VASCULAR BUDDING, A NEW TYPE OF BUDDING IN BOTRYLLUS [1, 2]

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In the early days of the investigation on compound ascidians, it was believed that botryllids propagate either by stolonial budding alone or by stolonial *and* palleal (peribranchial) budding. It was Seeliger (1907) who, in his monumental treatise on ascidians, denied once and for all the occurrence of stolonial budding in botryllids. According to him, the supposed stolonial budding in these ascidians is palleal budding which was misinterpreted. After Seeliger, all the workers on ascidians agreed that the only budding in botryllids is palleal (*cf.* Huus, 1937; Brien, 1948; Berrill, 1950; *et al.*), except perhaps E. C. Herdman, who maintained as late as 1924 that in *Botryllus* buds are occasionally formed from the blood vessels of the test.

Since 1950 we often have had opportunities to observe that, in *Botryllus primigenus* at least, buds are formed from blood-cells gathered at the base of ampullae. It is proposed to name this type of budding "vascular" as distinct from "stolonial," for in *Botryllus* the blood vessels are generally called test vessels and not stolons, and, further, the buds are formed from blood-cells, and not from the mesenchymatous septum as in stolonial budding.

In this paper the process of vascular budding will be described in some detail and a comparison will be made between this and the ordinary palleal budding.

Historical [3]

Following are the various views concerning budding in botryllids, arranged in chronological order.

Savigny (1816), in his description of the marginal tubes (test vessels) in *Botryllus,* seems to have regarded them as an apparatus for the production of buds; this view, which was more fully elaborated and established by Milne-Edwards (1842), was generally adopted until Metschnikoff (1869) demonstrated that in *Botryllus* gemmation takes place from the sides of the ascidiozooids, *i.e.,* budding is palleal. This view of Metschnikoff was fully confirmed by later investigators such as Della Valle (1881), Oka (1892), *et al.*

Ganin (1870), however, maintained that the buds can be formed also "auf langen Stolonen und weit entfernt von dem Körper der Ascidien . . ." (p. 517).

[1] Contributions from the Shimoda Marine Biological Station, No. 92.

[2] The cost of this research has been partly defrayed from the Scientific Research Expenditure of the Department of Education of Japan.

[3] Because the wartime literature is only poorly represented in Japan, we consulted Prof. N. J. Berrill of McGill University, Montreal. He assured us in a letter that since 1940 no paper had been published on the stolonial budding in *Botryllus.* We wish to express here our thanks to Prof. Berrill for his kind information.

Giard (1872) also stated that in botryllids buds might be produced from blood vessels as well as from the body-walls of the ascidiozooids. In 1891, he still insisted that the inability of the test vessels to produce buds had not been sufficiently demonstrated.

W. A. Herdman (1886) described for *Sarcobotrylloides wyvillii* (and *Collela pedunculata*) that buds were produced intravascularly from aggregations of blood-cells. He went so far as to make the stolonial budding one of the characteristics of the family Botryllidae.

Bancroft (1903b) studied an aestivating colony of *Botrylloides gascoi* at Naples and found that many buds were formed in blood vessels apparently independently of zooids. He believed, however, that they were developed from zooids, not from blood vessels. He states (p. 151), "As no evidence in favor of an intravascular nor intra-ampullar origin of the isolated buds was detected, I feel convinced that they were developed from the zooids of the original colony before these had degenerated entirely. The buds must have severed their connections with the parent zooids, and must have been carried into the yellow lobe that was then being formed." According to the same author the buds described by Herdman (see above), too, might have been produced elsewhere and have migrated into the vessels.

Seeliger (1907) denied once and for all the occurrence of the stolonial budding in botryllids. According to him, the budding once described as stolonial is in reality typical palleal budding. As to how misinterpretation has come into existence, he says (p. 999) : "Ich glaube, dass dieser Irrtum darauf zurückzuführen ist, dass die häufig in Rückbildung begriffenen Zooide, die bereits an Grösse abgenommen haben und mit den stoloähnlichen Mantelgefässen innig verwachsen sind, für neue Knospenanlagen gehalten wurden, die sich an und aus den erweiterten Gefässampullen entwickelt hätten."

E. C. Herdman (1924), however, could not reconcile herself to this view of Seeliger's and expressed herself in the following way (p. 4) : "It is quite possible, however, that occasionally certain swollen knobs on the blood vessels of the test may give rise to buds." She adds, however, that "it is certainly not usual in *Botryllus* and there is no definite proof that it has ever occurred."

