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# THE HEAD ACTIVATOR, A NEUROPEPTIDE, CONTROLS NERVE CELL DIFFERENTIATION IN HYDRA

BY

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ABSTRACT

Interstitial stem cells in hydra were induced to differentiate nerve cells by treatment of animals with extracts of hydra tissue. The activity in the extracts was shown to be due to a peptide (pGlu-Pro-Pro-Gly-Gly-Ser-Lys-Val-Ile-Leu-Phe) called the head activator. Head activator acts on stem cells in S phase to cause nerve cell commitment. Differentiation of committed precursors requires a second signal which can be mimiced experimentally by injurying tissue. In the absence of an injury stimulus, committed precursors accumulate in Gl. Injury of the tissue then causes differentiation of these cells within 5 hours. Reaction-diffusion models of pattern formation can generate concentration profiles of "free" head activator which match the observed pattern of nerve cell commitment along the hydra body column.

Key words: Hydra - stem cell - nerve cell - commitment - differentiation - neuropeptide.

## INTRODUCTION

Patterns of cell differentiation are a central feature of embryogenesis. However, despite considerable research over nearly 100 years, very little is known about how such patterns arise beyond a formal description that "prepatterns" or "gradients" of diffusible molecules control the differentiation patterns. This lack of information is due principally to the fact that the prepatterns are only fleetingly present at a particular point during embryonic development. As such they are difficult to study.

Hydroids offer a convenient solution to this problem. As adult organisms, they appear to retain the prepatterns characteristic of the embryonic state. Thus signals

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(morphogens) controlling patterns of cell differentiation continue to be present in the adult stage where they are accessible to experimental investigation. One system which has been used extensively for such investigations in recent years is the interstitial cell system in hydra (for review see Gierer, 1977).

## THE INTERSTITIAL STEM CELL SYSTEM IN HYDRA

Interstitial stem cells in hydra continuously differentiate nerve cells and nematocytes (Bode and David, 1978). Examples of these cell types are shown in Figure 1. Cell cloning experiments have shown that the stem cells are multipotent for nerve

