Development 102, 223-235 (1988)
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Development of the two-part pattern during regeneration of the head in hydra

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Summary

The head of a hydra is composed of two parts, a domed hypostome with a mouth at the top and a ring of tentacles below. When animals are decapitated a new head regenerates. During the process of regeneration the apical tip passes through a transient stage in which it exhibits tentacle-like characteristics before becoming a hypostome. This was determined from markers which appeared before morphogenesis took place. The first was a monoclonal antibody, TS-19, that specifically binds to the ectodermal epithelial cells of the tentacles. The second was an antiserum against the peptide Arg–Phe–amide (RFamide), which in the head of hydra is specific to the sensory cells of the hypostomal apex and the ganglion cells of the lower hypostome and tentacles. The TS-19 expression and the ganglion cells with RFamide-like immunoreactivity (RLI) arose first at the apex and spread radially. Once the tentacles began evaginating in a ring, both the TS-19 antigen and RLI+ ganglion cells gradually disappeared from the presumptive hypostome area and RLI+ sensory cells appeared at the apex. By tracking tissue movements during morphogenesis it became clear that the apical cap, in which these changes took place, did not undergo tissue turnover. The implications of this tentacle-like stage for patterning the two-part head are discussed.

Key words: hydra, regeneration, pattern formation, two-part pattern, head, monoclonal antibody.

Introduction

The basic structure of hydra is a hollow cylinder composed of two tissue monolayers, an ectoderm and an endoderm separated by a thin extracellular matrix, the mesoglea. At the base of the cylinder is a holdfast, which is simply a disc of ectodermal cells secreting adhesive substances. The head, however, is a more complicated two-part structure. The upper half is a smooth dome or cone, the hypostome, that opens to serve as a mouth. The lower half is the tentacle zone from which six or seven tentacles protrude.

When the head is removed from an animal a new head regenerates. Much has already been learned about the patterning process, such as the factors affecting its location, as well as information on its properties and kinetics as it develops (see Bode & Bode, 1984*b* for review). However, most analyses yielded information only on the presumptive head as a unit. An important question concerning this process is how the two parts of the head dome arise. Recent observations on tentacle regeneration in adult budding animals and the most common abnormalities that result have suggested a possible sequence of events (Bode & Bode, 1987).

The apical end heals over rapidly after decapitation. After one day of regeneration the first one or two tentacles are visible in some animals. These occasionally emerge from a small area at the apex. During the next two days new tentacles appear in more basal positions, forming a ring around the dome. Some of the early tentacles may remain above the ring, well within the hypostome area where the mouth would normally open. Occasionally regeneration is incomplete and only a single tentacle forms at the apical end.

The interpretation proposed for these results was that the patterning process begins at the apical tip where it advances to a stage that initiates tentacle formation. Ultimately this area progresses to a new level which leads to hypostome development. In the meantime, patterning continues to spread radially until the adjacent tissue attains the tentacle-forming level.

To visualize the pattern before morphogenesis took place, markers for the two head areas were used: a monoclonal antibody (TS-19) specific for tentacle ectodermal cells and an antiserum against Arg–Phe–amide (RFamide) which stained neurones specific to the upper and lower portions of the head. In this way, the early events in the development of the two-part pattern could be followed (preliminary description, Awad *et al.* 1987).

Materials and methods

Maintenance of animals

Hydra attenuata were used for all experiments. Cultures were maintained as described previously (Dunne *et al.* 1985). Animals were starved for one or two days before decapitation.

Immunocytochemistry

The monoclonal antibody, TS-19, was generated by S. Heimfeld (Heimfeld *et al.* 1985; Bode *et al.* 1986) according to procedures described by Oi & Herzenberg (1980) and modified for use with hydra by Dunne *et al.* (1985). Ascites fluid containing this antibody was produced with standard procedures (Yaross *et al.* 1986).

The binding pattern of TS-19 was visualized on live animals using a modification of the indirect immunofluorescence procedure described by Dunne *et al.* (1985). The animals were incubated in a 1:100 dilution of ascites fluid containing TS-19 in hydra medium for 2 min, washed twice in hydra medium, and then incubated with a 1:50 dilution of FITC-goat anti-mouse immunoglobulins (Antibodies Inc.) in hydra medium for 2 min. For examination with fluorescence microscopy, stained animals were relaxed in 2 % urethane in hydra medium for 2 min and then placed on glass slides in a drop of hydra medium. To prevent crushing the animal, ridges of silicone grease were placed around the drop before the cover slip was added. For photography, the animals were fixed immediately poststaining by a sudden immersion in 50 % ethanol and mounted as described above.

Rabbit antiserum 146II to the sequence Arg–Phe–amide (RFamide) was produced by Grimmelikhuijzen (1985). The spatial pattern of neurones in hydra showing RFamide-like immunoreactivity (RLI) was visualized using a whole-mount technique described by Dunne *et al.* (1985), and a procedure for staining RLI+ neurones as described by Grimmelikhuijzen (1985), with some minor modifications. Live animals were first relaxed in 2 % urethane in hydra medium for 2 min and then fixed for about 18 h at 0°C in Zamboni's fixative (0·15 M-NaCl, 0·1 M-phosphate, pH 7·2, containing 2 % paraformaldehyde, 15 % (v/v) saturated picric acid). RFamide antiserum was diluted 1:2000 with PBS–Triton–BSA (0·15 M-NaCl, 0·01 M-phosphate buffer, pH 7·2, 0·25 % Triton X100, 0·25 % bovine serum albumin, 0·01 % sodium azide). Incubation in the RFamide solution was carried out overnight at 0°C, followed by incubation in the secondary antibody for 1 h at room temperature. The secondary antibody was FITC-conjugated goat anti-rabbit immunoglobulins (Antibodies Inc.) which had been diluted 1:120 in PBS–NCS (PBS containing 10 % neonatal calf serum (Irvine Scientific), 0·1 % sodium azide).

Marking animals to follow tissue movements

India ink (Pelikan) diluted 1:2 with hydra medium was used as a vital stain to mark the tissue of the ectoderm and the endoderm of regenerating animals (Campbell, 1973; Marcum & Campbell, 1978; Otto & Campbell, 1983). To mark the endoderm, ink was injected through the mouth into the gastric cavity 5 h after feeding. There it was phagocytosed by the endodermal epithelial cells. To mark the ectoderm, ink was blown at the outer tissue layer with a micropipette where it penetrated the surface of the ectodermal cells producing small spots.

