + 0.84 \approx 37\%$ of the nerve cells in the regenerating tissue at 48 h differentiated from i-cells that migrated into the upper 2-region. Thus, $\sim 37\%$ of the total nerve cells in the tip at 48 h arose from cells which were not in that region at the time of head removal, adding to those produced by the 10- to 20-fold increase in the nerve cell commitment of the i-cells in the regenerating tip.

Table 2. Migration of labelled cells into unlabelled regenerating tissue

<table>
<thead>
<tr>
<th>Labelling Indices, %</th>
<th>Single i-cells</th>
<th>I-cell pairs</th>
<th>Nerve cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Upper 2-region (a)</td>
<td>58.9</td>
<td>37.5</td>
<td>30.8</td>
</tr>
<tr>
<td>Gastric region (b)</td>
<td>83.5</td>
<td>84.5</td>
<td>11.7</td>
</tr>
</tbody>
</table>

Animals were grafted and fragments (a) and (b) excised at 48 h as indicated in Fig. 3. Labelling indices are the mean values of 2 independent determinations, each of which analysed 8–12 grafts. From 88 to 250 cells of each type were counted per determination.

The elevated nerve cell commitment in the regenerating tip raises the question of what event during head regeneration causes the local increase in nerve cell commitment. One possibility is that the increased local nerve cell commitment results from wounding. If the increased local nerve cell commitment were simply a response to trauma, then any wounding of the hydra would be expected to produce a local accumulation of nerve cells. To test this possibility, groups of 10–15 hydra were carbon-marked in the 2-region such that the mark extended 15°–135° around the circumference of the animal (see Fig. 4). These animals were then cut through the mark to the gastric cavity parallel to the apical-basal axis of the hydra. Forty-eight hours after wounding, the ring of tissue containing the carbon mark was excised. This ring of tissue was then divided into 2 portions: the wounded half containing the carbon mark and the unmarked, unwounded half. The marked and unmarked fragments were then macerated separately. The nerve cell density of each sample was determined and compared with the nerve cell density in the apical tip of regenerating animals run concurrently. While the mean nerve cell density of the head regenerating tip in 3 such experiments was $0.243 \pm 0.028$, the mean nerve cell density of the wounded tissue, $0.08 \pm 0.023$, did not differ from that of the unwounded tissue, $0.082 \pm 0.005$. Thus wounding alone did not result in increased nerve cell formation.

Alternatively, nerve cell commitment has been found to correlate with nerve cell density in intact hydra (Yaross & Bode, 1978a). Thus the increase in nerve cell commitment which occurred in the regenerating tip could result from the local increased nerve cell density. If so, then the increase in nerve cell density should occur...
prior to or concurrent with the increase in nerve cell commitment. Therefore the nerve cell density was also measured in regenerating and control hydra at intervals after H1 removal. Nerve cell density of the regenerating tip did not increase above control levels until 24 h after H1 removal (Fig. 5). Since the increase in nerve cell commitment precedes rather than follows that of nerve cell density (cf. Fig. 1B), the possibility that the increase in nerve cell commitment was due to increased nerve cell density can be excluded.

**Fig. 4. Procedure for examining effect of wounding on nerve cell density (see text for explanation).**

**Fig. 5. Changes in nerve cell density during head regeneration. Each point represents 10 regenerating tip (—•—) or control (---○---) fragments analysed together.**

Nerve cell commitment has also been found to correlate with the axial position of the i-cells along the body column (David, 1975; Yaross & Bode, 1978a). The elevated nerve cell commitment during head regeneration therefore could result from the altered axial position of the regenerating tissue (Wolpert, 1969; Wolpert, Clarke & Hornbruch, 1972). Evidence supporting this interpretation will be considered below.

**DISCUSSION**

Head regeneration was found to be accompanied by an increase in the nerve cell commitment fraction in the regenerating tip. Within 3 h after head removal, the fraction of the i-cells destined to differentiate into nerve cells rose 10- to 20-fold. In
regenerating aggregates of dissociated cells, nerve cell commitment increased 2- to
3-fold.

In intact hydra, nerve cell commitment has been shown to be correlated with nerve
cell density as well as with the axial position of the stem cell (Yaross & Bode, 1978a).
Examination of the basis of the rapid elevation in nerve cell commitment in head
regenerating tissue has ruled out a number of possible explanations. The rise in
nerve cell commitment preceded the increase in nerve cell density as well as other
known changes in the cellular composition of regenerating tissue (Bode et al. 1973;
Yaross & Bode, 1978b). Therefore, the initial increase in nerve cell commitment
could not have been caused by alterations in the cellular composition. Neither was the
increased nerve cell differentiation a non-specific wound response.