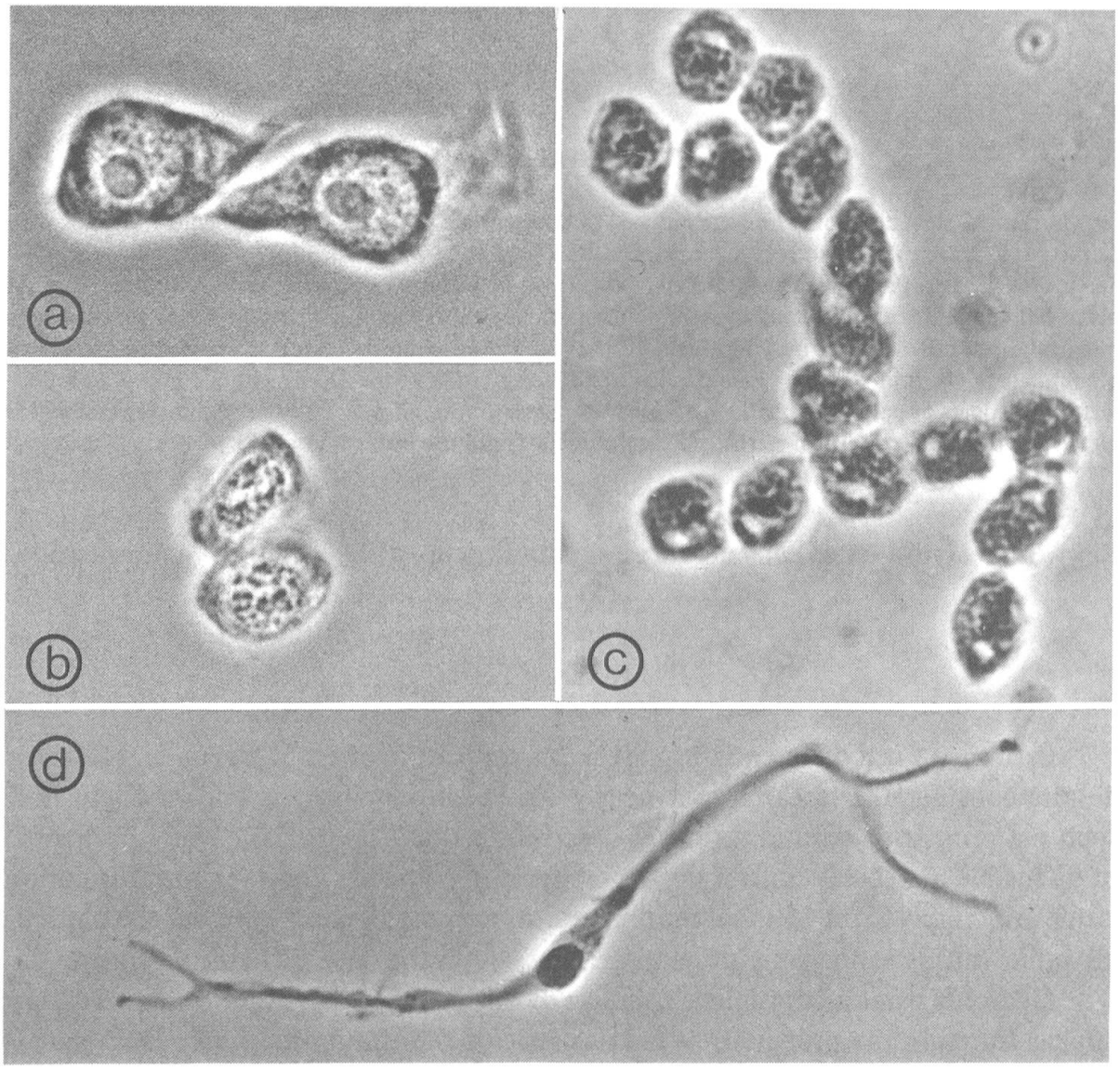


FIG. 1. — Interstitial stem cells *(a)*, committed nerve precursors *(b)*, differentiating nematoblasts *(c)* and differentiated nerve cell *(d)*. Cells were prepared by maceration of hydra tissue (David, 1973). Phase contrast; *a-c* ×1500, *d* ×1000.

and nematocyte differentiation (David and Murphy, 1977). Hence, stem cells must decide in each generation how to differentiate. The most interesting feature of interstitial cell differentiation, as a model for investigation of pattern formation, is that the decision for nerve or nematocyte differentiation depends on the position of the stem cell along the body column. Stem cells in the head and foot differentiate primarily as nerve cells; stem cells in the body column differentiate primarily nematocytes (David and Gierer, 1974; David and Challoner, 1974).

This spatial pattern of nerve and nematocyte differentiation appears to be controlled by morphogenetic signals localized in the regions of the body column. These morphogens constitute the "prepattern" and, in the case of stem cells, cause commitment to either nerve or nematocyte differentiation. To identify these morphogens we have developed an assay for committed nerve precursors and used it to search for molecules in extracts of hydra which control nerve cell commitment.

## ASSAY FOR COMMITTED NERVE PRECURSORS

The assay for committed nerve precursors is based on the premise that such cells will continue differentiation when removed from an environment causing nerve cell differentiation whereas uncommitted cells will not. Under such conditions, uncommitted cells proliferate to form more stem cells. To distinguished between committed and uncommitted nerve precursors, cells were transferred to the gastric region of hydra which does not stimulate nerve cell differentiation. Figure 2*a* shows schematically the procedure used to test nerve cell commitment. Tissue containing putative committed cells was dissociated and the cells were transferred to a culture system consisting of reaggregated hydra cells. Most of the transferred cells end up in areas of the aggre-