Results

Changes in the distribution of a tentacle-specific antigen during head regeneration

To determine where and when tentacle-like characteristics appear during the course of head development, a tentacle-specific monoclonal antibody was used. The antibody, designated TS-19, stains the entire tentacle intensely (Fig. 1A,B). The staining ends abruptly at the base of the tentacle and no stain is found in the hypostome (Fig. 1B) or adjacent body column (Fig. 1A). TS-19 also stains the lower peduncle lightly plus a ring around the basal disc (Fig. 1A). Histological sections indicate that the antigen is restricted to the apical surface of the ectodermal cells (data not shown).

The dynamics of TS-19 reappearance were followed during regeneration of the head. Animals were decapitated at either of two points (Fig. 4, inset), directly below the tentacles (group A) or at one-quarter the length of the body column (group B), to determine if axial level makes a difference in the type of pattern obtained. Groups of regenerates were stained every 4 h over a period of 3 days.

Seven stages were observed in the staining pattern of TS-19, and are presented schematically in Fig. 2. Stage 1 was characterized by the appearance of a small stained area at the apex of the animal (Fig. 3A). At stage 2 a larger area of the regenerating tip was stained and the staining intensity had increased (Fig. 3B). By stage 3 the tentacles were beginning to evaginate from within the stained area (Fig. 3C). The stain in the developing tentacles reached normal

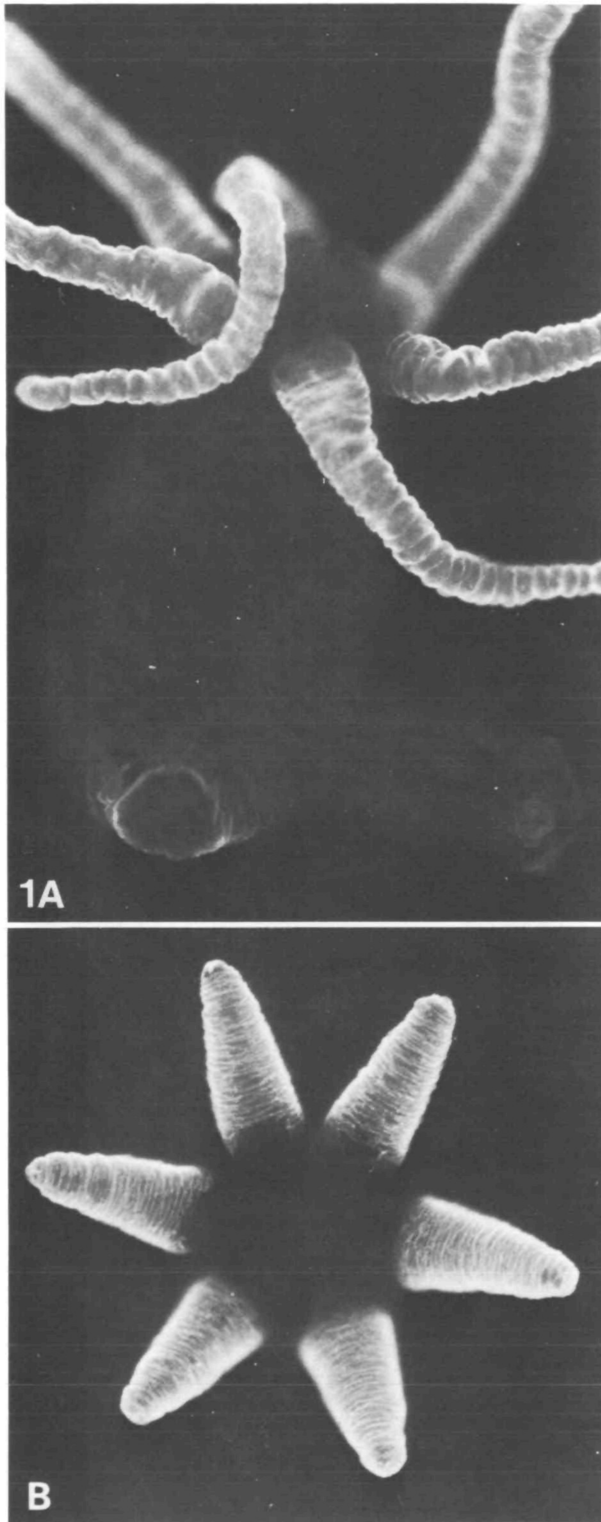


Fig. 1. *H. attenuata* stained with the monoclonal antibody TS-19. (A) Side view of a budding adult. (B) View of the head from above, looking down on the hypostome. ×45.

levels while in the area between the tentacles it began to fade (stage 4; Fig. 3D). At stage 5 the stain receded, often in a 'star' pattern with strings of cells connected to the evaginating tentacles (Fig. 3E). During stage 6 the nontentacle cells gradually lost the last of their staining capacity (Fig. 3F). Stage 7 was the resumption of the normal pattern. The hypostome had formed and was free of fluorescence. The tentacles were intensely fluorescent, although still adding to their length and occasionally to their number.

The animals decapitated at level A and at level B showed the same pattern of staining as the head developed, differing only in the time of onset of staining. TS-19 was first expressed in most level A animals by 20 h, while in group B this occurred 8 h later (Fig. 4). At each time point thereafter the animals in group A were on average one stage ahead of those in group B. (The time line in Fig. 2 refers to group B animals, for comparison with the neurone appearance described in the next section.)

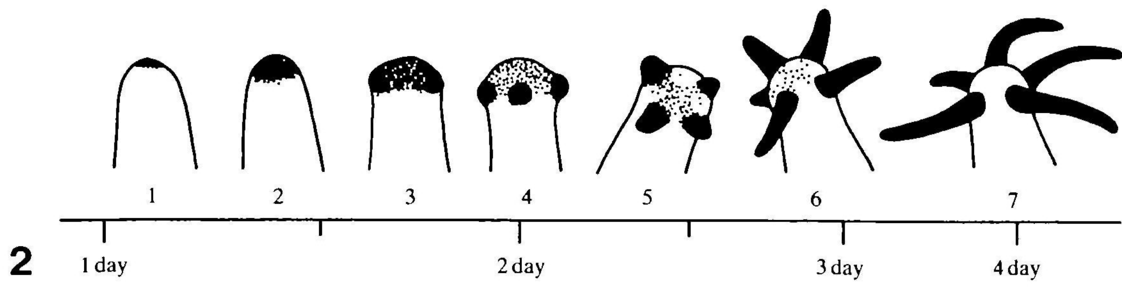
Buds have a similar staining pattern as they develop, except that the hypostome stain simply fades from the hypostomal area (Fig. 1A) rather than retreating in a 'star' pattern. Thus, the general pattern of head development as visualized by TS-19 suggests that the most apical region first takes on tentacle-like characteristics. 'Tentacleness' spreads to the level of the future tentacle ring, finally localizing in the new tentacles, and eventually disappears from the remainder of the head.

