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Formation of pattern in regenerating tissue pieces of *Hydra attenuata*

IV. Three processes combine to determine the number of tentacles

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Summary

The tentacles in hydra have characteristics of both spacing patterns and number-regulating patterns in that their number under some circumstances changes with the size of the animal and under others does not. To determine which type of processes could yield these results, an extensive analysis was undertaken of the size parameters pertinent to tentacle formation. To do this pieces of tissue, varying in shape and spanning a 30-fold size range, were excised and allowed to regenerate into complete animals. Three separate mechanisms were found to combine which resulted in the final number of tentacles: (1) the spread of the two-part head pattern to produce a competent band of tissue of a given size where the tentacles could form; (2) initiation of tentacle-forming centres by a spacing mechanism and (3) growth of the tentacles to a size proportional to the size of the animal.

Key words: hydra, pattern formation, regeneration, spacing pattern, tentacles.

Introduction

Repeating elements are ubiquitous in development, and include such disparate structures as body segments, digits and hairs. In principle, repeating patterns can be separated into two quite distinct categories, number-regulating patterns (Cooke, 1981) and spacing patterns (Wolpert, 1971), depending on how they are affected by changes in the size of the tissue in which they form. In a number-regulating pattern, as the name implies, the number of elements is maintained more or less accurately as the overall size is changed. To accomplish this the size of the individual elements is altered, as in the somites (Cooke, 1979; Flint, Ede, Wilby & Proctor, 1978; Veini & Bellairs, 1983). In a spacing pattern, the repeating elements retain their size and an average distance between them. Thus, increases or decreases in the size of the tissue result in proportionally more or fewer elements being formed. The many examples include feather primordia (Davidson, 1983), insect bristles (Held, 1979*a*), and *Acetabularia* hairs (Harrison, Snell, Verdi, Vogt, Zeiss & Green, 1981).

Hydra are radially symmetric animals with one row of repeating structures, the tentacles, emerging from the lower half of the head dome. The number of tentacles can vary within a population, and in *H. attenuata* is distributed around a mean of six to seven. A variety of evidence indicates that higher tentacle numbers appear on average in larger animals (e.g. Peebles, 1897; Parke, 1900; Shostak, 1979, 1982) and the increase in number is related to an increase in the circumference of the animal (O'Hearn & Lenhoff, 1982; Wanek, 1983). On the other hand, a common observation in a steady-state culture is that the mean tentacle number of attached mature buds does not differ significantly from that of their parents, although the buds are one-quarter adult size. Also, when animals are bisected transversely and allowed to regenerate, the average number of tentacles formed does not differ significantly from the original number (Shostak, Medic, Sproull & Jones, 1978).

Thus, the tentacles of hydra have characteristics of both spacing and number-regulating patterns. Because very different patterning mechanisms have been postulated for these two categories, a precise knowledge of how size affects the number of tentacles is necessary to determine which mechanisms might be involved. To obtain a broad size range for analysis, the impressive regeneration abilities of hydra were utilized. Tissue from the body column was cut into pieces of varying sizes and shapes, and allowed to regenerate. The numbers of tentacles, their size and their spacing were then analysed in animals spanning a 30-fold size range.

Methods

Preparation of regenerates

Adult *Hydra attenuata* with two to four buds were used in all experiments as a source of tissue for regeneration. Procedures for the maintenance of animals in culture, for the excision of pieces of tissue from the body column and for the estimation of the size of the excised pieces were as described previously (Bode & Bode, 1980). To remove just the tentacles from an animal, each tentacle was pulled taut with a forceps until the tissue of the head showed as a thickening at the proximal end. The tentacle was cut just distal to this area. Regeneration was allowed to proceed for 6 or 7 days, unless stated otherwise. The animals were not fed during the course of the experiments.

Analysis of regenerates

Abnormal regenerates (Bode & Bode, 1980) with more than one head or more than two extra tentacles on the body column were not used for analysis. The number of tentacles per head in each regenerate was counted using a dissecting microscope. To measure the diameter of the heads, measurements were made with an ocular micrometer across the head at the level of the tentacles. The size of individual animals was determined in whole mounts stained with 0·7 % silver nitrate. The numbers of ectodermal epithelial cells along the length of the body column and around the circumference at three or four locations were counted, and the number of cells per body column calculated (Bode & Bode, 1984*a*). No regenerates with zero tentacles remained intact through the staining process. To determine the number of cells in their body columns, measurements of live animals were made with an inverted microscope (Bode & Bode, 1984*b*) and these were converted to ectodermal cell counts using a calibration curve. Determination of the number of ectodermal epithelial cells per tentacle, the number spanning the tentacle bases and the number separating the tentacles were as described previously (Bode & Bode, 1984*a*).

Results

Description of tentacle regeneration

Tissue can be excised from the body column in a variety of sizes and shapes. The tissue rounds up and heals, and during the first day the epithelial cells rearrange to form shells ranging from spherical to cylindrical, depending on the initial shape of the tissue (Bode & Bode, 1984*b*). During the next four days the head dome forms, the tentacles evaginate and the body column elongates to its final shape.