To sum up, it is today a well established fact that in botryllids buds are generally formed from the sides of ascidiozooids. Further, being organs for blood propulsion and respiration and perhaps also for excretion of test matrix, the ampullae are never transformed into new zooids. The problem is whether or not under certain circumstances buds are also formed from the walls of the blood vessels or the ampullae.

Materials and Methods

The following observations were made principally on living colonies of *Botryllus primigenus* Oka, occurring in the vicinity of the Shimoda Marine Biological Station, Shimoda, Japan. As is well known, the zooids in *Botryllus* are generally grouped into systems. In *Botryllus primigenus*, however, they are independent of one another, and each opens through its own atrial opening (*cf.* Oka, 1928).

To facilitate observation, colonies were fixed on glass slides. These, except at the time of observation, were set out in the bay. The technique employed for fixing the colonies was the same as that described in the paper of Oka and Usui (1944). The colonies grew well on glass slides.

Various developmental stages of the bud were observed and sketched under a binocular (magnification: × 72) and an ordinary microscope (magnification: × 100). Since the buds are formed from blood-cells, vital staining of these was tried with methylene blue and neutral red.

Observations on living materials were supplemented with examination of sections. In this case, strong Flemming's fluid was used as the fixative and the sections were stained with Heidenhain's haematoxylin and eosin. Staining with thionin (1% aqueous solution) was also tried.

All the observations were made at, or upon material obtained at, the Shimoda Marine Biological Station. It is our present duty to acknowledge our indebtedness to the Director and staff of that station for providing us facilities for carrying out this investigation. Thanks are also due to Miss Yoshiko Oshima who helped us in various ways.

Observations

Developmental cycle in the colony of *Botryllus*

In *Botryllus,* buds (palleal buds) appear very precociously, so that the constituting members of a colony are not single individuals, but aggregates of individuals belonging to three successive generations. They have been given the name "units" (Watanabe, 1953). Generally, a unit consists of more than three individuals. As a rule, two pairs of buds are formed by each individual, though not all of them develop. In the following text, as well as in Figure 1, each generation is represented by only one individual. An individual has a definite life-span. Its life has been divided into 11 developmental stages by Berrill (1941a).

A unit shows four different combinations of stages.

On the first day, a bud of stage 1 is seen on the lateral wall of a bud of stage 6, which in its turn is connected with a zooid of stage 9 by means of the connecting