Pattern of appearance of nerve cells specific to the upper and lower portions of the head during regeneration

To obtain a more complete picture of the development of the two-part head pattern, spatial and temporal information on the appearance of hypostome characteristics was needed as well. This was provided by an antiserum to the dipeptide RFamide, which binds two subsets of neurones with different distributions within the head (Grimmelikhuijzen, 1985).

Epidermal sensory cells serve as a marker for the hypostome. They are found only at the apical tip, clustered around the mouth region (Kinnamon & Westfall, 1981). A large number of them exhibit RFamide-like immunoreactivity (RLI+; Fig. 5A,B). A corresponding marker for the lower portion of the head is RLI+ ganglion cells. They are located in the basal portion of the hypostome (Fig. 5A,B), between the tentacles (Fig. 5C), in the tentacles (Fig. 5D), in the region just below the head, and in the lower peduncle.

These two types of RLI+ neurones reside in the ectoderm of the animal and are easily distinguished by their morphology. The cell bodies of the epidermal sensory cells are elongate and extend from the basal to the apical edge of the ectoderm (Westfall, 1973; Fig. 5A). Their processes project from the basal end of the cells near the mesoglea and extend down the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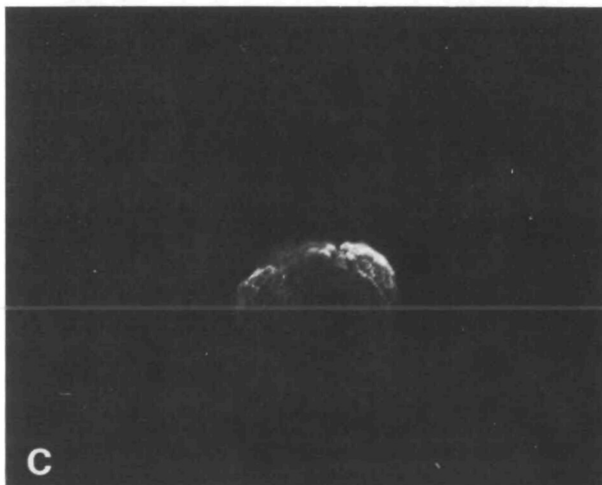
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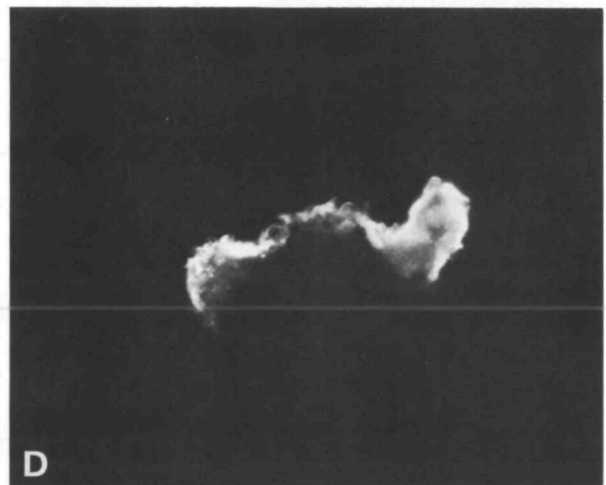
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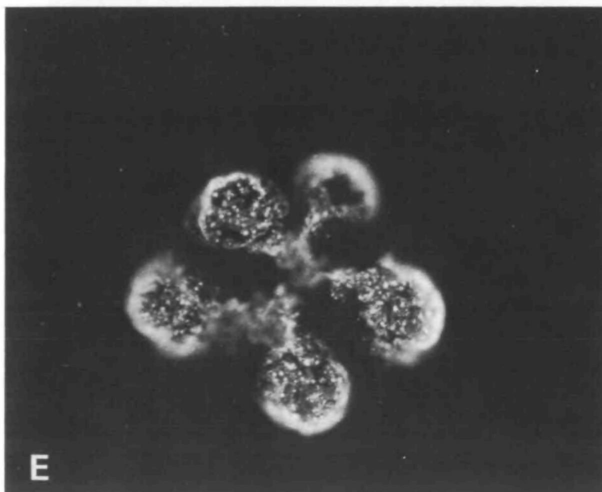
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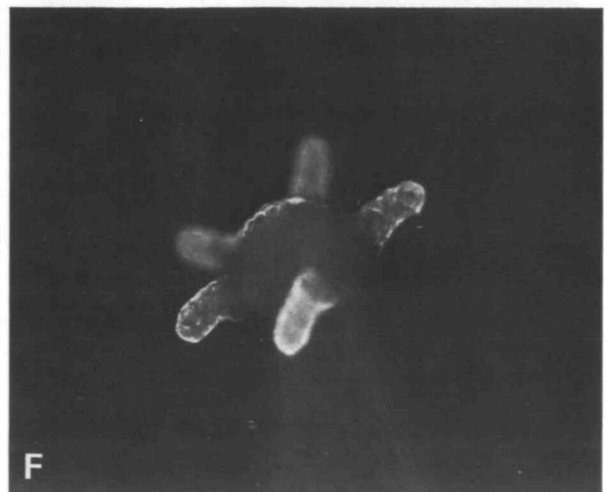
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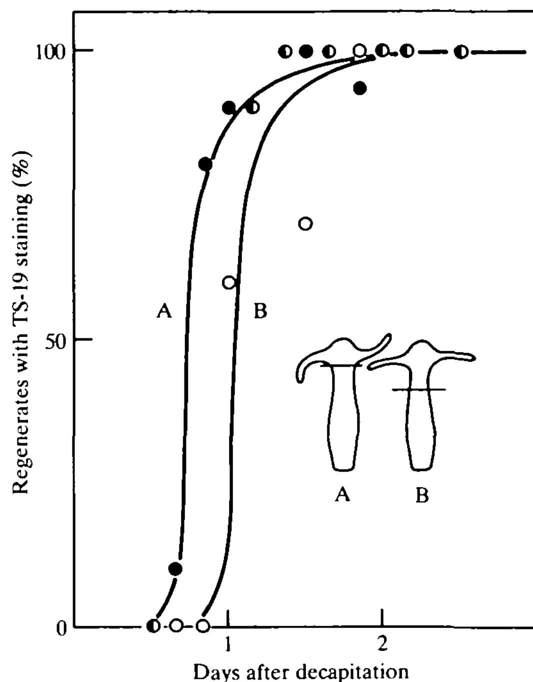