Fig. 1 shows the details of tentacle development in individual regenerates of whole, half, quarter and eighth body columns. Addition of tentacles did not follow any regular pattern. Tentacles appeared either sequentially or in groups. For example, the regenerate in Fig. 1G had a single tentacle at 2 days, the second and third appeared by 3 days, the fourth by 4 days and the fifth by 5 days. In contrast, all six ring tentacles appeared between day 1 and day 2 in the regenerate shown in Fig. 1D. Typically new tentacles would emerge between existing ones, wherever space was available. However, frequently tissue was added between neighbouring tentacles as development progressed and tentacles emerged where space for them was not evident at an earlier time (e.g. Fig. 1A–C, between arrows). An extreme example is shown in Fig. 1E, where four tentacles grew in what was originally a single space (between arrows).

The rate of tentacle addition was followed for a period of 10 days. The average number of tentacles per animal increased rapidly during the first 3 days, and then more slowly over the next 3 days (Fig. 2A). Thereafter, only a small percentage of the animals added additional tentacles.

Interestingly, the diameter of the head when the first tentacles appeared was similar regardless of the size of the excised tissue (Fig. 2B; also compare Fig. 1A–H1). It was only thereafter that the size of the head increased in regenerates equal to or larger than a quarter of a body column. Tissue apparently moved into the head dome and the extent of the increase in size was related to the size of the excised tissue. After 3 days, the diameter of the head decreased in all animals, as the cells of the head dome became increasingly columnar. By 6 days the size had stabilized, with only slight further decreases due to starvation.

The tentacle zone appeared to move during the development of the head (Fig. 1). The first tentacles sprouted within a small area at the apical tip of the regenerate. Subsequent tentacles emerged in a ring below this area. Most of the early tentacles were displaced basally with the tentacle zone by the movement of tissue into the head (e.g. 1 and 2 in Fig. 1B,C and E). Occasionally, however, the initial tentacles were left behind in their original positions (Fig. 1D,F, indicated by arrows). Tentacles were also found just below the final tentacle ring, as if the tentacle zone had reached a more basal position and then retreated. (With the exception of the first day of tentacle appearance, only the ring tentacles were included in tentacle number counts.)

Effect of tissue shape on the number of tentacles regenerated

To understand what influences the final number of tentacles, it was necessary first to determine whether or not the tentacles tend to arise at certain positions due to some predisposition of the tissue. The tissue of the body column is a double-layered epithelium in the form of a cylindrical shell. The tissue is not uniform either circumferentially or along the length. Areas of squamous and columnar epithelial cells alternate around the endoderm of the body, and in the head the squamous areas are associated with the positions of the tentacles (Kanajev, 1926). Along the length of the body, developmental gradients affecting head regeneration have been found (see Bode & Bode, 1984*c*, for a recent review). It is possible that the inclusion of more or less of one of these dimensions might affect the number of tentacles regenerated. To examine this possibility, pieces of body column with approximately the same number of cells but different fractions of the donor circumference and length were excised (Fig. 3) and allowed to regenerate for 6 or 7 days.

Head regeneration progressed in the manner described above in all types of pieces. The final numbers of tentacles per head within each size group, halves, quarters or eighths, were very similar regardless of the original shape (Fig. 3). For example, despite the very different ratios of length to circumference of the eighths, each type of piece formed into regenerates having an average of close to four tentacles per head. Thus, the relative extent of either body dimension in the excised piece did not appear to play a role in the number of tentacles formed by the head.

Relationship between tissue size and the number of tentacles regenerated

To distinguish between models for spacing patterns and models for number regulation, it was necessary to determine the relationship between the size of the tissue and the number of elements formed. For this purpose, the entire possible range of sizes was used. At the lowest end of the range were the smallest pieces of tissue able to regenerate a head and foot, equivalent to 1/32 of a body column. At the upper end were adults with just the tentacles removed. The number of ectodermal epithelial cells in the body column of the regenerates was used as the criterion for size (Bode & Bode, 1984*a*).

The relationship between tentacle number and animal size is shown in Fig. 4. Over the first sixth of the size range, the numbers of tentacles rose rapidly from zero to six with small increases in regenerate

