Budding in hydra: the role of cell multiplication and cell movement in bud initiation

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SUMMARY

The work described in this paper is concerned with the role of cell multiplication and cell movement in relation to the initiation of buds in hydra.

Hydra starved for 6 days do not initiate new buds; in such animals the mean mitotic index is only 10% of that in well-fed animals. When starved animals are re-fed, there is a rapid rise in mitotic index which reaches a maximum 12 h after feeding and thereafter declines. This cell division causes an increase in the cell population of about 30% in the 24 h following the meal. New buds are initiated at 24–72 h, i.e. at some time after the major part of the cell multiplication.

Cell division occurs in all parts of the axis to more or less the same extent and there is no sign of a growth zone in the budding region. However, the cell population in the budding zone of re-fed animals shows a significantly greater increase than in other parts of the axis and this can only be accounted for if it is assumed that cells have moved into this region from other parts of the axis.

Some cell multiplication is a necessary prerequisite for bud initiation, but grafting experiments with starved animals suggest that division \textit{per se} is not necessary; the important factor seems to be the increase in size resulting from division.

The mechanics and causes of the cell movement which results in bud initiation are briefly discussed. It is suggested that changes in intercellular adhesion may be important.

INTRODUCTION

Budding is one of the most obscure developmental processes in hydra. Although we have a rudimentary understanding of the factors involved in the individuation of the axial pattern in regenerating hydra, and several models have been devised which can account, in a more or less satisfactory fashion, for the principal observations (reviewed: Wolpert, Hicklin & Hornbruch, 1970; Webster, 1971 our understanding of budding is much less advanced and none of the proposed models incorporates an adequate explanation of the phenomenon. It is clear, however, that an understanding of budding, as well as being of intrinsic interest, will provide valuable insights into the organization of developmental fields, since the formation of a bud can be regarded as the formation of a secondary, autonomous and apparently identical individuation field within the primary field.

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In actively growing *Hydra littoralis*, buds are initiated at the proximal end of the digestive zone just above the peduncle. The first sign of bud formation is an increase in the optical density of the endoderm in the budding zone. This is followed by the formation of a small conical protuberance which increases in diameter and rapidly elongates to form a tube. Tentacle rudiments appear at the distal end of the tube and, at about the same time, a constriction appears at the proximal end. Eventually a small complete hydra detaches from the parent. The process of bud individuation can, therefore, be divided into three phases: initiation, elongation and regionalization.

Burnett (1961, 1962, 1966) originally argued that the initiation and elongation of the bud are caused by cell division localized in the budding region, i.e. this region is essentially a meristem or ‘growth zone’. It is now clear that this view, at least in part, is incorrect. First, it has been shown that cell division occurs more or less uniformly along the axis of the hydra and there is no sign of any localized ‘growth zone’ (Campbell, 1967a; Clarkson & Wolpert, 1967). Secondly, Clarkson and Wolpert showed that the elongation of extant buds can continue in animals in which cell division has been inhibited by γ-irradiation, and they suggested that elongation occurs as a consequence of cell movement, a view which is consistent with observations that the cells of the bud are derived from those of the parent and that the bud grows at the expense of parental tissue (Campbell, 1967b; Shostak & Kankel, 1967; Burnett, 1961; Clarkson & Wolpert, 1967).

With regard to initiation, however, Clarkson & Wolpert noted that no new buds were initiated in γ-irradiated animals, a finding consistent with the common observation that budding does not occur in starved animals (in which cell division might be expected to be reduced or absent) and that a certain amount of growth is a necessary prerequisite for budding (Burnett, 1961; Li & Lenhoff, 1961; Shostak, 1968; Shostak, Bisbee, Ashkin & Tammariello, 1968).

The position with regard to the precise role of cell multiplication in bud initiation, as opposed to elongation, is, therefore, somewhat confused. The work described in this paper clarifies the issues to some extent by providing data on the amount, location and time of occurrence of cell multiplication in relation to bud initiation. It also shows that cell division *per se* is not a prerequisite for initiation but that increase in size is.