Fig. 4. Onset of TS-19 staining in regenerates decapitated at two axial levels: (A) Just under the tentacles and (B) at one-quarter the length of the body column. Each point represents 10 animals.

hypostome (Fig. 5B). In contrast, the cell bodies of the ganglion cells are more spherical and are located at the base of the ectoderm. Ganglion cells are multipolar, with processes that project from any part of the cell body and extend in all directions (Fig. 5C,D).

To follow the reappearance of these two subsets of neurones during regeneration of the head, animals were decapitated at one-quarter the distance along the body column (Fig. 4B in inset). The more basal level for cutting was chosen to ensure that all RLI+ neurones in the upper part of the body column were eliminated. Groups of regenerating animals were stained every 12 h over a 5 day period.

The seven stages observed in sensory and ganglion cell reappearance are drawn schematically in Fig. 6. Initially, no RLI+ neurones were visible (Fig. 7A). The first RLI+ neurones appeared at the apex of the regenerating tip (stage 1, Fig. 7B). They were dimly stained, but identifiable as ganglion cells by their shape and their location at the base of the ectoderm. Also, although the processes were short and immature, the cells were clearly multipolar. At stage 2 their numbers had increased and the processes were longer. Stage 3 was characterized by the beginning of tentacle evagination and further spread of the ganglion cells. By stage 4 the processes of the ganglion cells had extended sufficiently to form the beginnings of a net throughout the presumptive head (Fig. 7C). Occasional cells were now visible at the apex, which were more elongate, and were tentatively identified as sensory cells (Fig. 7C, arrows). At stage 5 sensory cells were clearly present at the apex, distinguishable by their cell bodies which extended through the ectoderm (Fig. 8A,B). By stage 6 the numbers of sensory cells increased and their processes could be seen. At stage 7 the numbers of ganglion cells at the apical end had decreased noticeably and the sensory cells filled the apex (Fig. 8C). The processes of the sensory cells were well-developed and extended down the hypostome. The ganglion cells were located mainly in the tentacles and adjacent areas (Fig. 8D).

The time course of appearance of RLI+ cells is shown in Fig. 9. The first ganglion cells appeared at 1·5 days, and no sensory cells were evident. At 2·0 days some sensory cells were visible in many animals. Sensory cells were present in all animals by 2·5 days.

Development of the head pattern as revealed by RLI+ neurones follows a course similar to that found with the TS-19 antigen. Ganglion cells, typically found in the lower portion of the head and on the tentacles, appear first at the apical tip. Gradually sensory cells replace them, marking the tip as the mouth area of the hypostome. Thus, the apical tip passes through a stage of 'tentacleness' before it progresses on to its final hypostome expression.

Pattern of tissue movement into the head during regeneration

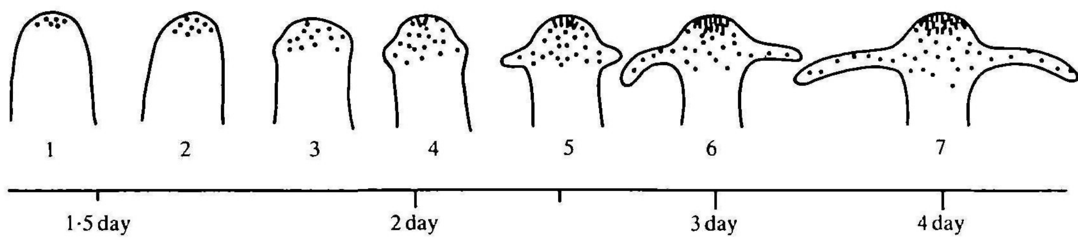
One explanation for the loss of TS-19 staining and the change in nerve distribution is a turnover of tissue at the regenerating tip. It is conceivable that the apical tissue with tentacle characteristics gradually passes onto the tentacles as they evaginate, to be replaced by tissue moving in from the body column which has (or develops) hypostome characteristics. This would be consistent with the 'star' pattern of disappearance of the TS-19 antigen.

To determine whether the changes at the apical tip resulted from tissue movements during morphogenesis, the tissue was marked. First, to visualize the front of tissue as it moves into the growing head, the endoderm of animals was stained with India ink (Campbell, 1973; Marcum & Campbell, 1978). One day later, stained and unstained animals were cut at one-quarter the length of the body column and

Fig. 2. Schematic representation of the seven stages in TS-19 expression during regeneration of the head (see text). The line below indicates the time course for animals decapitated at one-quarter the length of the body column (Fig. 4B in inset).

Fig. 3. Reappearance of the TS-19 antigen during regeneration of the head. The developing head is seen from the side in A–D and F and from the top in E. ×45.

5A B C D



reciprocal grafts were made (Fig. 10). After healing for 2–3 h the animals were decapitated under the tentacles. The advancement of contrasting tissue was monitored as regeneration progressed over a period of 6 days.

Two examples illustrating the typical pattern of endoderm movement are shown in Fig. 11. As the tentacles began to evaginate, the border between the dark and light tissue moved up the body column toward the head. Once at the head, it passed between the tentacles and continued to move out along their length. However, the border did not advance any further into the hypostome. The apical cap remained basically intact with only small losses and gains of cells at its edges. (Note the abortive attempt at tentacle formation at the apex in Fig. 11B.)