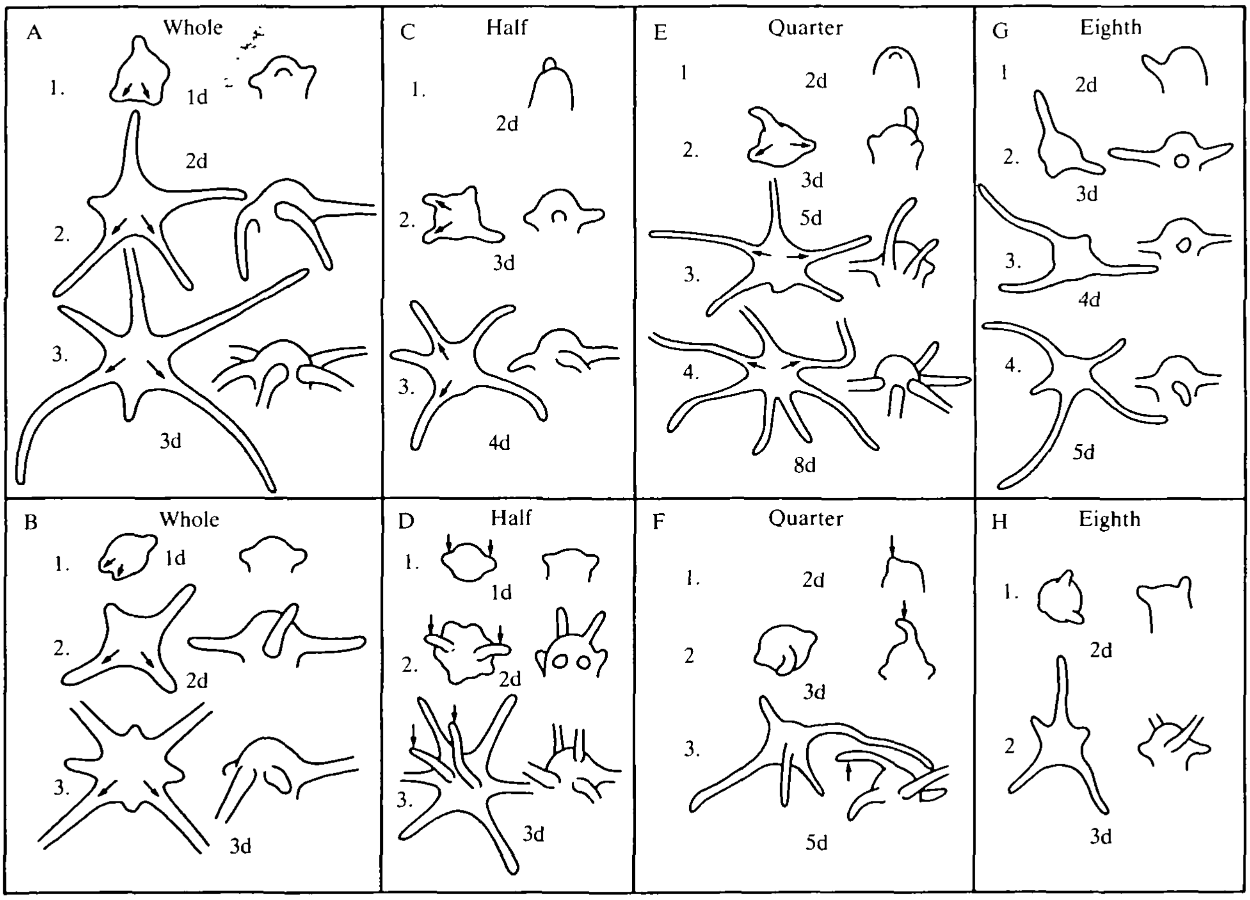

Fig. 1. Sequence of tentacle development in individual regenerates of whole, half, quarter and eighth body columns. The pictures were drawn to scale using a dissecting microscope with an ocular micrometer. In each case the top views are on the left and the side views on the right. Only the regenerate shown in F added an additional tentacle to the ring during subsequent development. The arrows are explained in the text.

size. Throughout the remainder of the size range, the tentacle number increased very slowly, never exceeding eight. Considerable variability was evident in the overlap of tentacle numbers found at any given size.

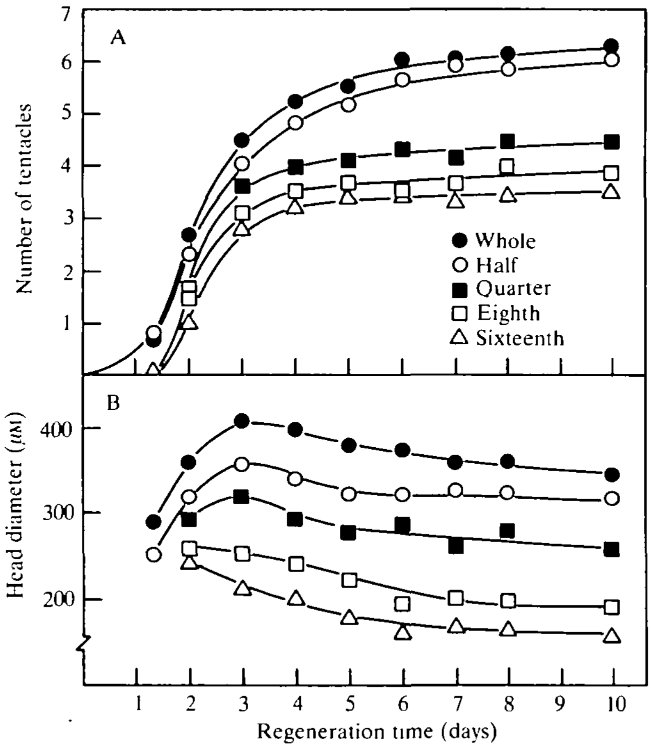

Fig. 2. The rate of tentacle addition (A) and the change in head diameter (B) during regeneration of whole, half, quarter, eighth and sixteenth body columns. The number of samples per point was 17–35.