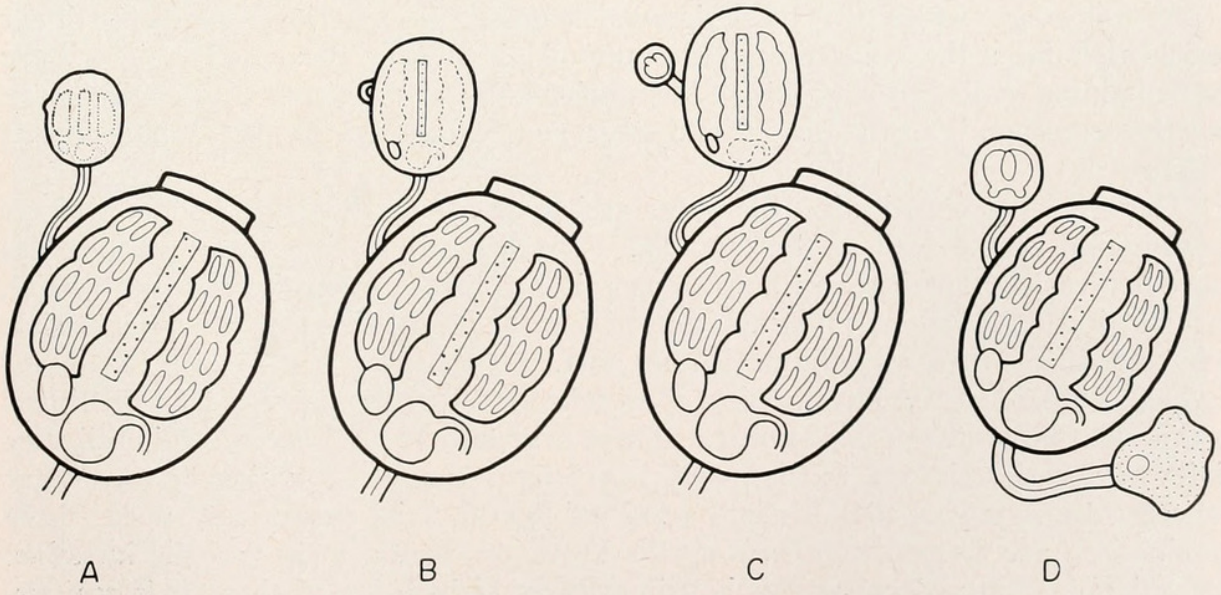


Figure 1. Developmental cycle of the unit in the colony of *Botryllus* (semi-diagrammatic). A, Phase A; B, Phase B; C, Phase C; D, Phase D.

vessel, *i.e.*, the colonial circulatory system. The combination of stages is 1–6–9. This phase is called phase A (Fig. 1, A).

On the second day, the bud of stage 1 has developed into a bud of stage 3. The bud of stage 6, on the other hand, has grown into a bud of stage 8. This latter is attached to the parent zooid which is still in stage 9. The combination of stages is 3–8–9. The phase is called phase B (Fig. 1, B).

Similarly, the phase on the third day is called phase C (combination of stages: 4–8–9) (Fig. 1, C), and that on the fourth day phase D (combination of stages: 5–9–11) (Fig. 1, D).

On the fifth day, the bud of stage 5 has grown into stage 6 with a new bud of stage 1 formed upon it. It is attached to the zooid of stage 9. The zooid of stage 11 has completely disappeared. The unit thus returns to phase A and starts a new cycle.

In each unit, the four phases are regularly repeated. Since all the units in a colony are exactly coordinated, we can also speak of the phases of the colony as a whole. A colony has four successive phases, which constitute a developmental cycle.

Formation of the vascular bud and its further development

The formation of the bud is initiated by gathering of particular blood-cells (diameter *ca.* 3–4 μ; see below) under the epidermis at the base of ampullae (Fig. 6). The number of cells is about 15–20. An intensive cell division follows, and, in one hour or so, a mass of cells (diameter *ca.* 20 μ) is formed (Figs. 2, 7). Since an ampulla is about 100–110 μ in diameter, the mass occupies about one-fifth of its width.

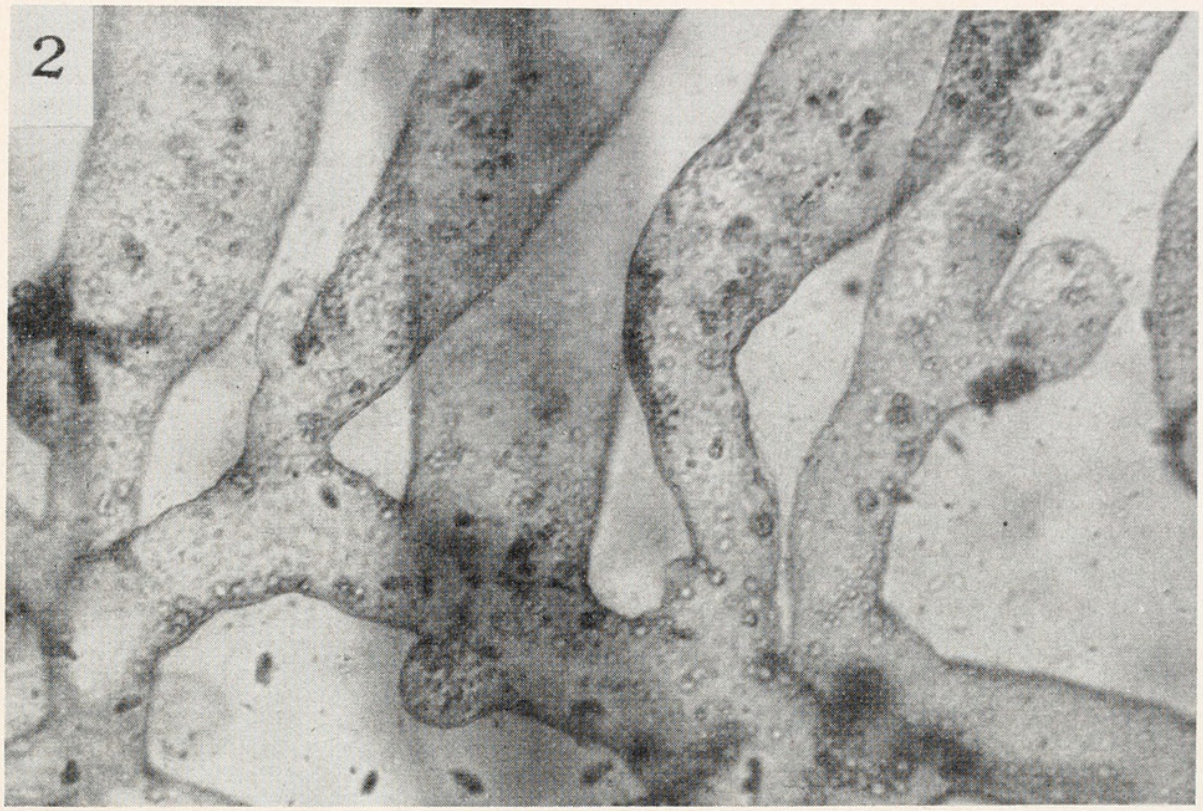
Active cell division continues, and, in two or three hours, a blastula-like structure is formed (Figs. 3, 8), At the same time, the side-wall of the ampulla that lies over the blastogenic mass begins gradually to protrude, and, in four to five hours, becomes distinctly visible as a bud (diameter 40–50 μ) (Figs. 4, 9).