**MATERIALS AND METHODS**

*Hydra littoralis* was used for all experiments. Details of culture method are given in Webster & Wolpert (1966). For experiments on bud initiation, animals were starved for 6 days before use. After 3 days of starvation hardly any new buds are initiated and after 6 days virtually all buds have detached from the parents. Starved animals were induced to produce buds by feeding once with freshly hatched *Artemia* nauplii and then incubating at 26 °C; such animals will be referred to as re-fed hydra.
1. **Measurement of mitotic index**

Mitotic indices were measured in squash preparation of whole animals, either unfixed or fixed briefly (5-10 min) in acetic-ethanol (1:3). Squashes were stained in lacto-acetic orcinol (see Darlington & La Cour, 1962) for 1–2 h. 1000 nuclei were counted in each preparation and the number of nuclei in mitosis (metaphase, anaphase and telophase) determined as a percentage of the total number of nuclei.

2. **Measurement of cell number**

A method was devised for estimating the number of cells in hydra based upon the unpublished observations of O. K. Wilby that when animals are sonicated in a sucrose solution the nuclei and nematocysts remain intact.

Batches of 10 hydra were suspended in 0.5 ml of hydra medium (‘M’) containing 2 M sucrose and sonicated for 5 s using a Branson sonicator (power setting 8). Samples of the suspension were removed and the nuclei counted in a haemocytometer using phase-contrast illumination. Duplicate counts were made of each preparation and the number of nuclei (= cells) per hydra calculated.

Preliminary experiments showed that there was a linear relationship between the number of hydra sonicated and the number of nuclei counted, indicating that the method is satisfactory for comparative purposes. We have not attempted to determine whether it gives accurate absolute values.

3. **Measurement of DNA synthesis**

Hydra were incubated in [3H]thymidine (specific activity 17.4 Ci/mM; obtained from the Radiochemical Centre, Amersham) at a concentration of 20 µCi/ml. Reduced glutathione (1 x 10^-5 M) was included in the incubation medium to induce the feeding reflex and promote isotope uptake (Clarkson, 1969a).

Animals were placed in isotope 5 h after feeding and incubated for 24 h at 26 °C. Samples of 10 animals were prepared for the determination of isotope incorporation following the methods of Clarkson (1969a).

Since the animals were placed in [3H]thymidine within a few hours of feeding and hence had ingested, but might not have broken down, large quantities of *Artemia* DNA, protein, etc., we felt that it would probably be more meaningful to express isotope uptake as counts per minute per hydra, rather than in terms of DNA or protein, when comparing starved and re-fed animals. Since the size of hydra is not constant, this method of expressing the results gives rise to a certain amount of variability, though not enough to make the measurements useless.

Hydroxyurea (Sigma; 0.75 mg/ml) was used to inhibit DNA synthesis (Clarkson, 1969b).

4. **Determination of the axial pattern of cell division**

This was studied using autoradiographs prepared from hydra labelled with [3H]thymidine.
Animals were re-fed and then immediately incubated in [3H]thymidine (30 μCi/ml) as above for 20 h at 26 °C. Following incubation the animals were washed in 'M' and the hypostome and tentacles removed and discarded. The remainder of the axis was divided into three regions of equal length: distal digestive zone, proximal digestive zone (including budding zone) and peduncle. These regions were fixed briefly (5 min) in acetic-ethanol diluted 1:4 with distilled water and then squashed on slides under coverslips. The preparation was immediately frozen on a block of solid CO₂, the coverslip prised off and the slide plunged into acetic-ethanol (full strength) where fixation was allowed to continue for 1–2 h. Slides were then hydrated and covered with Kodak AR10 stripping film. After drying they were exposed for 8 days at 4 °C, developed in Kodak D19 b for 4 min, fixed, dried and lightly stained in Ehrlich's haematoxylin. Six preparations were made of each region of the axis and the number of labelled nuclei per 1000 nuclei counted. Results were expressed as thymidine index, i.e. percentage of labelled nuclei. The autoradiographs were very clean; labelled nuclei had 10–20 grains compared with a background count of 0–2 grains over an equivalent area.