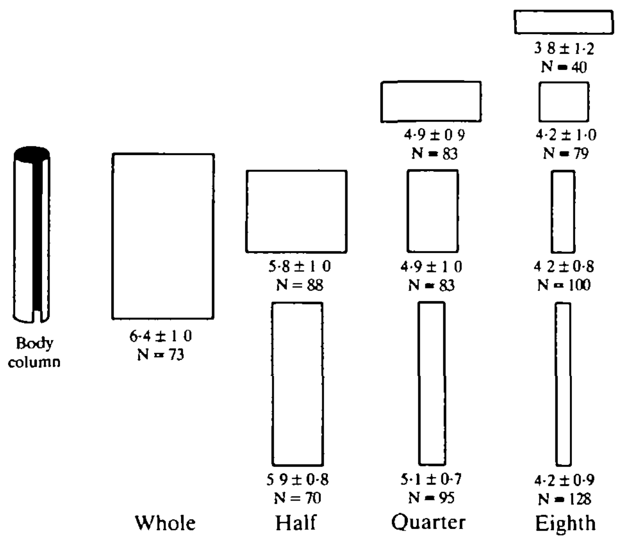

Fig. 3. The effect of tissue shape on the number of tentacles regenerated. The head, foot and buds were removed from the body column, which is depicted opened up into a rectangle. This was cut further into halves, quarters and eighths of different shapes. The mean number of tentacles, the standard deviation and the sample size (N) is given for each type of piece.

Particularly striking was that the higher tentacle numbers, especially six, could be found over an extremely broad range of sizes. However, since the average number of tentacles was not invariant, the tentacles could not be a number-regulating pattern.

The relationship between circumference and tentacle number

Since the tentacles formed around the head wherever space was available, the number of tentacles should be related to its final circumference, especially if they are a spacing pattern. Measurement of the head was carried out using cell number rather than diameters (as in Fig. 2B). The shape of the epithelial cells (although not the volume) changes with size, becoming increasingly squamous and wider in small animals (Bode & Bode, 1984*b*). Thus, the number of cells is a more sensitive measure of size than linear distance across the surface. Since the line of cells around the head is interrupted by the tentacles, the number of cells around the body column just below the head was used. The circumference of the body column and the circumference of the head are equivalent over the size range (Bode & Bode, 1984*b*).

A complex relationship between circumference and number of tentacles was evident (Fig. 5). If the tentacles were a simple spacing pattern, the average circumference divided by the average number of tentacles (or spacing, Fig. 6) would be a constant, and the number of tentacles would increase at a rate directly proportional to the increase in circumference. Assuming some variation in the spacing, the distribution of tentacle numbers would fall between the two lines shown in Fig. 7. Only in the first third of the size range were most of the data in the predicted region (stippled area, data from Fig. 5). Some of the smallest animals and many of the larger animals had fewer tentacles than expected.

First, an unexpectedly broad range of tentacle numbers was found in animals with the minimal head size. Animals with zero (Bode & Bode, 1984*b*), one or two tentacles had the same range of circumferences. However, as suggested by both Fig. 5 and Fig. 8, the smallest circumferences could easily accommodate as many as three or four tentacles. Thus, head size was unlikely to be the limiting factor in producing zeros, ones and most of the twos. A possible explanation for the very low tentacle numbers is simply that the animals were so small that tissue may have been too scarce to form any more. Some of the tentacles that did form were incomplete, composed of only a few cells.

Second, tentacle numbers greater than eight were not found. If the spacing of the tentacles remained the same over the size range, tentacle numbers up to 16 should have been observed. Instead, after an

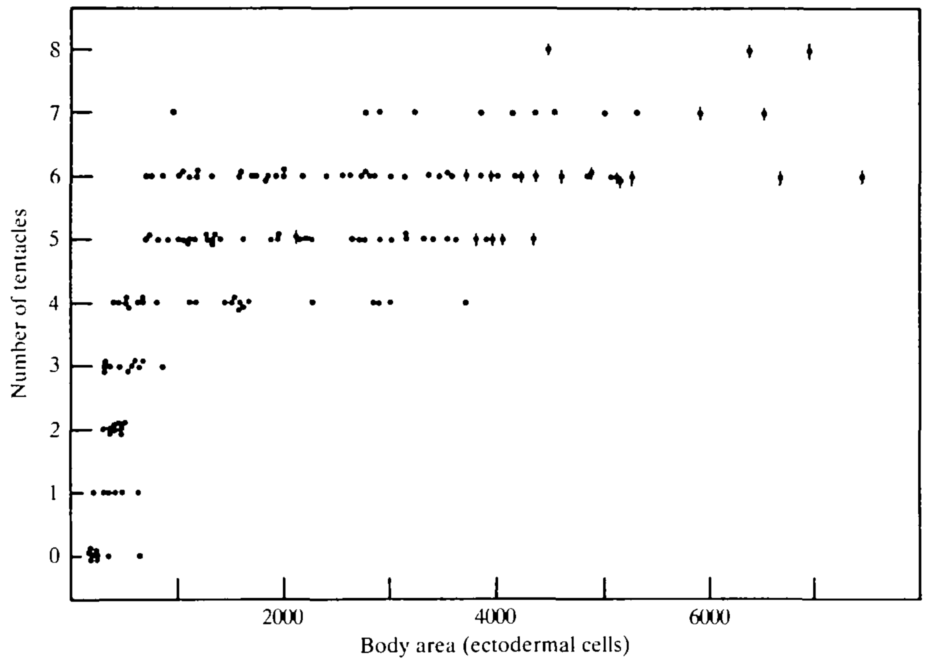

Fig. 4. The relationship between tissue size and the number of tentacles regenerated. The number of ectodermal epithelial cells in the body column of the regenerates was determined as described in Methods and plotted with respect to the number of tentacles. Each point represents a single animal. The points with vertical lines indicate regenerates with only the tentacles removed.

initial rise the same numbers continued to form as the circumference increased. This suggests that the spacing must also have increased.