To observe the movement of the ectoderm, where the TS-19 antigen and the RLI+ neurones reside, spots of India ink were used as markers in a second regeneration series. The general pattern was found to be the same, with some details of the pathways clarified. Tissue that moved up directly beneath an evaginating tentacle, continued out along its underside (Fig. 12A). Tissue that moved into the tentacle zone between two tentacles split and passed onto the side and upper surface of both (Fig. 12B). Newly arrived tissue did not penetrate the apical cap and any increase in size came apparently from cells that remained at its edge (Fig. 12C,D). The tissue at the apical end remained stationary throughout (Fig. 12D).

In other words, the evaginating tentacles recruited tissue almost exclusively from the body column, and not from the apical tip. The apical region underwent little if any tissue turnover. This is similar to the movement of tissue in the normal animal during steady-state growth (Campbell, 1967). Therefore, the changes in antibody staining and neurone type at the apex must have all occurred within the same tissue.

Since the epithelial cells of the apical tip do not move and there is no evidence that mature nerve cells migrate (Heimfeld & Bode, 1984), the disappearance of the ganglion cells must be explained. One possibility is that they died due to incompatibility with a

Fig. 5. RLI+ neurones in the head of a normal animal. (A) Tangential views of a ganglion cell (arrow) and several sensory cells (upper right). Apex is towards the upper right, out of the photograph. (B) Distribution of RLI+ neurones in the hypostome. Sensory cells are found around the mouth at the apex of the hypostome (upper right). Ganglion cells are located in the lower part of the hypostome (base of a tentacle at lower left). (C) Ganglion cells in the tentacle zone (base of a tentacle at middle right). (D) Ganglion cells in the tentacles. ×350.

Fig. 6. Schematic representation of the seven stages in RLI+ nerve net formation during regeneration of the head (see text). Animals were decapitated at one-quarter the length of the body column (Fig. 4B in inset). The line below indicates the time course of regeneration. The circular symbols represent the ganglion cells and the oblong symbols represent the sensory cells. The nerve processes were omitted for the sake of clarity.

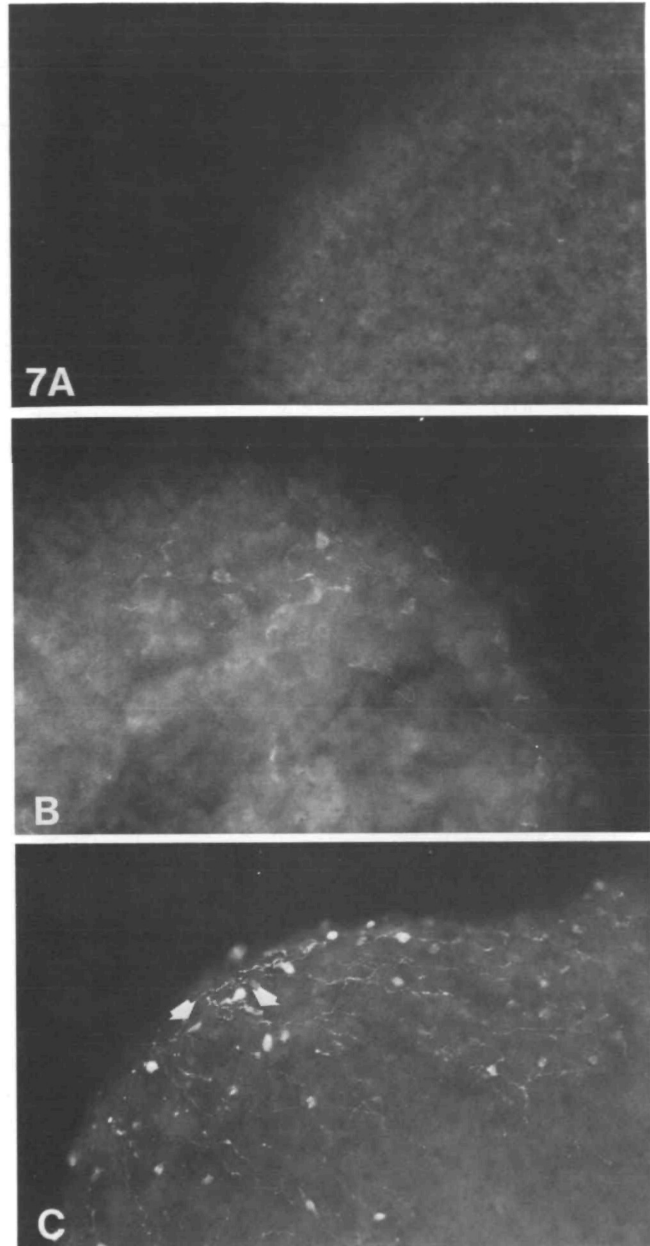


Fig. 7. Reappearance of RLI+ neurones during regeneration of the head (see text). The photographs are side views of the apex of regenerating animals. (A) No RLI+ neurones visible. (B) A few RLI+ ganglion cells at the apex. (C) Spread of RLI+ ganglion cells. Possible RLI+ sensory cells at apex (arrows). Beginning tentacle in upper right corner. (A,B) ×350; (C) ×245.