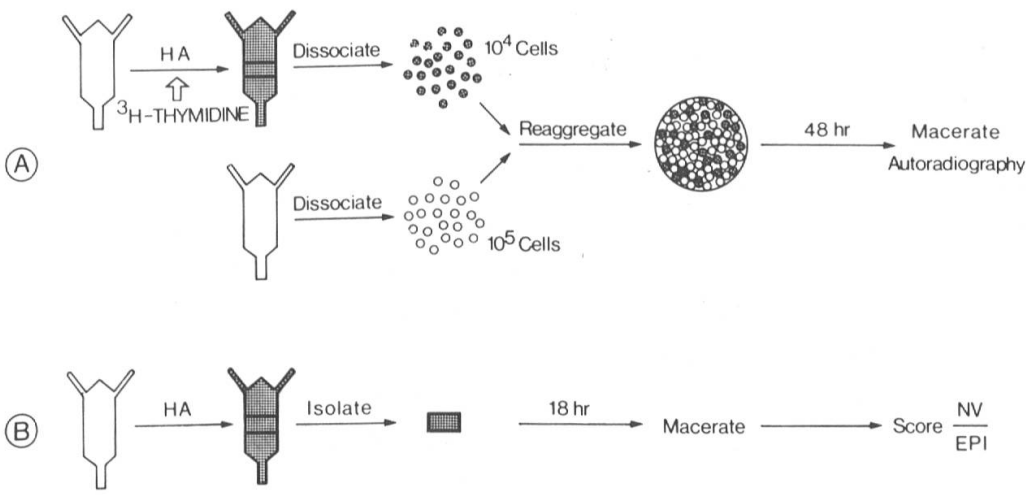


FIG. 2. — Procedures for assaying committed nerve precursors: (A) dissociation/reaggregation; (B) explantation. See text for details. HA: head activator.

gate which form gastric tissue during regeneration. Cells which differentiate nerves under these conditions are defined as "committed" (Venugopal and David, 1981*a*; Yaross *et al.,* 1982); uncommitted stem cells form clones of proliferating cells (David and Murphy, 1977).

Because the dissociation/reaggregation technique is time consuming, we have also developed a simplified explantation technique which achieves the same effect (Venugopal and David, 1981*a*). In this procedure (Figure 2*b*) tissue pieces are explanted from the animal and incubated in medium. Such pieces round up and regenerate. Since most of the tissue in the regenerate is gastric region (as in the aggregates), this procedure also tests the ability of nerve precursors to differentiate in the absence of signals stimulating nerve differentiation. Committed nerve precursors continue differentiation in such explants.

## FACTORS CAUSING NERVE CELL COMMITMENT

To identify factors causing nerve cell commitment, hydra were incubated for 12-24 hours in extracts prepared by sonication of hydra tissue. Following incubation, pieces of tissue were explanted, incubated for 18 hours to permit nerve cell differentiation and scored for increased numbers of nerve cells. Such extracts, at a concentration equivalent to 1 hydra/ml, stimulate nerve cell commitment (Holstein *et al.,* 1985). Following methanol extraction and subsequent removal of lipids with petroleum ether, all the biological activity is recovered in the methanol phase. Figure 3 shows that this extract stimulates nerve cell commitment at a concentration equivalent to the extract of 0.25 hydra/ml.

Based on earlier work of Schaller (1973), which demonstrated the presence in methanol extracts of a peptide stimulating head formation, it appeared likely that the factor causing nerve cell committment was this peptide (Schaller, 1976*a*). The peptide, which occurs in nerve cells in hydra (Schaller and Gierer, 1973), was named the "head activator" based on its morphogenetic activity. It has recently been sequenced (pGlu-Pro-Pro-Gly-Gly-Ser-Lys-Val-Ile-Leu-Phe) and can now be prepared synthetically (Schaller and Bodenmüller, 1981). Figure 4 shows that the head activator stimulates nerve cell commitment in the concentration range 0.1-10 pM.