5. Determination of the axial pattern of tissue mass

The ‘tissue mass’ at any point on the axis is the product of cell number × cell size. It was determined by measuring cross-sectional areas of ectoderm and endoderm in serial transverse sections. Hydra were allowed to relax and then flooded with cold 10 % acrolein in distilled water (Gauthier, 1963). Fixation was allowed to continue overnight, after which the animals were dehydrated, embedded and serially sectioned transversely at 10 μm. The total number of sections obtained from each animal (omitting the hypostome and tentacles) was counted and this number divided into 20 groups of equal size; each group, therefore, represented 5 % of the axial length of the animal. An outline drawing of the ectoderm and endoderm of a section from the middle of each group was made on paper of uniform thickness using a Zeiss drawing apparatus; the drawings were cut to separate ectoderm and endoderm and the pieces of paper weighed. This weight represents the cross-sectional area of ectoderm and endoderm expressed in arbitrary units. Five animals were processed in this way and the means of the cross-sectional areas for each 5 % of the axis obtained.

6. Grafting experiments

Experiments were performed to increase the length of hydra by inserting extra pieces of axis. A rod grafting technique described in Wilby & Webster (1970) was used, and the grafted pieces were sometimes stained by the methods described in the same paper.
**Bud initiation in hydra**

![Graph showing mitotic index, cell number, and incidence of buds in re-fed hydra at different times after feeding.](image)

Fig. 1. Mitotic index, cell number and incidence of buds in re-fed hydra at different times after feeding, in the absence (●—●) and presence (○—○) of hydroxyurea. The number adjacent to each point refers to the number of determinations, the point is the mean of these. The vertical lines represent two standard deviations. (a) Mitotic index; (b) number of cells (= nuclei) per hydra; (c) incidence of buds in a representative batch of ten hydra.

**EXPERIMENTS**

1. *Cell division and the initiation of budding as a consequence of feeding*

Well-fed hydra growing logarithmically in mass culture initiate new buds at regular intervals, and in such animals the mitotic index (measured 15 h after the last meal) was 1.3 ± 0.19 (mean and s.d. of 10 measurements). After 6 days of starvation, bud initiation had ceased and the mitotic index had fallen to 0.12 ± 0.09. When such animals were fed there was a dramatic increase in the mitotic index within 6 h of feeding and this reached a maximum (1.6 ± 0.12) at 12 h, thereafter declining steadily (Fig. 1a). Stage 1 buds (Clarkson & Wolpert, 1967)
The maximum mitotic index, therefore, occurred some time before bud initiation, and at the time when the majority of buds were initiated the proportion of cells in mitosis was actually declining. This observation of a temporal separation between cell division and bud initiation in itself suggests that, although cell division may be a necessary prerequisite for budding, the actual initiation of a new bud is not caused by a high rate of mitosis whether this occurs generally throughout the animal or in a localized growth zone.

The increase in mitotic index following feeding resulted in an increase in the size of the cell population and from nuclear counts of animals prepared by the sonication method it was possible to obtain a measure of this increase (Fig. 1b). The results are in agreement with those obtained by measuring the mitotic index in that the rapid increase in cell numbers occurred in the first 24 h after feeding and thereafter the rate of increase declined. After 24 h the number of cells per hydra had increased by about 30% and after 72 h by about 46%. Once again, the temporal dissociation of the rapid increase in cell number and the onset of budding suggests that the morphological changes which occur in bud formation are not a direct consequence of cell multiplication.

2. The axial pattern of cell division

The measurement of mitotic index and cell number indicates that cell division occurs as a consequence of feeding but provides no information as to whether this occurs to a greater extent in one part of the axis rather than another.

This was investigated by looking at the proportion of isotopically labelled nuclei in three regions of the axis after incubating 6-day starved animals in \( ^3 \text{H} \)thymidine for 20 h after re-feeding. Results are shown in Table 1. It is apparent that the proportion of labelled nuclei is not very different in the three regions examined. Although the thymidine index in the distal digestive zone is significantly higher than in the proximal digestive zone \( (P < 0.01 \text{ by Student’s } t \text{ test}) \), the latter, which includes the budding zone, is not significantly different from the peduncle as regards the proportion of labelled nuclei. The average proportion of labelled nuclei in the three regions is 33%.