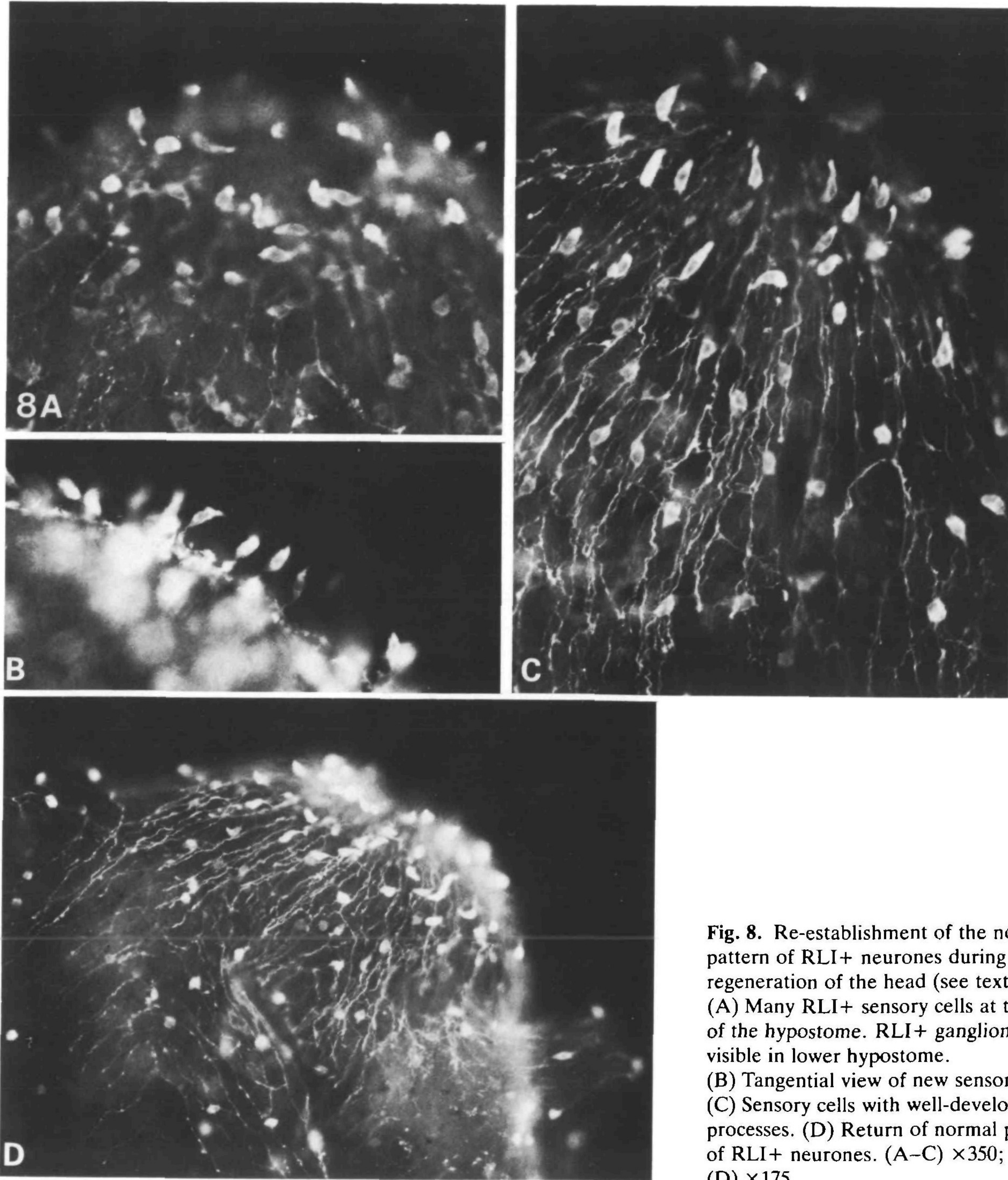


Fig. 8. Re-establishment of the normal pattern of RLI+ neurones during regeneration of the head (see text). (A) Many RLI+ sensory cells at the apex of the hypostome. RLI+ ganglion cells visible in lower hypostome. (B) Tangential view of new sensory cells. (C) Sensory cells with well-developed processes. (D) Return of normal pattern of RLI+ neurones. (A–C) ×350; (D) ×175.

changed environment (shown for nematocyte intermediates; Yaross & Bode, 1978). More likely they have changed phenotype, becoming either sensory cells or simply RLI− ganglion cells (Koizumi & Bode, 1986; Bode *et al.* 1986; Koizumi *et al.* 1988).

Discussion

Patterning the head as a unit during regeneration

Considerable information has been obtained on the regenerating head as a unit. The position of the presumptive head within the body tissue is influenced by two gradients: a stable gradient of head-forming potential, the head activation gradient, and a labile gradient emanating from a head, the head-inhibition gradient (Webster, 1966; Hicklin *et al.* 1973; Macwilliams, 1983*a*,*b*). After decapitation, the initiation of a patterning process can be detected within 2 h. The head activation level rises rapidly at the apical end, as measured by its increasing ability to induce secondary heads when transplanted into a host animal. By 6–10 h it reaches a stage when it is 'determined' and

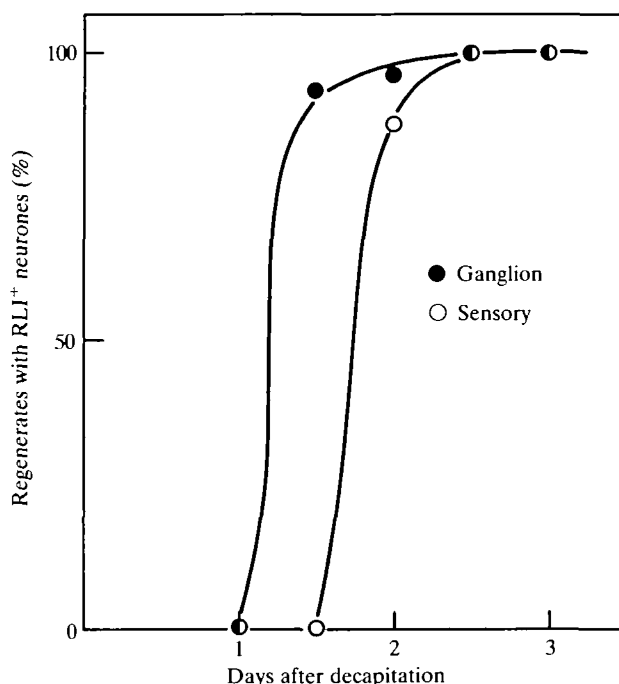


Fig. 9. First appearance of RLI+ ganglion cells (closed circles) and RLI+ sensory cells (open circles) during regeneration of the head. Each point was obtained from two to four separate experiments and represents 20–40 animals.

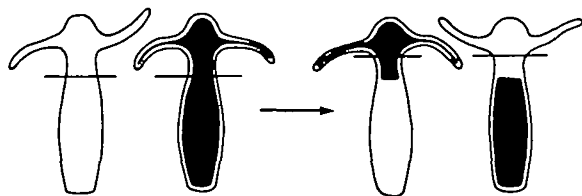


Fig. 10. Diagram illustrating the preparation of reciprocal grafts of animals with carbon-marked endoderm. The horizontal lines indicate the point of cutting.

can no longer be stopped by head inhibition if transplanted (Webster & Wolpert, 1966; Hicklin *et al.* 1975). Thereafter, the activation process can be followed with a head-specific monoclonal antibody, CP-8, as it spreads from the apex to eventually encompass all the tissue that forms the head (Javois *et al.* 1986). The point at which the pattern ceases to expand is regulated, since the size of the regenerated head is related to the overall size of the tissue (Bode & Bode, 1980, 1984*a*).