To determine the spacing over the size range, the average circumference was divided by the average number of tentacles in six different size classes (data from Fig. 5). As shown in Fig. 9, the spacing of the tentacles increased linearly over the size range by one cell for every nine-cell increase in circumference. Thus, the number of tentacles that formed in larger

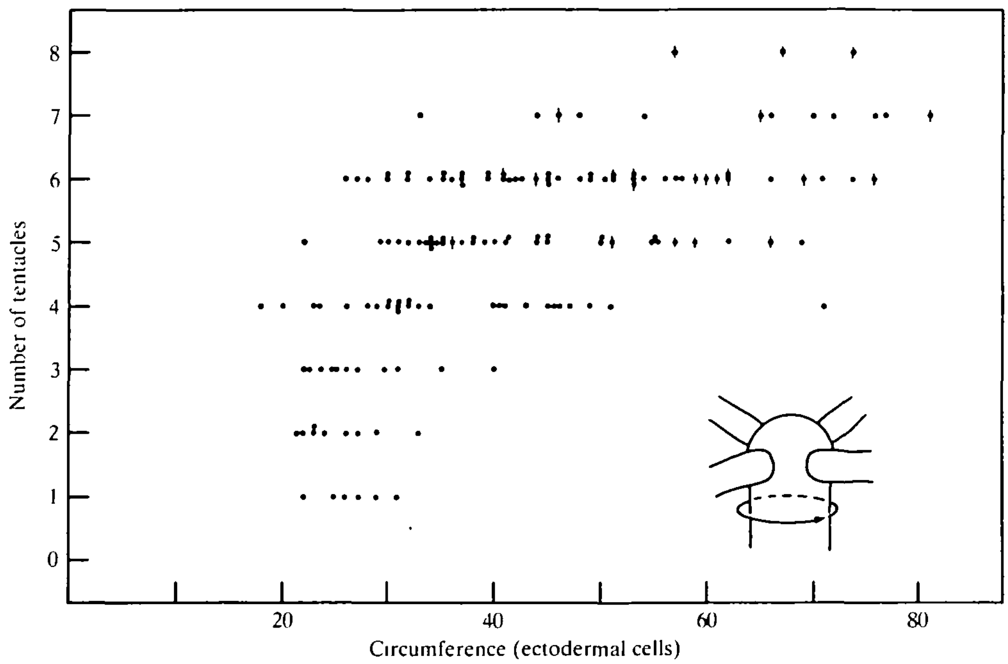

Fig. 5. The relationship between circumference and the number of tentacles regenerated. The number of ectodermal epithelial cells around the upper fifth of the body column (see inset) is plotted with respect to the number of tentacles. The symbols are as in Fig. 4.

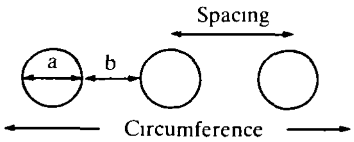

Fig. 6. The spacing of the tentacles. The circumference divided by the number of tentacles gives the spacing, which is the distance from the centre of one tentacle to the centre of the next. The two components that contribute to the magnitude of the spacing are the diameter of the tentacle bases (a) and the distance between them (b).

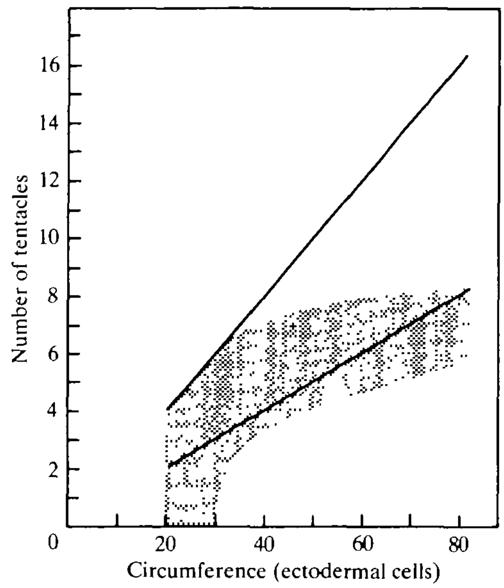

Fig. 7. The expected distribution of tentacle numbers (between the two lines) if the average spacing of the tentacles remained constant. The distribution of tentacle numbers obtained experimentally (from Fig. 5) is indicated by the stippled area.

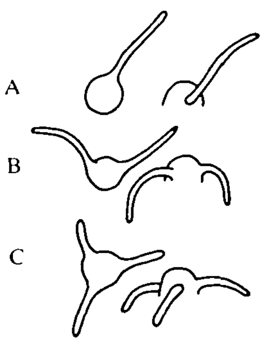

Fig. 8. Regenerates with one, two or three tentacles. The animals regenerated from 1/32 of a body column, the smallest size capable of regeneration. The animals were drawn to the same scale as those in Fig. 1.

