REGENERATION OF BUDS IN BOTRYLLUS [1]

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The process of budding in the colonial ascidian, *Botryllus schlosseri,* has been carefully analyzed by N. J. Berrill (1941a) and recently by Sabbadin (1955). The new buds (Z_3) arise from the atrial epithelium and epidermis of large buds (Z_2) in which internal structure is nearly complete but which are still attached to the parent (Z_1). The disc-like thickening of the atrial epithelium increases in cell number and area until a certain size, called the maximum disc, is reached. It then folds out into a hemisphere and finally to a closed sphere attached to the large bud by a stalk. Three generations are thus present and connected together at one time. The sphere then goes through a process of expansion, folding, and evagination to form the internal structure of the new zooid. The bud continues to grow until it reaches a size nearly equal to the parent, at which time the latter degenerates and the bud becomes functional. There is considerable variation among colonies in the number of buds formed and the number which reach maturity.

Berrill (1941a, 1941b, 1945) has shown that in young colonies the diameters of the maximum disc and sphere stages are less than half those of older colonies and that they gradually increase with each successive generation. The size of the adult zooid is closely related to the size of the bud and hence to the number of cells initially present. A sphere with a diameter of 0.035 mm., for example, becomes a zooid with a length of 1.1 mm., while a sphere of 0.080 mm. becomes a zooid of 2.6 mm. Sabbadin (1956a, 1956b) showed that the growth of the bud is conditioned not only by its initial dimensions, but also by the quantity of food made available to it by the regression of the parent zooid and the duration of its growth period. In his experiments all but one bud was removed from each zooid in the experimental colonies. These buds and the zooids from them attained greater maximum length than corresponding buds and zooids in control colonies. Sabbadin concludes that the buds on one zooid compete for food made available by the parent as it regresses. This does not explain, however, the gradual increase in size of the zooids with each generation. It was deemed of interest to determine whether the size of the zooid depends directly on the number of cells present in the early bud or is determined in some other manner by the parent. In order to study this problem, buds were damaged at an early stage and the amount of regeneration as shown by the final size was noted.

This degeneration of the parent zooid has generally been thought to be due to the increasing need of the bud for space and nourishment (Berrill, 1935). To test

whether this is true the buds were removed in an attempt to prolong the life of the zooid.

MATERIALS AND METHODS

Adult colonies of Botryllus were collected from the dock in Eel Pond at Woods Hole, Massachusetts, and placed in finger bowls on a table of running sea water. Each day a few of the tadpole larvae were released from the colonies and these were collected and placed in Syracuse dishes for approximately 12 hours. After the tadpoles had attached to the glass, the Syracuse dishes were inverted in wooden racks placed in tanks of running sea water. The tadpoles metamorphosed in less than one day, forming oozoids with large right buds which became the first blastozooids. Under these conditions at $22.5 \pm 0.5°$ C. the adult zooid persisted for from 4 to 6 days, with as much as 24 hours' variation between two colonies in the same dish. The experimental colonies were examined every day or two with a binocular dissecting microscope and rough sketches were made to follow the fate of individual zooids and buds.

Buds were removed by cutting through the stalk with a needle sharpened to a blade. With care this could be done with very little damage to the parent, but sometimes the latter was damaged severely and disappeared. To determine whether the new buds originated from the same area as the destroyed buds or from bud primordia posterior to it, all the small buds (Z_3) were cut off 220 large buds (Z_2) in 31 colonies. The site of formation of the new buds was then observed. In 5 colonies an attempt was made to keep the parent zooids from degenerating by constantly removing new buds as they appeared.

Buds were damaged with sharpened steel needles inserted through the tunic. An effort was made to destroy half or more of the forming bud. Although the amount of actual damage varied from bud to bud, in most cases at least half of the bud was destroyed. Frequently, part of the bud was torn away and could be seen sticking to the needle. In preliminary experiments on 21 colonies, buds ranging from the sphere stage to those with some internal structure present were damaged. These were watched to see whether they reached maturity, but no measurements were made to determine if they were full size. In order to examine the effect of destroying approximately half of the cells at a stage before the closed sphere, both right and left buds were damaged when the atrial epithelium had begun to fold out into a hemisphere (between stages 2 and 3 of Berrill, stage 2 Sabbadin). The length of the zooid which formed these buds was then measured with an ocular micrometer and compared with the length of undamaged zooids. The width of the zooids varied in different colonies of the same age and seemed to decrease as the number of zooids around the cloaca increased; therefore, no measurements of width were made.