Division of the pattern into hypostome and tentacle zone

One of the many remaining questions involves the development of detail within the 'activated' region. How the pattern progresses through the division into hypostome and tentacle zone is suggested by the results. The most apical region, the eventual hypostome area, transiently exhibits characteristics associated with a tentacle-forming area. This was shown by markers that appear before morphogenesis begins. In fact for a brief time the area may actually be competent to form tentacles, since in adult animals the first one or two tentacles occasionally begin to evaginate there (Bode & Bode, 1987; Fig. 12B). Because the apical tissue remains stationary, these changes first to 'tentacleness' and then to hypostome cannot be explained by tissue movements. This implies that it is the underlying patterning process which is undergoing the transformation.

This type of pattern progression provides an explanation for the outcome of incomplete regeneration. Under several circumstances, one or two tentacles are all that form. This is observed in decapitated animals in which head regeneration is partially inhibited by buds, and in induced secondary axes partially inhibited by a host head (described in Webster, 1971; Rubin & Bode, 1982). Isolated tentacles not associated with a head are found after regeneration of tissue with certain shapes (Bode & Bode, 1980), and after regeneration of aggregates of cells (Gierer *et al.* 1972). A regeneration-deficient mutant, *Reg-16* (Sugiyama & Fujisawa, 1977), occasionally produces only one or two tentacles, if regeneration is initiated at all. In these animals only RLI+ ganglion cells are found at the apical end and RLI+ sensory cells are absent (O. Koizumi, unpublished results). All these observations are consistent with the idea that the tissue rises to a tentacle level of competence, and remains there.

Implications of the tentacle zone stage for models of pattern formation

Three models have been applied to hydroids to explain how the different apical structures might arise during regeneration of the head. The fact that the apical tip passes through a stage characteristic of the tentacle zone eliminates one of them, but is consistent with the other two.

The first model is a cascade mechanism, in which a series of structures is patterned in a sequential fashion (Rose, 1967; see also Cooke, 1982). The patterning process is initiated upon removal of all apical structures. Apical structure 1 would always be patterned first. This process would eventually inhibit itself, preventing further tissue from becoming part of structure 1. The patterning of structure 2 would then be triggered, and the process repeated. For the

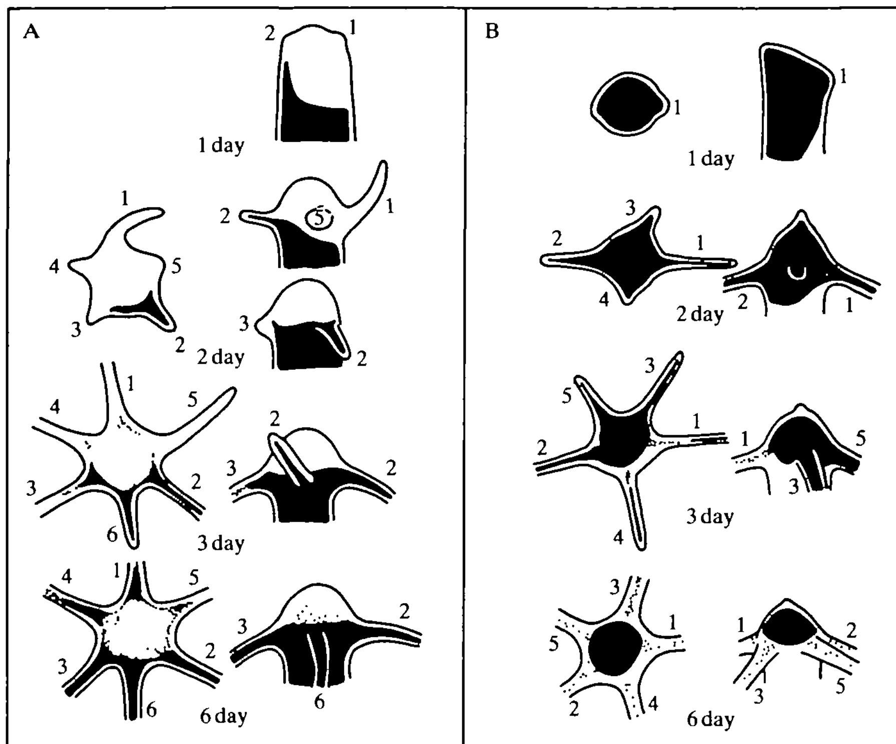


Fig. 11. Tissue movement into the head during regeneration as indicated by the boundary between unmarked and carbon-marked endoderm. The regenerates were drawn to scale using a dissecting microscope with an ocular micrometer. The series on the left are top views looking down on the hypostome and those on the right are side views. The tentacles are numbered in order of appearance for purposes of orientation.