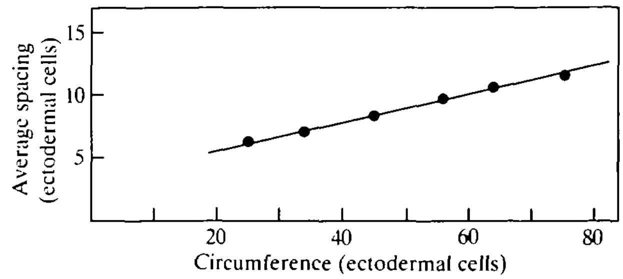

Fig. 9. The increase in the average spacing of the tentacles as the circumference of the regenerates increases. The data from Fig. 5 were divided into six size groups. For each group the average circumference was divided by the average tentacle number to determine the average spacing. Animals with one or two tentacles were omitted from the calculation of the first point (see text).

animals was limited because the spacing of the tentacles became larger.

The distance between tentacles is a constant

Further measurements were made to determine what was responsible for the change in spacing with size. The spacing of the tentacles depends upon two components, the distance between neighbouring tentacles and the diameter of the tentacle bases (Fig. 6). First, measurements were made to determine how regular the distance between tentacles was and whether it changed with the size of the animal. The number of ectodermal cells in a line between the bases of neighbouring tentacles was counted and the results are plotted in Fig. 10. Over most of the size range the distance between tentacles was quite constant, as would be expected for a spacing pattern. It averaged six cells and varied between four and eight. Clearly, the distance between the tentacles was not responsible for the increase in spacing in large animals.

In fact, large gaps were found almost exclusively in the smallest animals with one or two tentacles and in some of the animals with three tentacles. Animals with two tentacles averaged twice as many cells between tentacles (12) and those with one tentacle had a distance of almost four times the number of cells (23) uninterrupted by tentacles, supporting the idea that tentacles were missing.

The size of the tentacles is directly correlated with the size of the animal

Since the distance between tentacles did not change with size, the increase in spacing must have been due solely to increases in the size of the tentacles themselves. The total amount of tissue in the tentacles was found to vary in direct proportion to the number of cells in the body column (Bode & Bode, 1984*a*). Since it is the size of the tentacle bases that directly affects

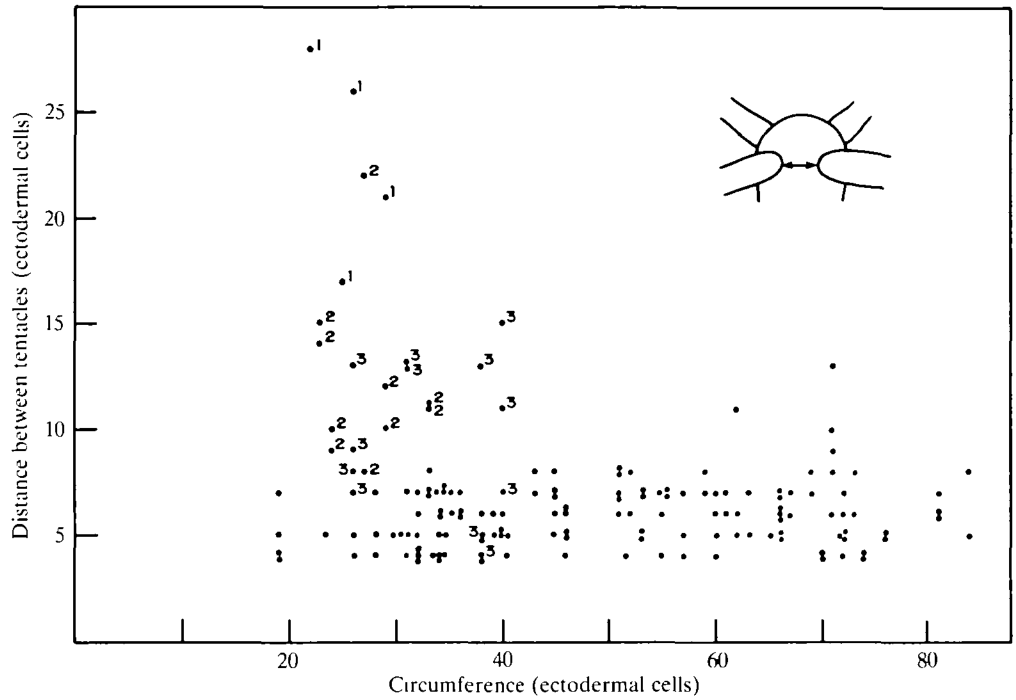

Fig. 10. The constant distance between tentacles. The number of ectodermal epithelial cells in a line between the bases of neighbouring tentacles (see inset) is plotted with respect to the upper body circumference. One to four intertentacular spaces were counted per animal and each space is represented by one point. For animals with one, two or three tentacles, the number of tentacles is indicated next to the points.