To investigate whether head activator could account quantitatively for the nerve commitment activity in hydra extracts, the concentration of head activator in methanol extract was determined using a radioimmune assay for the peptide (Bodenmüller and Zachmann, 1983). Based on these determinations it is possible to express the activity of methanol extracts in terms of head activator content; maximally active extracts contain about 0.1 pM head activator (Figure 3). Comparison of Figure 3 and 4 indicates that essentially all the activity in methanol extract can be accounted for by its

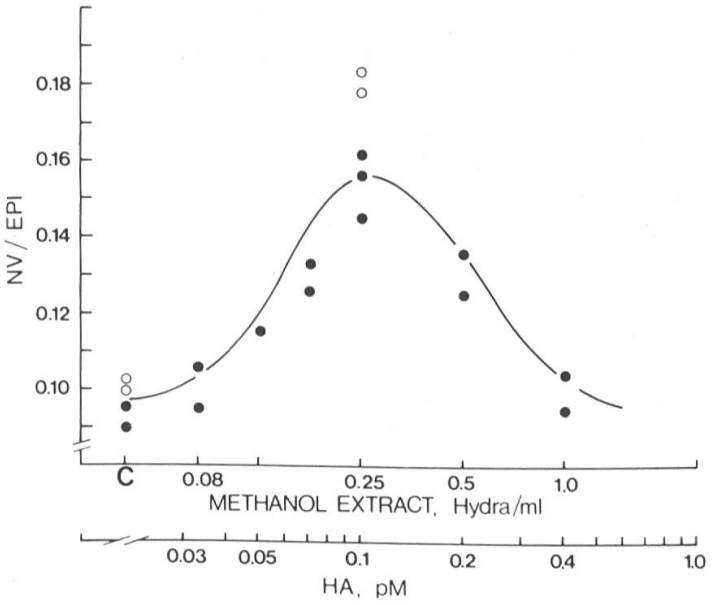


FIG. 3. — Induction of committed nerve precursors by methanol extract. Hydra were treated for 18 hours with methanol extract. Pieces of the body column were then explanted, incubated for 18 hours in hydra medium, macerated and scored for nerve cells and epithelial cells (NV/EPI). Each point represents an independent determination; open and closed symbols represent two different methanol extracts. The extract concentration is expressed in terms of hydra/ml. The concentration of head activator (HA) in the extract is based on the results of a radioimmuneassay for the head activator.

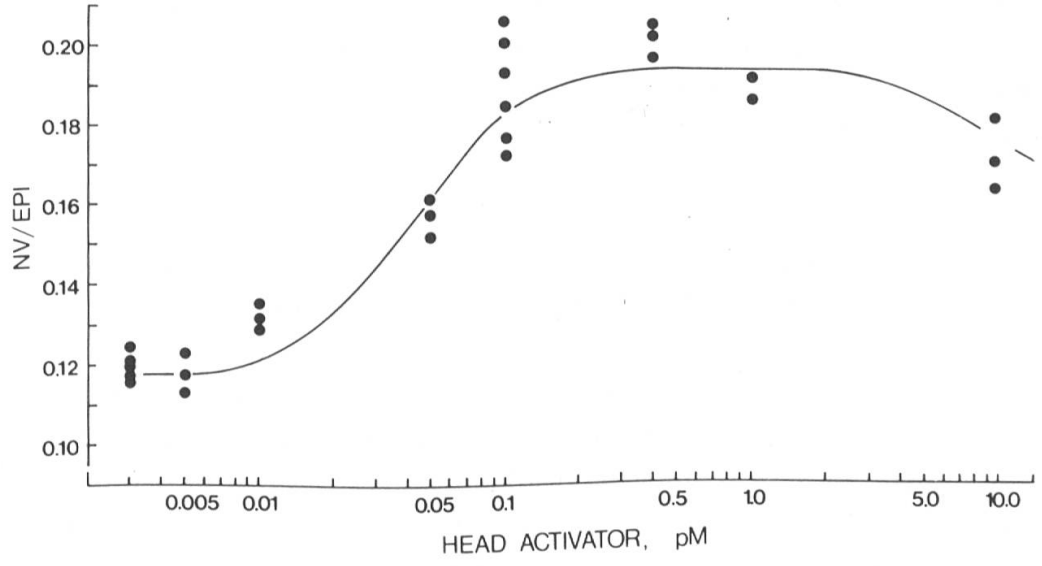


FIG. 4. — Induction of committed nerve precursors by pure head activator. Each point represents an independent experiment. See Fig. 3 for experimental details.

content of head activator. Furthermore, treatment of methanol extract with an antibody which specifically binds head activator removed the nerve cell committing activity from the extract (Holstein *et al.,* 1985). Hence we conclude that head activator is the principal factor causing nerve cell commitment in hydra.