<table>
<thead>
<tr>
<th>% labelled nuclei ± S.D.</th>
<th>Distal digestive zone</th>
<th>Proximal digestive zone (incl. budding zone)</th>
<th>Peduncle</th>
</tr>
</thead>
<tbody>
<tr>
<td>38.9 ± 3.5</td>
<td>32.2 ± 2.6</td>
<td>29.3 ± 4.4</td>
<td></td>
</tr>
</tbody>
</table>

appeared at 24 h on some animals and the proportion of budding animals increased over the next 24 h (Fig. 1c).
Fig. 2. Axial distribution of 'tissue mass' represented as mean cross-sectional area (arbitrary units) in 6-day starved animals (bottom), and re-fed animals, 24 h after feeding (top). Δ---Δ, ectoderm of starved animals; O---O, endoderm of starved animals; ▲—▲, ectoderm of re-fed animals; ▼—▼, endoderm of re-fed animals. Each point is the mean of measurements made on five different animals. On the abscissa the numbers refer to 5% lengths of the axis; 1 is the sub-hypostomal region, 20 is the basal disc. The budding zone (positions 13–18) is somewhat exaggerated in size, measured in per cent axial length, in this plot of mean cross-sectional areas; this is a consequence of the variability in the position of this region in individual animals as a result of differential contraction during fixation.

The results from the autoradiography studies suggest that the increase in cell number which occurs as a result of cell division occurs in all parts of the axis to more or less the same extent, possibly somewhat greater in the most distal region. These results are in agreement with those obtained by other workers who have examined the axial pattern of cell division in well-fed, actively growing hydra. Campbell (1967a) in a careful study found no pronounced variation along the axis in either mitotic index or the proportion of nuclei which had incorporated [³H]thymidine. Clarkson & Wolpert (1967) found no pronounced axial variation in either mitotic index or the gross incorporation of [³H]thymidine into DNA. These results, together with those obtained in the present work, suggest very strongly that there is no localized region of high mitotic activity corresponding to the site of bud initiation, and provide further evidence that cell division is not a direct cause of bud initiation.
Table 2. Mean cross-sectional areas of ectoderm and endoderm in starved and re-fed animals in two regions of the axis

<table>
<thead>
<tr>
<th>Axial positions (see Fig. 2)</th>
<th>1-12</th>
<th>13-18</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ectoderm</td>
<td>0.14</td>
<td>0.13</td>
</tr>
<tr>
<td>Endoderm</td>
<td>0.19</td>
<td>0.22</td>
</tr>
</tbody>
</table>

6-day starved animals
Animals 24 h after re-feeding
(per cent increase shown in brackets)

| 6-day starved animals       | 0.26 (86%) | 0.32 (68%) | 0.40 (131%) | 0.50 (127%) |

3. The axial pattern of 'tissue mass' in starved and re-fed animals

The distribution of 'tissue mass' along the axis was studied by measuring the cross-sectional area of ectoderm and endoderm in serial transverse sections. The results for 6-day starved animals and for such animals 24 h after feeding are shown in Fig. 2 and in Table 2. In starved animals the cross-sectional area of both cell layers is virtually uniform along the axis. In re-fed animals the cross-sectional area of both cell layers shows an overall increase which is particularly marked in the budding region (axial positions 13-18).

The cross-sectional area of the two cell layers is a measure both of cell number and of cell size. We obtained an estimate of cell size in re-fed animals by counting the number of nuclei in sections from two regions of the axis in the five serially sectioned animals, calculating the mean number, and from this figure and that for the corresponding cross-sectional area calculated the mean area of the cells in arbitrary units. There was no difference in mean cell size between the digestive zone and the budding zone; the mean cell sizes were: for axial positions 6-7, ectoderm, 4.6 ± 0.8, endoderm, 9.8 ± 1.2; for axial positions 13-16 (the budding region), ectoderm, 4.2 ± 1.4, endoderm, 9.8 ± 0.4.

From these measurements it is apparent that the large increase in cross-sectional area of the budding zone of re-fed animals, as compared with the digestive zone of the same animals, is the result of an increase in the number of cells in the region. The axial pattern of cross-sectional areas shown in Fig. 2 is, therefore, a measure of the axial distribution of cells. This pattern is virtually identical to that pictured by Campbell (1967a) which he obtained by counting cells in actively growing, 'steady state' hydra.