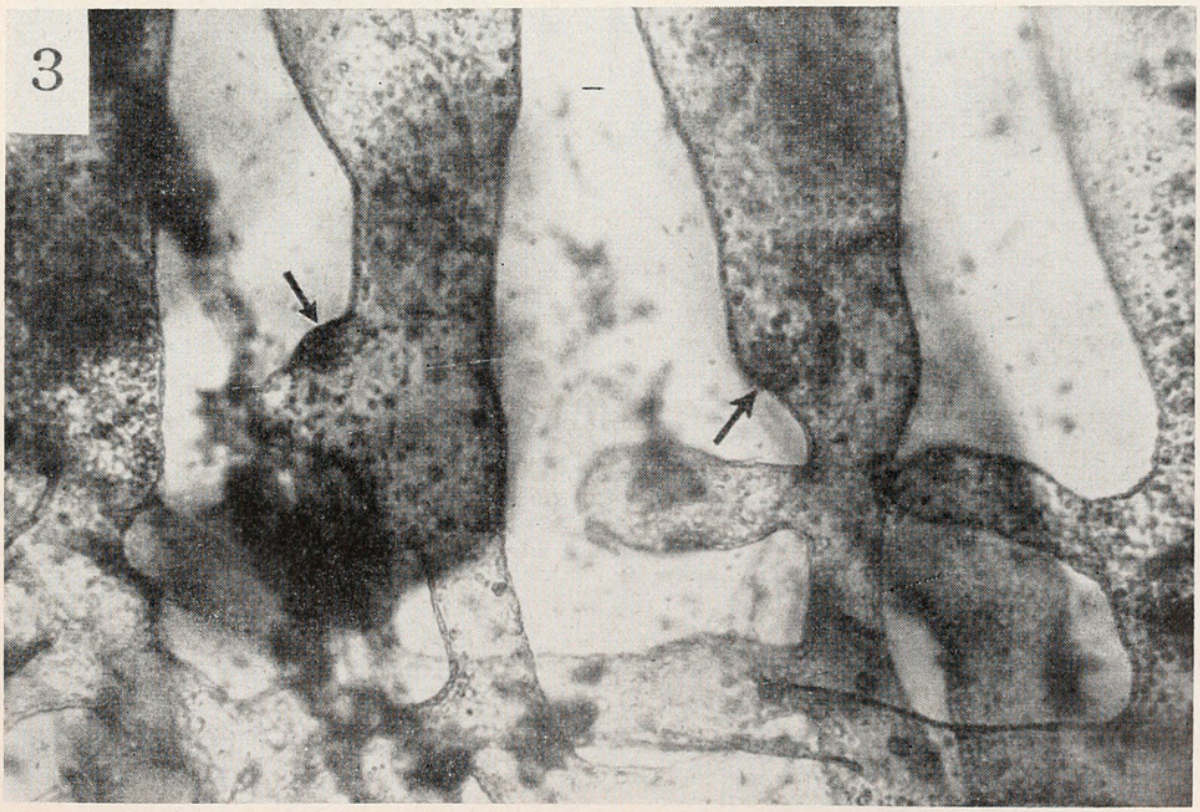
At this stage, which will be designated stage 3, the anterior wall of the inner vesicle that faces the epidermis of the ampulla is two or three cells thick, while the remaining walls are very thin. Morphologically, the vascular bud of this stage exactly corresponds to the palleal bud of stage 3 (diameter *ca.* 48 μ) except that it has no ova.