Assuming that all the available head activator molecules are bound by hydra cells during the assay, it is possible to estimate the number of molecules required per cell to cause nerve commitment. If all cells bind head activator, about 1500 molecules are required; if only stem cells bind head activator, about 50.000 molecules are required. Such estimates are only approximate since at present nothing is know about the binding characteristics of the head activator receptor. Nevertheless, they indicate that relatively few molecules are required to cause nerve cell commitment.

## HEAD ACTIVATOR CAUSES COMMITMENT IN S PHASE

Previous work has shown that nerve cell commitment occurs in S phase *in vivo* (Berking, 1979*a*; Venugopal and David, 1981*b*; Yaross *et al.,* 1982). These experiments utilized head regeneration as a natural signal to induce nerve cell differentiation. They showed that committed nerve precursors, assayed by the techniques shown in Figure 2, appear within a few hours after the start of head regeneration and that newly differentiated nerve cells appear in large numbers from 12-24 hours later. From the kinetics of nerve cell differentiation relative to the precursor cell cycle it was possible to conclude that commitment occurred in S phase.

Similar experiments have been carried out using head activator to stimulate nerve cell commitment. The kinetics of nerve cell differentiation following head activator treatment are essentially identical to the results with head regeneration. Thus head activator also causes commitment in S phase. This result was confirmed by blocking cells in S phase with hydroxyurea, an inhibitor of DNA synthesis. Under these conditions, nerve cell commitment was completely inhibited.

## SECOND SIGNAL REGULATES DIFFERENTIATION OF COMMITTED NERVE CELLS

From the results on nerve cell commitment one would expect treatment of hydra with head activator to cause differentiation of new nerve cells. This turns out not to be true. Explantation of tissue after head activator treatment in required to cause differentiation of the committed nerve cells. Without explantation, the committed nerve cell precursors accumulate in treated tissue but do not differentiate. Figure 5 demonstrates this effect. During head activator treatment no new nerve cells differentiate. However, by 5 hours after explantation, committed precursors have differentiated

to nerve cells. Longer treatment with head activator induces more committed nerve precursors and hence more new nerve cells after explantation.

The nature of the signal provided by explantation is not known. However, it does not appear to be removal of head and foot since simply wounding tissue by placing cuts in the gastric region is sufficient to cause differentiation of the committed precursors. Thus a more likely possibility is that wounding releases a positive signal into the tissue which causes differentiation.