It will be remembered that the nuclear count experiments indicated an increase in the size of the total cell population of re-fed hydra as a result of cell multiplication of about 30% (Fig. 1b). Even if all the cell division occurred in the budding zone, this increase is not sufficient to account for the large increase in cell number in this region. Moreover, the autoradiographic measurements of
Fig. 3. Transverse section of re-fed hydra at axial position 7 (see Fig. 2).

Fig. 4. Transverse section of the same animal at axial position 13, part of the presumptive budding zone. Note the columnar ectodermal cells (arrow) and the accumulation of endodermal cells at the same point on the circumference.

Fig. 5. Transverse section of a different animal at axial position 14. The columnar ectodermal cells are again present (arrow) and the accumulation of endodermal cells is more pronounced.

Fig. 6. Photograph of a 6-day starved hydra taken 72 h after an extra piece of axis had been grafted in, thereby increasing the length of the animal; the graft is distinguishable by its lighter colour. A bud has formed just below the proximal graft-host junction.
the proportions of cells which have undergone division or DNA synthesis indicate that the budding zone does not differ significantly in this respect from other regions of the axis. The increase in the size of the cell population in the budding zone must, therefore, be a result of movement of cells into this region from other parts of the axis.

Assuming that cell multiplication occurs uniformly throughout the digestive zone and the budding zone, it can be estimated from the figures in Table 2 that about 21% of the ectodermal and endodermal cells produced in the former region have moved into the latter region by 24 h after re-feeding.

Photographs of sections from the digestive and budding regions of re-fed animals can be seen in Figs. 3, 4 and 5; it is very clear that, in the endoderm at least, the increase in cross-sectional area in the budding region is not uniform around the circumference of the animal but is predominantly on one side; this is presumably the site from which the bud will develop. The cells of the ectoderm at this point show a characteristic columnar configuration compared with the point diametrically opposite where they are flattened.

Finally, it may be noted that re-fed animals show an increase in length as compared with starved animals. This was measured by counting the number of serial 10 μm sections. The mean length of starved animals was 2.08 ± 0.53 mm; of re-fed animals 2.85 ± 0.47 mm, an increase of about 37%.

4. Is cell division a necessary prerequisite for bud initiation?

(a) The effect of an inhibitor of DNA synthesis on cell division and bud initiation

The fact that starved animals do not bud and have a very low mitotic index compared with re-fed animals strongly suggests that cell division is a necessary prerequisite for bud initiation, even if not directly responsible for the formation of the new bud.

This supposition is strengthened by the results of experiments in which animals were treated with hydroxyurea (H.U.), a substance which inhibits DNA synthesis (Clarkson, 1969b) and, presumably, cell division. At a concentration of H.U. of 0.75 mg/ml, hydra survived and remained healthy for about 4 days, ample time in which to observe the effect of cell division and bud initiation.

Six-day starved animals were placed in H.U. immediately after feeding and the mitotic index, cell number and incidence of buds determined at various times as described above. Results are shown in Fig. 1. It can be seen that there is no mitosis whatsoever in H.U.-treated animals, and also no increase in cell number. Budding is severely inhibited (see also Table 4) and in many experiments no buds at all were produced.

The inhibitory effect of H.U. on DNA synthesis was confirmed by measuring the incorporation of [3H]thymidine over a period of 24 h in (i) 6-day starved animals; (ii) re-fed animals; (iii) re-fed animals in H.U. Results are shown in Table 3. It can be seen that the thymidine incorporated into re-fed animals
Bud initiation in hydra

Table 3. Incorporation of $[^3]H$thymidine into 6-day starved, re-fed and hydroxyurea (H.U.) treated hydra (3 experiments)

<table>
<thead>
<tr>
<th></th>
<th>Starved hydra</th>
<th>Re-fed hydra</th>
<th>Re-fed hydra + H.U.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thymidine incorporation (cpm/hydra)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>225</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>97</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>29</td>
<td>190</td>
<td>47</td>
</tr>
<tr>
<td>Mean</td>
<td>29</td>
<td>171</td>
<td>32</td>
</tr>
</tbody>
</table>

Table 4. The effect of hydroxyurea (H.U.) on bud initiation in re-fed hydra treated at different times after feeding