RESULTS

a) Degeneration of parent zooids

In no case observed did an adult zooid persist beyond 24 hours of the time of degeneration of other zooids of the same age. When all the buds were removed from a zooid, that zooid degenerated at the same time as the rest of the zooids in that colony or in other colonies, whether new buds formed or not. The five col-

onies in which new buds were constantly removed as they appeared degenerated and disappeared within 24 hours of the time of degeneration of control colonies.

b) Formation of new buds

If the large bud which normally occurred on the right side of an oozoid was removed, a bud then appeared in four cases out of five on the left side of the oozoid and became a normal blastozooid. In later generations, if all the large buds (Z_2) with complete internal structure were cut off (4 colonies), the colony degenerated. If, however, only approximately half of the larger buds were cut off (5 colonies), those remaining proceeded to maturity, and in addition a few new buds appeared. When all the small buds (Z_3) at the sphere stage were cut off 220 large buds (Z_2) in 31 colonies, a total of 72 new buds appeared, an average of one bud for every three parents. There was a great deal of variation among the colonies, with anywhere from zero to seven buds produced by the six or seven parents. Of the new buds, 26 appeared on the left side of the blastozooids and 29 on the right side. Of the latter 6 definitely were from the same area as the destroyed bud, 18 were probably from this area, and 5 appeared posterior to the destroyed bud. The origin of the other 17 buds was impossible to determine. Most of these were first seen in the midst of a degenerating colony quite separate from any blastozooid.

c) Bud regeneration after damage

In preliminary experiments in which 107 buds ranging from the sphere stage to those with some internal structure present were damaged, 55 reached maturity. The rest of the buds became progressively smaller and eventually disappeared. In 36 colonies in which 361 hemispheres were damaged, 40% reached maturity as compared to 78% in 8 control colonies with 124 hemisphere stages.

TABLE I

Size regulation of zooids in partially damaged colonies

Colony number	No. of zooids in colony	No. of zooids damaged	Average length of *all* zooids in colony, in mm. ±S.D.	Average length of *damaged* zooids in colony, in mm. ±S.D.	Average length of *undamaged* zooids in mm. ±S.D.
A12c	12	5	1.5 ± .1	1.6 ± .1	1.5 ± .1
A12e	7	3	1.8 ± .1	1.8 ± .2	1.9 ± .1
A12h	21	8	1.9 ± .1	2.0 ± .1	1.8 ± .1
A12k	13	6	1.6 ± .1	1.6 ± .1	1.6 ± .1
A12l	17	4	1.4 ± .1	1.5 ± .1	1.4 ± .1
B2d	25	4	1.8 ± .2	1.8 ± .1	1.8 ± .2
A5a	8	3	1.8 ± .1	1.8 ± .2	1.9 ± .1
A5e	15	13	1.6 ± .1	1.6 ± .1	1.6 ± .1
A5i	7	6	1.8 ± .1	1.8 ± .1	1.9
A1a	16	16		1.8 ± .2	
A5b	6	0			1.4 ± .1
A5g	13	0			1.6 ± .1
A5h	13	0			1.7 ± .1
A6a	7	0			1.8 ± .1
A6b	11	0			1.7 ± .1

TABLE II

Size regulation of zooids in experimental and control colonies

Colony number	No. of Z_1	Length of Z_1 in mm. $\pm$S.D. at time of experiment	No. of Z_2	Length of Z_2 in mm. $\pm$S.D. 1–2 days after reaching maturity	No. of Z_3	Length of Z_3 in mm. $\pm$S.D. 1–2 days after reaching maturity	Length of Z_3 in mm. $\pm$S.D. 3–4 days after reaching maturity
			Experimental Colonies				
A3e	9	1.7 ± .1	8	2.0 ± .2	12	2.0 ± .2	2.4 ± .3
A7a	5	1.8 ± .1	8	2.0 ± .1	12	2.2 ± .1	2.8 ± .1
A8a	7	1.7 ± .1	4	1.7 ± .1	4	1.9 ± .1	2.4 ± .1
A10a	4	1.6 ± .1	4	—	8	2.2 ± .1	—
A10e	—	—	6	1.7 ± .1	10	1.8 ± .1	—
Total	25	1.7 ± .1	30	1.9 ± .2	46	2.1 ± .2	2.6 ± .3
			Control Colonies				
A3d	6	1.8 ± .1	8	1.8 ± .2	13	1.8 ± .1	—
A7b	5	1.6 ± .2	9	1.8 ± .1	15	1.9 ± .1	2.3 ± .2
A8a	7	1.8 ± .1	10	2.0 ± .1	17	2.0 ± .1	2.6 ± .1
A10b	5	1.8 ± .1	5	1.8 ± .1	11	2.1 ± .1	—
A10f	—	—	5	1.7 ± .1	9	1.8 ± .2	—
Total	23	1.7 ± .1	37	1.8 ± .2	65	1.9 ± .2	2.4 ± .2

d) *Size regulation in damaged buds*

In the first experiments, only some of the hemispherical buds in each colony were damaged with the idea of using the others as controls. The data for 15 such colonies are given in Table I. In colonies A12h and A12l the damaged left buds did not survive, so the right buds measured received the full food supply from the parents. In all other colonies as many damaged left buds survived as undamaged, so the supply of food did not affect the results. The colonies are not all of the same size or age at the time of the experiments, so the average length for different colonies cannot be directly compared, but the average lengths of damaged and undamaged zooids in the same colony show no significant difference.