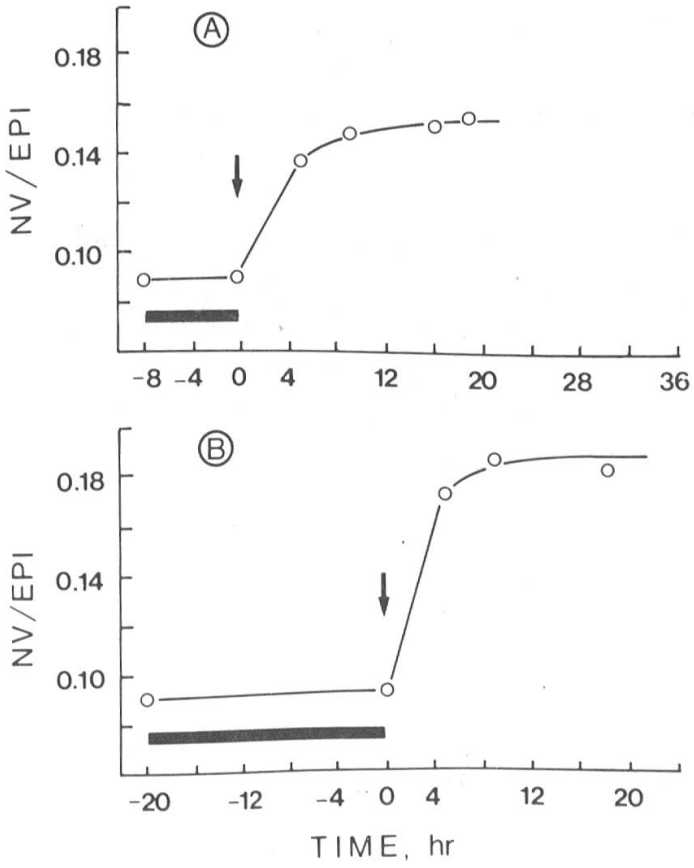


FIG. 5. — Kinetics of nerve cell differentiation in explants of hydra treated with head activator. Hydra were treated for 8 hours (A) or 20 hours (B) with methanol extract. Pieces of the body column were explanted (arrow), incubated for various times, macerated and scored for nerve cells and epithelial cells (NV/EPI).

It is interesting to note that, in terms of their effects on nerve cell differentiation, wounding and bud initiation appear to be very similar. Berking (1979*b*) demonstrated that committed nerve precursors accumulate in bud anlagen prior to bud initiation and that these cells differentiate synchronously to nerve cells within 5 hours after the onset of bud evagination. Hence wounding, although an unnatural stimulus, appears to mimic or release a natural signal in tissue.

## CELL CYCLE LOCALIZATION OF THE BLOCK TO NERVE CELL DIFFERENTIATION

The results in Figure 5 indicate that, in normal animals, committed nerve precursors accumulate at a block somewhere between the time of commitment in S phase and terminal differentiation. Since nerve cells have $2n$ nuclear DNA contents (David and Gierer, 1974), this block could lie either before or after cell division in the precursor cell cycle. Two pieces of evidence indicate that the block lies in Gl after cell division of the precursor cells (Holstein and David, 1985): (1) A wave of interstitial cell mitoses is not observed between explantation and nerve cell differentiation. (2) A population of small interstitial cells (Figure 1) with Gl nuclear DNA content accumulates in head activator treated animals. This population disappears within 1-2 hours after explanation and just prior to nerve cell differentiation. Furthermore careful observations indicate numerous morphologial intermediates between the small interstitial cells and differentiated nerve cells.

Figure 6 summarizes schematically the temporal features of nerve celle differentiation as derived from the experiments discussed above. Head activator interacts during S phase with uncommitted stem cells to cause commitment to the nerve differentiation pathway. Committed precursors then proceed through G2 and mitosis before arresting in Gl. Following explantation (or injury) arrested cells complete nerve cell differentiation within about 5 hours.

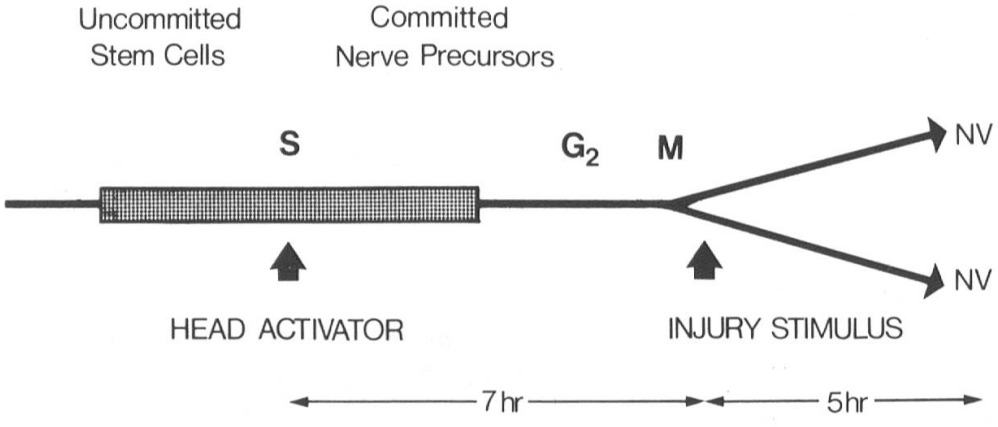


FIG. 6. — Temporal sequence of events during nerve cell differentiation from uncommitted stem cells. Arrows indicate the time of action of head activator and the injury stimulus. S, G2, and M are cell cycle phases; Nv: nerve cell.