<table>
<thead>
<tr>
<th>Time after feeding when placed in H.U.</th>
<th>No. of animals which have produced buds 48 h after feeding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated controls</td>
<td>50</td>
</tr>
<tr>
<td>20 h</td>
<td>50</td>
</tr>
<tr>
<td>Immediately</td>
<td>50</td>
</tr>
</tbody>
</table>

placed in H.U. is about 18 % of that incorporated into control animals and is virtually identical to that incorporated into 6-day starved animals. The results, therefore, confirm that H.U. inhibits DNA synthesis, but do not rule out the possibility that other synthetic processes were being affected in the above experiments.

Although H.U. inhibits bud initiation, it has no effect on the elongation of extant buds. This observation supports the conclusion of Clarkson & Wolpert (1967), based on γ-irradiation experiments, that DNA synthesis, and hence cell division, plays no role in bud elongation. This observation in itself suggests that H.U. is acting fairly specifically as an inhibitor of cell division. More convincing evidence, however, comes from experiments in which re-fed hydra were placed in H.U. 20 h after feeding, i.e. a few hours before the first animals show signs of budding and a few hours after the peak of cell division (see Fig. 1). The results of this experiment are shown in Table 4 together with the results of control experiments, i.e. those in which re-fed animals were not treated with H.U., or treated with H.U. immediately after feeding. It is clear that, although immediate treatment of hydra severely inhibited budding, treatment which was delayed for 20 h had a significantly ($P < 0.05$ by $\chi^2$ test) reduced inhibitory effect. This observation is suggestive evidence that the inhibitory effect of H.U. on bud initiation is a consequence of its effect on cell division rather than upon some other unknown process, and supports the idea that cell division is a necessary prerequisite for bud initiation.
(b) Grafting experiments using starved animals

The results of the above experiment raised the question whether cell division per se is the prerequisite for bud initiation (i.e. do cells have to go through one or more rounds of division before a bud can be formed?) or whether the important factor is merely an increase in the number of cells per hydra. An attempt was made to answer this question by carrying out experiments in which the size of 6-day starved animals was increased by grafting. The animals remained unfed during the course of the experiments and there is no reason to believe that simple surgical operations stimulate cell division (see Clarkson, 1969a).

Graft combinations were of two types. In the first set produced by method 1 (Fig. 7a) the graft had the same polarity as the host (normal polarity). In the second set produced by method 2 (Fig. 7b) the polarity of the graft was reversed with respect to the host (reversed polarity). The grafts with reversed polarity should be identical to those with normal polarity (apart from the polarity of course) since combinations effectively equivalent to the latter could be produced by inserting the graft with normal polarity using method 2; a few graft combinations that were produced by this method behaved identically to those produced by method 1. In the initial experiments all the grafts were produced by method 2 but the polarity was not controlled. In all experiments, the length of the animals was increased by an amount equivalent to about half the length of the digestive zone. The results from all graft combinations – normal, reversed and unknown polarity – are shown in Table 5.

None of the control animals (i.e. starved and ungrafted) produced buds within 7 days. All the graft combinations, except 2, with grafts of normal polarity behaved identically and showed no change in form or development of any sort for as long as 7 days after grafting. The two exceptions each produced a bud from a point about \( \frac{3}{4} \) down the axis; these developed normally and eventually detached from the parent. The graft combinations with reversed polarity
behaved in a very variable fashion. 24–48 h after grafting nearly all produced basal discs at the distal graft–host junction. At the proximal graft–host junction many animals (ca. 50 %) produced a single or double ring of tentacles from the distal and/or proximal component of the combination. Many animals (ca. 30 % overall), however, produced a bud (rarely two) which was initiated about 24–72 h after grafting at a point below the proximal graft–host junction (i.e. from the presumptive budding region of the host) and developed and detached in a normal manner (see Fig. 6). The results obtained with reversed-polarity grafts varied from one experiment to another; in some batches virtually all the animals produced buds, in others none did so, although all had been starved for the same period of time. It is possible that the variability might have been due to some of the animals not being ‘adult’ at the onset of starvation, though care was taken to select only the largest animals for grafting. The majority of graft combinations with unknown polarity produced buds in a comparable manner to those with reversed polarity. It should be noted that simply grafting small pieces from the distal part of the axis (e.g. the sub-hypostomal region) to more proximal levels did not result in bud initiation (30 grafts).