In later experiments, all the hemisphere stages in a colony were damaged and these colonies were compared with control colonies. Three generations were present at the time of the experiment: the parent zooids (Z_1), the large buds (Z_2), and the hemispherical buds (Z_3). Each of these was measured as it in turn reached maturity. In both experimental and control colonies, most of the left buds reached maturity, so the food supply was about the same for all buds. The data for these experiments are given in Table II. No significant difference can be seen between the experimental and control colonies.

DISCUSSION

If the degeneration of the adult zooid is due only to the increasing need of the growing buds for space and nourishment, removal of all the buds in a colony ought

to have prolonged the life of the zooids. In this study, any attempt to postpone degeneration of the zooid in this way met with failure. No zooid was observed to persist more than 24 hours longer than other zooids of the same age even if its buds were continually removed. Sabbadin (1956b), however, found that when all but one bud was removed from each zooid, that zooid had a prolonged stage of functional maturity. Perhaps removal of all buds was a shock to the zooid and partially caused its regression; however, it seems likely that adult regression will occur without the presence of buds. At the same time the buds may play an important part in the process by their increasing need for nourishment.

There is some question as to the origin of new buds after removal of these already growing. Blastozooids have two potential budding areas, one on the right side and one on the left, although frequently only the bud on the right side reaches maturity. At times, a third bud may be formed posterior to the bud normally found on the right side (see Watterson, 1945, and Sabbadin, 1956a, for a discussion of the number of buds usually formed). It would appear that a certain amount of atrial epithelium is set aside for bud formation; after that is used no more buds can be formed. Frequently in these experiments, when buds were removed from the blastozooid, new buds were formed at the same area as the buds were destroyed. Sabbadin (1956a) reports that he never observed buds arising "de novo" after removal of buds present. Sometimes, however, after he had removed buds in the hemisphere stage, he saw fragments adhering to the atrial side of the parent zooid, and these fragments formed new buds. This is a possible explanation of the present results although every effort was made to remove the entire bud intact. In these experiments all the buds removed were at least in the closed sphere stage and many were quite large and visibly separated from the parent though still attached by the stalk. It would take considerable powers of regeneration for fragments of such buds to form a whole new zooid.

The 17 buds whose origin it was impossible to determine might possibly be cases of vascular budding (Oka and Watanabe, 1957). They arose during or after the regression of the adult zooids, so a vascular origin seems likely. They were not observed, however, until they were large enough to obscure their point of origin.

Although development of ascidians from egg to tadpole is determinate (Conklin, 1905), the adults have remarkable powers of regeneration (Berrill, 1951). Zhinken (1939) has shown that while tadpoles have little ability to replace lost parts, the oozooid has acquired considerable regulative powers. The present study would indicate that buds also have the ability to regenerate lost tissues from the earliest stages onward.

Berrill has shown (1941b, 1941c, 1945) that the size of the maximum disc and sphere stages increases with succeeding generations and that the size of the adult zooid is clearly related to the number of cells or the diameter of the maximum disc and sphere. This might suggest that the parent determines the size of the new zooid by the number of cells which are initially incorporated into the early stages of the bud. If this were true, then destroying some of these cells would have resulted in smaller adult zooids. However, using length as an index of zooid size, it was found that there was no decrease in size of the zooids damaged at the hemisphere stage. After a bud was damaged it either disappeared completely or reached the predetermined size. Thus the size of the adult does not appear to depend directly on the number of cells originally present since these cells can be replaced. Either the

parent zooid retains control over the growth of the bud or the bud has "received instructions" as to the size it should attain and follows them by regenerating lost tissue and then continuing to grow. Sabbadin (1956c) has shown that zooids with the position of the digestive tube reversed may appear if the growth of the bud is delayed at an early stage. The buds on these abnormal zooids showed a marked tendency to be the same as their parents unless the parent has started to regress before organogenesis is complete. This would indicate that the parents do retain control over the growth and organogenesis of their buds.

SUMMARY

1. The degeneration of the adult zooid of *Botryllus schlosseri*, which normally occurs when the buds become functional, occurred even after all buds were removed.

2. All stages of the growing buds of Botryllus have considerable regenerative ability.

3. Buds damaged in the hemisphere stage became adult zooids with the same length as undamaged zooids of the same age. Control over the size of the adult zooid appears to be maintained during the growth of the bud.

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