The increase in assayed nerve cell commitment also did not result solely from an
influx into the regenerating tip of cells committed to nerve cell differentiation else-
where in the animal. The grafting experiments presented here demonstrate an
increase in the nerve cell commitment of the i-cells already present in the regenerating
tip at the time of head removal. Thus 37% of the total nerve cells present in the
regenerating tip at 48 h result from migrating cells (Table 1). As some nerve cells
are already present in the tissue at the time of head removal, 37% of the total nerve
cells represents ~50% of the newly formed nerve cells in the regenerating tip (see
Appendix D, Yaross, 1978). Therefore ~50% of the new nerve cells result from
migrating cells, while the remainder, ~50% are the product of local i-cell commitment.
These figures are further supported by calculations which indicate that the measured
i-cell density and the nerve cell commitment fraction in the regenerating tip when
migration has been excluded (0.065) can account for about 68% of the new nerve
cells formed during the first 48 h following head removal (Appendix D, Yaross,
1978). Thus a significant fraction of the increase in nerve cell density, about one half
to two thirds is due to a local increase in i-cell commitment to nerve cell differentiation.
In addition, migration cannot be responsible for the increase in nerve cell commit-
ment in regenerating aggregates. Since the aggregates represent a closed system into
which cells committed elsewhere cannot migrate, the increased nerve cell commitment
fraction clearly indicates increased stem cell commitment.

Existing evidence suggests that the increase in nerve cell commitment results
directly from the change in the axial position of the i-cells. By removing the H1
region, the cells of the regenerating tip effectively have been moved from the mid-
gastric region to the presumptive head. Although the regenerating tip physically
resembles a head only 2-3 days after decapitation, it acquires the morphogenetic
properties of a head more rapidly. Within a day after head removal, the apical tip,
like the head but unlike the gastric region, has the capacity to induce the formation
of a head when transplanted into the body column of another animal (Webster &

The 10- to 20-fold increase in nerve cell commitment which occurs when the
regenerating tip undergoes this change in position parallels the regional differences
within normal animals: nerve cell commitment in the head of an intact hydra is 20
to 30 times greater than in the gastric region (Yaross & Bode, 1978a; David, 1975).
Furthermore, this abrupt increase in nerve cell commitment in the regenerating tip occurs concurrently with the changes in the morphogenetic properties of the tissue. Thus we conclude that in the regenerating tip, the primary influence on i-cell commitment to nerve cell differentiation is positional.

This leads to the question of the basis of position-dependent differentiation. The conclusion that nerve cell commitment is position-dependent during head regeneration appears to conflict with the observation in intact hydra that nerve cell commitment behaves as a function of nerve cell density. However, this apparent discrepancy may not be real. There is evidence suggesting that the correlation of nerve cell commitment with nerve cell density in the intact animal and the positional effect on nerve cell commitment in regenerating tissue may in fact have the same basis. Head activator, a substance which is primarily localized in the nerve cells of hydra (Schaller & Gierer, 1973), causes increased nerve cell differentiation in both intact and regenerating hydra (Schaller, 1976a). Head activator is predominantly maintained in the intact animal in a stored form and is most likely released under normal conditions in minute amounts (Schaller & Gierer, 1973). During head regeneration, however, large amounts of head activator are released by the regenerating tissue during the initial 8 h after head removal (Schaller & Gierer, 1973; Schaller, 1976b). The sudden release of a large amount of head activator by the nerve cells in the regenerating tip and the steady release of minute amounts by the greater number of nerve cells in the head could be equivalent in their effects on i-cell differentiation. Both could result in nerve cell commitment markedly above that occurring in the normal gastric region. Thus a single mechanism, responsible for both the nerve cell density effect seen in intact hydra and the positional effect seen in regenerating animals, may be the basis of position-dependent nerve cell differentiation.

Another positional effect on i-cell differentiation behaviour in the regenerating tip concerns nematocyte differentiation. The number of cells in the nematocyte pathway also changes in head regenerating tissue. There is a 5- to 10-fold drop in nematocyte precursors per epithelial cell during the first 48 h following head removal (Yaross & Bode, 1978b). Thus there is a major shift in the behaviour of i-cells and their progeny in the regenerating tip. This shift, which occurs only in that portion of the body column which regenerates the new head, rather than throughout the hydra, alters the local i-cell differentiation behaviour from the high nematocyte production, low nerve cell production characteristic of gastric region tissue to the high nerve cell production, low nematocyte production normally found in the head (Bode et al. 1973; David & Challoner, 1974; Yaross & Bode, 1978a).

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