The results of these experiments indicate that buds can be induced to develop in starved hydra by increasing the length of the axis and, therefore, that cell division per se is not a necessary prerequisite for bud initiation. However, the results also suggest that the situation may be complex since either the polarity of the graft or the method of grafting used to increase size seems to have a significant effect on whether or not bud initiation occurs.

**DISCUSSION**

1. **Cell multiplication and bud initiation**

The cessation of bud initiation in starved hydra is associated with a reduction of the mean mitotic index to a value which is about 10 % of that in well-fed animals. When starved hydra are re-fed there is a rapid rise in mitotic index, which reaches a maximum 12 h after feeding and thereafter declines. This cell division causes an increase in the cell population of the animal of about 30 % by 24 h after the meal. New buds are initiated at 24–72 h, that is, at some time after the major part of the cell multiplication has occurred. Cell division seems
to take place in all parts of the axis to more or less the same extent and no sign of any 'growth zone' in the budding region could be detected. However, the cell population in the budding zone shows a significantly greater increase than in other parts of the axis, and it is only possible to account for this if it is assumed that cells have moved into this region from other parts of the axis. The process of bud initiation, therefore, seems to involve some sort of cell movement, and this phase of bud development appears to be comparable to the phase of elongation (Clarkson & Wolpert, 1967) with regard to the cellular activity involved.

Although bud initiation, in the sense of a localized increase in the size of the cell population, does not seem to be immediately dependent upon, or caused by, cell multiplication, the experiment in which cell division was blocked with hydroxyurea suggests that some cell multiplication is a necessary prerequisite for initiation. The grafting experiments with starved animals show, however, that cell division per se is not necessary; the important factor seems to be the increase in size which results from cell division. Detailed interpretation of this experiment is somewhat complicated by the fact that the manner in which the grafting is done seems to influence the result.

2. Some problems raised by the observations

The first problem concerns the nature of the cell movement which results in an accumulation of cells in the budding zone. Campbell (1967c) has shown, using isotopically labelled grafts, that both ectoderm and endoderm move as coherent cell sheets during normal growth. The movement of these cell sheets during bud initiation could, at least in part, be a consequence of an increase in adhesion between the cells of each sheet in the budding region (see Gustafson & Wolpert, 1963, 1967). Such a change would cause the cells to pack more tightly together, thus increasing the cell density, and would also cause an increase in the thickness of the cell sheets and a change in their curvature. The histological appearance of re-fed hydra, and of the ectodermal layer in particular, is consistent with this interpretation (see Figs. 4 & 5) since the cells become columnar and close-packed, and the curvature and thickness of the sheet change quite markedly. The change in curvature would, we suppose, result in the formation of the small conical protuberance which is the first sign of a developing bud. It is worth noting in this context our unpublished observation that, when hydra are placed in media which cause cell disaggregation (e.g. EDTA), the developing bud is always the last part of the animal to disaggregate. This suggests that the cells in the bud are more strongly adhesive than those in other parts of the animal.

A second problem concerns the cause of cell movement and accumulation. Why do cells not accumulate in the budding region of starving animals? What are the changes which occur following the increase in size, whether this is caused by normal growth or grafting? It is worth noting, in connexion with this question, the complementary observation of Burnett (1961) that bud initiation
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can be suppressed if the hypostome is moved nearer to the budding zone by removing parts of the digestive region.

The first event in bud initiation may be the formation in the budding zone of an organizing region comparable to the hypostome. It is known that the tip of a very early bud possesses organizing properties, and can re-orientate cells and cause morphogenetic movements when transplanted (Li & Yao, 1945). If this is, in fact, the first event in the development of bud, then both our own and Burnett’s (1961) observations suggest that bud initiation, i.e. the formation of an organizing region, is dependent upon either the budding zone being a certain distance from the hypostome, or the animal being a certain size. We are, at present, attempting to distinguish between these alternatives, and also to characterize the events which precede the formation of a secondary organizing centre in the budding zone.

REFERENCES


(Manuscript received 1 June 1971)