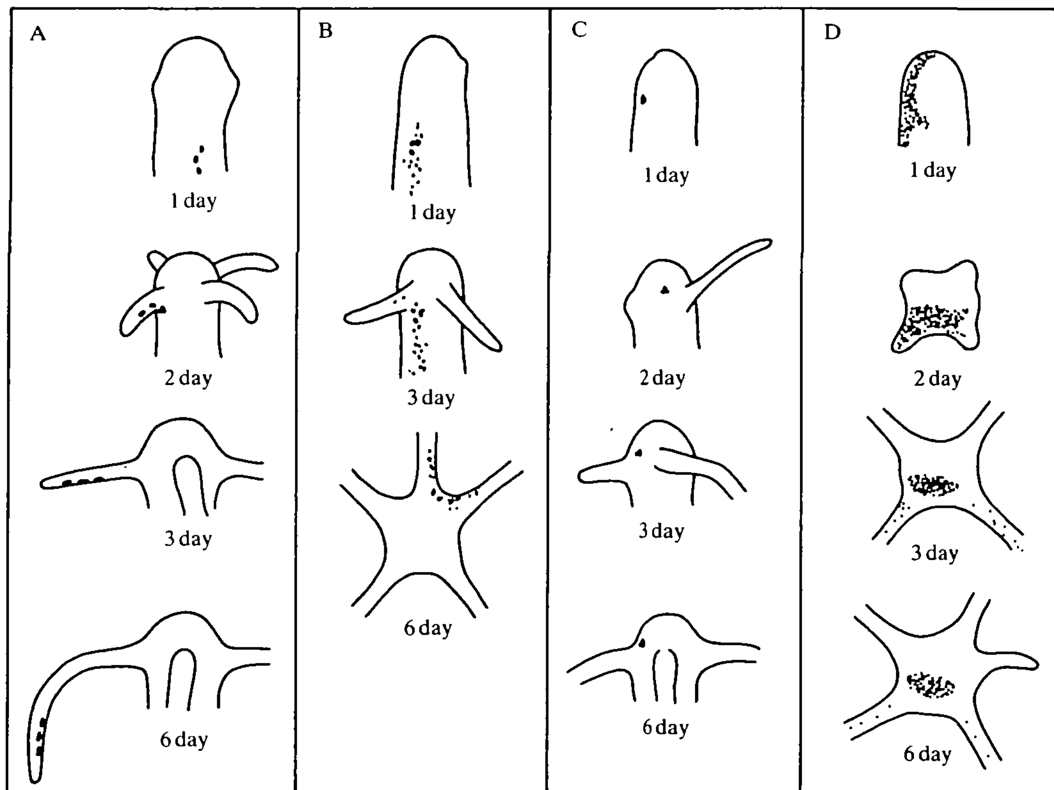


Fig. 12. Detailed pattern of tissue movement during regeneration of the head. The spots in the ectoderm were made with India ink. The regenerates were drawn to scale using a dissecting microscope with an ocular micrometer. Either top views or side views are shown.

regeneration of a head in hydra this would mean that the hypostome would be established first, and only thereafter would the patterning of the tentacle zone be initiated. This is at variance with the progression of the pattern observed experimentally. Also, incomplete regeneration with such a mechanism should

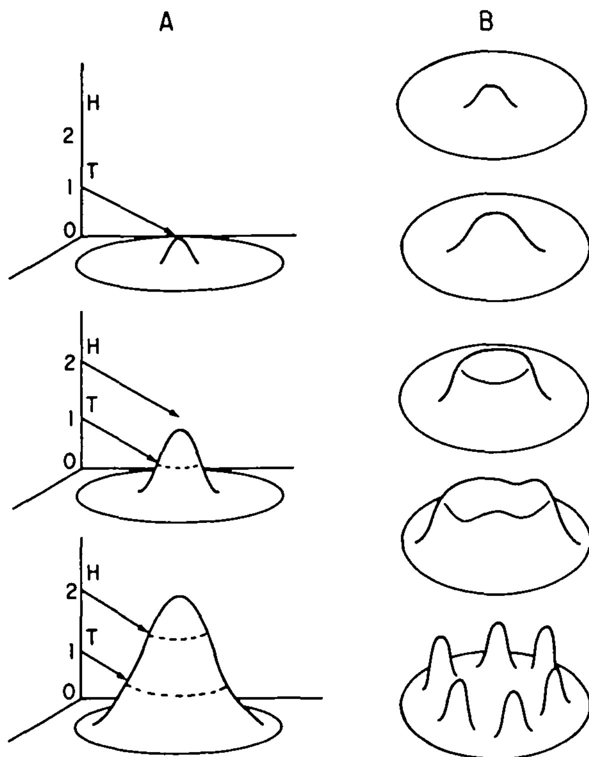


Fig. 13. Profiles of concentrations of hypothetical morphogens during regeneration of the head. (A) Two-step interpretation of a rising and spreading morphogen peak. Between concentration levels 1 and 2 'tentacle zone' (T) is expressed. Above concentration level 2 'hypostome' (H) is expressed. (B) Translocation of morphogen peaks signalling 'tentacle' (after Lacalli, 1980, fig. 1). A peak appears first in the centre. This transforms into a crater and finally into a ring of peaks.

yield just a hypostome and no tentacles instead of the opposite.

The other two models predict the transient tentacle zone stage. The second is a positional information model (Wolpert, 1971) which suggests that the same patterning process produces both areas of the head. In the simplest form this could be accomplished by a gradient of morphogen or morphogen sources (e.g. Gierer & Meinhardt, 1972; Wolpert *et al.* 1974). Interpretation of the gradient would occur in a stepwise fashion; 'hypostome' forms at the highest level, 'tentacle zone' forms at somewhat lower levels, and still lower levels remain body column (Berking, 1979; Bode & Bode, 1980, 1987).

Upon decapitation, the morphogen concentration would begin to rise at the apical tip ('activation increase'; Fig. 13A). Eventually the concentration would reach a level which would be interpreted as tentacle zone. This would be only transitory and normally the concentration would continue on to hypostome levels, while the adjacent areas would reach tentacle levels. The resulting pattern in two dimensions would be a circular area (hypostome) surrounded by a ring (tentacle zone). If the process is arrested before the morphogen concentration reaches the hypostome level, only a small area of tentacle zone would result at the apex, leading to the evagination of a median tentacle (or tentacles). A secondary process would still be required to initiate tentacle-forming centres within the tentacle zone.

The third model suggests that the hypostome and tentacles are initiated by different morphogen peaks. A distinctive property of this patterning process is a translocation of morphogen peaks as the area in which the pattern is forming grows or changes (Tyson's modification of the Brusselator; Lacalli, 1980). After decapitation, a peak signalling tentacle competence appears first at the apex (Fig. 13B). As the area expands (or the wavelenth of the peaks decreases) the central peak disappears and reappears as a ring of peaks. Such a prepattern would be isomorphic with the formation of the tentacle ring, or a median tentacle if the process was arrested at the earlier stage. A later peak of another morphogen would be needed at the apex to signal hypostome formation.

Conclusions

Since it is clear that the head has parts, unlike the basal disc, knowledge of the patterning process must go beyond a description of the properties of an activated area. This activated area is ultimately partitioned into different subareas, which have unique size relationships with one another and the remainder of the animal. The present results yield information about the first division within the activated area to give hypostome and tentacle zone. Further questions concern the mechanisms which (1) regulate the spread of the activated area, thereby controlling the size of the head dome (Bode & Bode, 1984*a*), (2) are responsible for spacing the tentacles within the tentacle zone (Bode & Bode, 1987), and (3) regulate the amount of tissue recruited into the tentacles so that it is proportional to the amount in the body column (Bode & Bode, 1984*a*).

The research was supported in part by a grant from the National Institutes of Health (GM29130).

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(Accepted 24 September 1987)