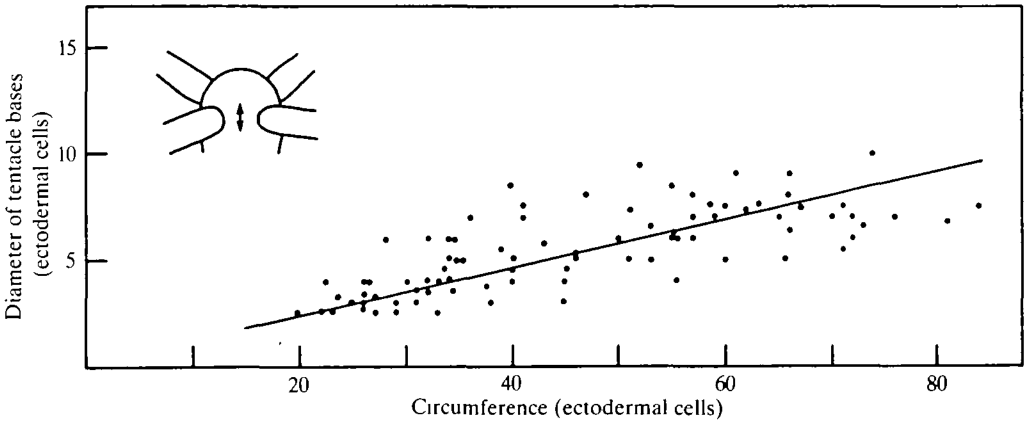

Fig. 11. The increasing diameter of the tentacle bases. The number of ectodermal epithelial cells in a line from the top to the bottom of the tentacle bases (see inset) is plotted with respect to the upper body circumference. Each point represents an average of at least three counts in a single animal.

the spacing of the tentacles, their diameters were measured. The number of ectodermal cells in a line from the top to the bottom of the bases was counted in groups of regenerates of various sizes. The data are presented in Fig. 11. The diameter of the bases increased by one cell for every nine-cell increase in circumference. This is the same as the rate of increase for the spacing of the tentacles (compare with Fig. 9). Therefore, it is the size of the tentacles that is the limiting factor in the number of tentacles that can regenerate as the animals increase in size.

Discussion

The establishment of the two-part head pattern

Regeneration of the head in hydra is a morphallactic process in which body epithelial cells rearrange to form a hypostome and tentacles. The positions of the tentacles as they evaginate mark the progression of the two-part head pattern during the early stages of regeneration (Fig. 12). Spread of the underlying pattern and its expression in tissue movements appear to be overlapping processes.

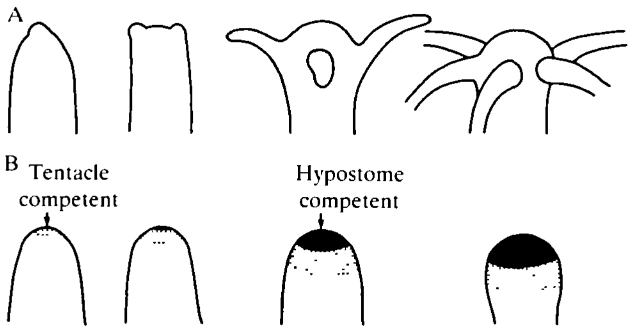

Fig. 12. The spread of the two-part head pattern. (A) The positions of the tentacles as they evaginate. (B) The development of the underlying head pattern suggested by the tentacle positions. The light shading represents the tentacle-competent area and the dark shading the hypostome-competent area.

Irrespective of the size or the shape of the excised tissue, the first one or two tentacles evaginate from a small area at the apex of the regenerate (Fig. 12A). This suggests that initially the pattern consists solely of a small competent zone for tentacle formation at the tip (Fig. 12B). By the following day, tentacles are emerging in a ring around a central domed area. Presumably the tip area has advanced to a second level of competency which signifies hypostome, while the tentacle zone spreads radially below it to form a ring. Almost concurrently, tissue is recruited into the hypostome- and tentacle-competent areas, enlarging them and displacing most of the early tentacles basally into the ring where new tentacles are emerging. The patterns of tissue movement into the new head structures are not unlike those occurring continuously in normal animals during growth and steady-state tissue turnover (Campbell, 1967; Otto & Campbell, 1977; Bode & Flick, 1976).

The spread of the head area ceases by the third day, perhaps in response to limits imposed by a mechanism for size regulation. Occasionally a tentacle is found just below the final ring, suggesting that the tentacle zone may at some points reach a more basal position and then recede during the proportioning process. The final size of the head dome is correlated with the size of the animal, although not exactly. The proportioning is allometric in that the number of epithelial cells in the hypostome and tentacle zone increases by only a factor of four as the number in the body increases by a factor of 20 (Bode & Bode, 1984*a*).

Generally, the head is treated as a single unit in pattern formation models. An activated area, which is the presumptive head, is generated by a reaction–diffusion mechanism which has some capability for size regulation (e.g. Gierer & Meinhardt, 1972; Gierer, 1977; MacWilliams, 1983). To obtain the two-part pattern it has been suggested that a graded concentration of activator or activator sources is interpreted as a step function (Berking, 1979; Bode & Bode, 1980). Peak values would specify hypostome and lower values would specify tentacle zone. During regeneration, the activation level rises and would first reach tentacle-forming levels in a small area at the apical tip. Subsequently, this area would attain hypostome levels and begin to spread laterally. Tentacle-forming levels would always surround it where the concentration falls off to a low enough value. (For an alternative model see Lacalli, 1980.)

The apparent dynamics of the two-part head pattern as it develops may explain some aberrant regeneration. Occasionally one or two median tentacles will form instead of a complete head (Webster, 1971; Bode & Bode, 1980). This occurs in situations where head inhibition may be emanating from another head or many buds (for a recent review see Bode & Bode, 1984*c*). Apparently in such cases the patterning process is arrested at an early stage when only a small area of activation at the tentacle-forming level is present. Measurements of activation in the tissue surrounding median tentacles indicate that its level is considerably below that of the hypostome but above that of the body column (Rubin & Bode, 1982).