## PATTERN OF HEAD ACTIVATOR CONTROLS PATTERN OF NERVE CELL DIFFERENTIATION

Using the nerve cell committment assay, Venugopal and David (1981*c*) demonstrated that nerve cell commitment is strongly patterned along the body column: most stem cells in head are committed to nerve cell differentiation whereas in the gastric region only relatively few stem cells are committed to this pathway (Figure 7). Since nerve cell commitment is caused by head activator, it is now possible to infer that head tissue contains high levels of "free" head activator whereas gastric tissue does not. Thus the head activator is assymetrically distributed along the body column.

Regeneration studies provide further evidence that the pattern of "free" head activator controls nerve cell commitment *in vivo*. Nerve cell commitment increases sharply during head regeneration at a cut surface in the gastric region (Venugopal and David, 1981*a*). Such gastric tissue normally has low levels of "free" head activator and expresses a low rate of nerve cell commitment (see above). Following removal of the head to initiate regeneration, head activator is released between 2-4 hours at the cut surface (Schaller, 1976*b*). Commitment of new nerve cells begins at about the same time (Venugopal and David, 1981*a*). Hence, also in this case, the pattern of nerve cell commitment is controlled by head activator.

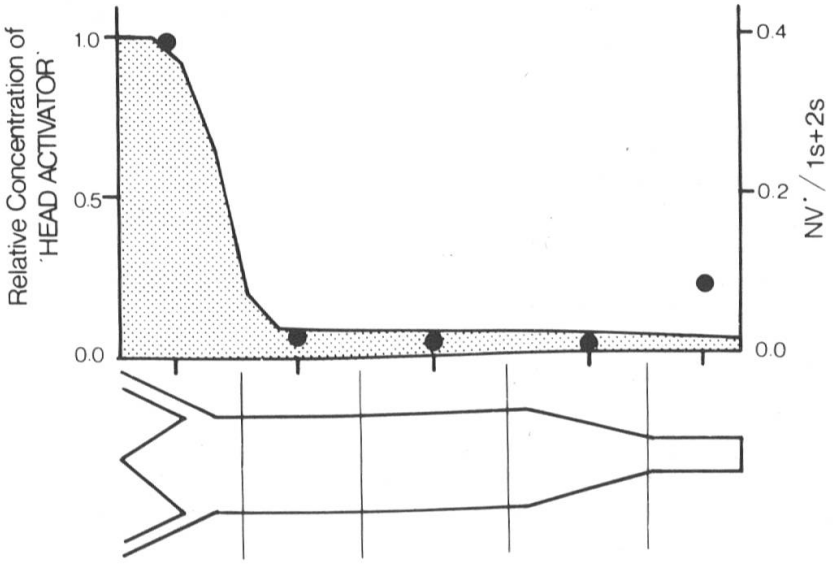


FIG. 7. — Comparison of the concentration profile of a hypothetical head activator generated by a reaction-diffusion mechanism with the rate of nerve cell commitment at different positions along the body column of hydra. The head activator profile (shaded area) was calculated by computer simulation using MacWilliams's version of the Gierer-Meinhardt model (modified from Fig. 13*c* of MacWilliams (1983)). The experimental points (closed circles) indicate the rate of nerve cell commitment in the stem cell population (expressed as the number of newly differentiated ($^3$H-thymidine labeled) nerve cells (NV) divided by the size of the stem cell population (1s+2s). The experimental values are taken from Fig. 3*b* of Venugopal and David (1981*c*).

The present experiments provide the first clear evidence for a model in which a spatial pattern of cell differentiation is controlled by a spatial pattern of a diffusible molecule (the "prepattern"). In this case the head activator is the essential molecule in the prepattern controlling nerve cell commitment. Although the present experiments do not indicate how the head activator concentration profile is established, there are several mechanisms available to establish such prepatterns (Gierer and Meinhardt, 1972; MacWilliams, 1983). Figure 7 shows one such example in which the concentration profile of a "head activator" has been generated by a Gierer-Meinhardt reaction-diffusion mechanism. The profile closely parallels the observed pattern of nerve cell commitment and hence the pattern of "free" head activator which was inferred from it.

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