The mode of tentacle emergence resembles a spacing pattern

During the initial patterning process, areas at the apical end become hypostome-competent or tentacle-competent. Within the tentacle-competent area tentacles begin to evaginate. These evagination centres always arise at some distance from one other or from tentacles already present. All available competent areas, including the new ones formed by the growth of the head, are eventually filled with tentacles. At the completion of the process the distance between tentacles is very regular, ranging from 4 to 8 cells in animals of all sizes, except the very smallest. Thus, it appears that the tentacles develop in a fashion that is very suggestive of a spacing pattern.

It is the regular distance between repeating elements that is the critical characteristic of spacing patterns. A secondary patterning mechanism, which operates locally within a competent area, is thought to commit certain cells to become a specific structure while inhibiting the competency of adjacent cells. Reaction–diffusion models have been used to generate the spacing pattern for the tentacles (e.g. Turing, 1952; Gierer & Meinhardt, 1972; Gierer, 1977). Peaks of activator would arise autocatalytically to specify the tentacle loci. An inhibitor produced by the reaction maintains a minimal space between them. Wherever the distance between elements exceeds

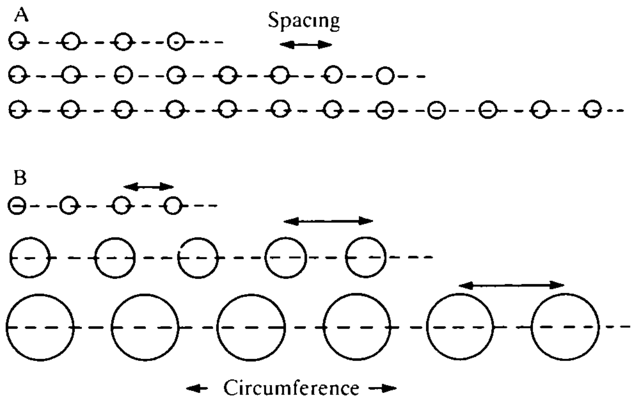

Fig. 13. Comparison of a simple one-dimensional spacing pattern (A) with the pattern observed for the tentacles (B). The circles represent tentacles and dashed lines indicate the circumference of a cylinder that has been opened up and laid flat. The spacing, or distance between tentacle centres, is indicated by the arrows.

the range of the inhibitor, the process repeats spontaneously.

Subsequent to the initiation of the tentacle loci, a second mechanism comes into play that affects the distance between the edges of tentacles throughout the life of the animal. This is the cell rearrangements that occur continuously during regeneration or normal tissue growth and turnover. Cells pass through the intertentacular spaces onto the hypostome and tentacles. This places a mechanical restraint on how close a newly evaginating tentacle can come to an established one and those too close together tend to merge. This tissue flow undoubtedly provides a fine-tuning mechanism, adjusting some of the randomness in the spacing that might arise during the patterning process (similar to the bristle pattern in the leg of *Drosophila*, Held, 1979*b*).

The limiting effect of tentacle size on tentacle number

In the simplest form of a spacing pattern the size of the repeating elements remains unchanged, and the relationship between the size of the competent area and the number of elements is directly proportional. In the case of a one-dimensional pattern around a cylinder, the number would be proportional to the circumference. For example, if the circumference is doubled or tripled, the number of elements would also be doubled or tripled (Fig. 13A).

The tentacles, however, are a spacing pattern with growth superimposed. At each tentacle locus, tissue evaginates. The amount of tissue that is displaced onto the tentacles during the process is directly proportional to the total amount of body tissue present. As a result, the bases of the tentacles take up an increasingly significant portion of the circumference as overall size increases (Fig. 13B).

The ability to adjust the size of a repeating structure in accordance with the size of the animal is a characteristic of number-regulating patterns. In ideal number regulation, the size of the elements would be controlled precisely so as to always produce the same number of elements. If the tissue area doubles, then the size of the elements would double so that the number does not change. While such tight control does not occur in the tentacle pattern, size regulation does reduce dramatically the number of tentacles that can be accommodated around the head in animals at the upper end of the size range.

On the other hand, in animals at the lowest end of the size range the proportion of tissue left in the body column after the head dome is formed may be too small for some of the presumptive tentacles to evaginate. Consequently, zero, one or two tentacles result and large gaps remain around the head. These animals are at the limit of tissue sizes that can successfully regenerate into an animal. Any further reduction in tissue results in very defective animals or no regeneration at all.

The final number of tentacles is a function of three processes

In conclusion, three processes appear to be involved in the development of the head, which when combined with the vagaries of chance determine the number of tentacles that will arise. The first is the spread of the head pattern which leads to a competent band of tissue of a given size within which tentacles can arise. The second is a spacing mechanism that generates the tentacle loci and maintains an average distance between them. The third is the growth of the tentacles to a size that is in direct proportion to the body tissue. The spacing mechanism dominates in the lower third of the size range, where the size of the tentacles is still insignificant relative to the distance between them. This gradually gives way to the dominance of size regulation as the tentacles take up increasingly more space around the head as they form, so that higher tentacle numbers rarely occur.

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