The cell layer constituting the outer vesicle or ectoderm of the bud is a direct continuation of the wall of the ampulla. The layer is at first closely applied to the inner vesicle, but later an ample space is formed between the two into which various kinds of blood-cells migrate, thus giving the impression that the inner vesicle is floating in the middle of the projecting part.

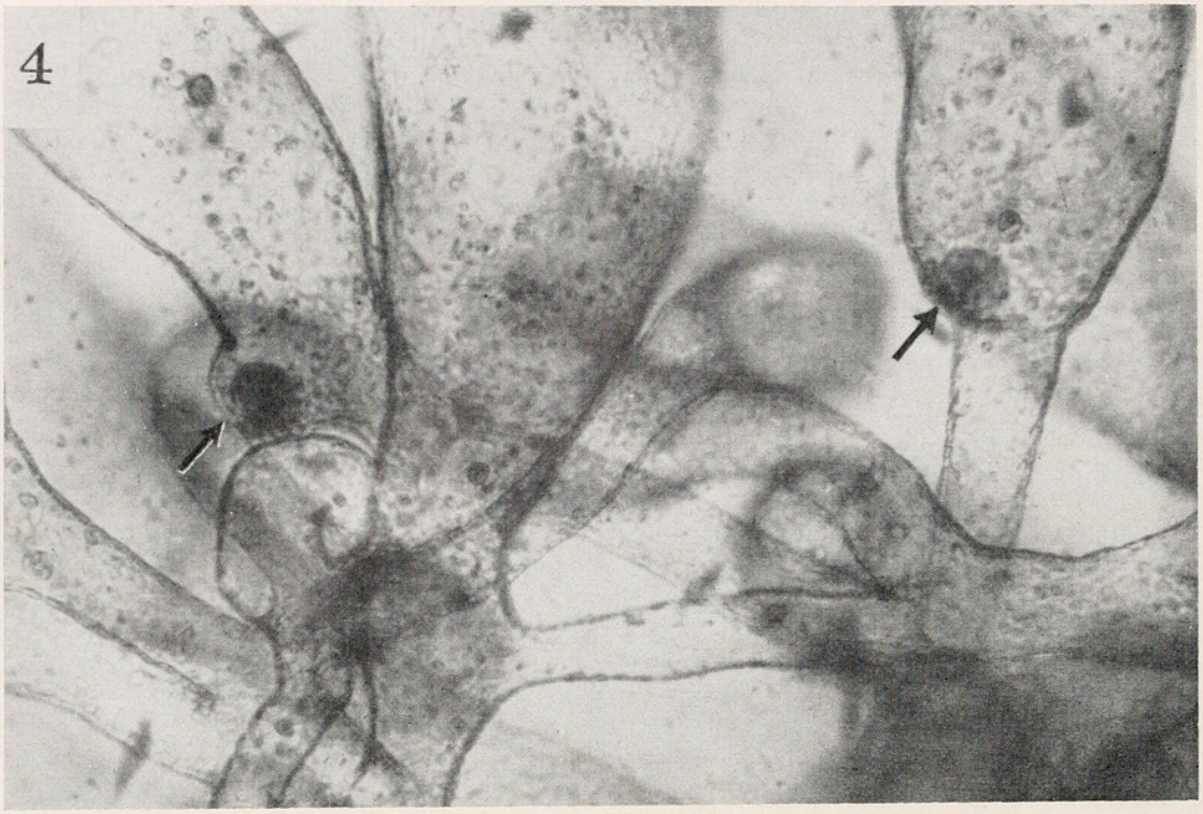
Development of the inner vesicle is as follows. As the anterior wall continues to expand, two vertical folds appear. These gradually extend backwards until they divide the vesicle into a median and two lateral chambers, which become later the central pharyngeal chamber and a pair of lateral atrial chambers. Next, three evaginations are formed, representing the heart, the neural mass and the intestine, respectively. Later development is primarily an elaboration of these unit regions.

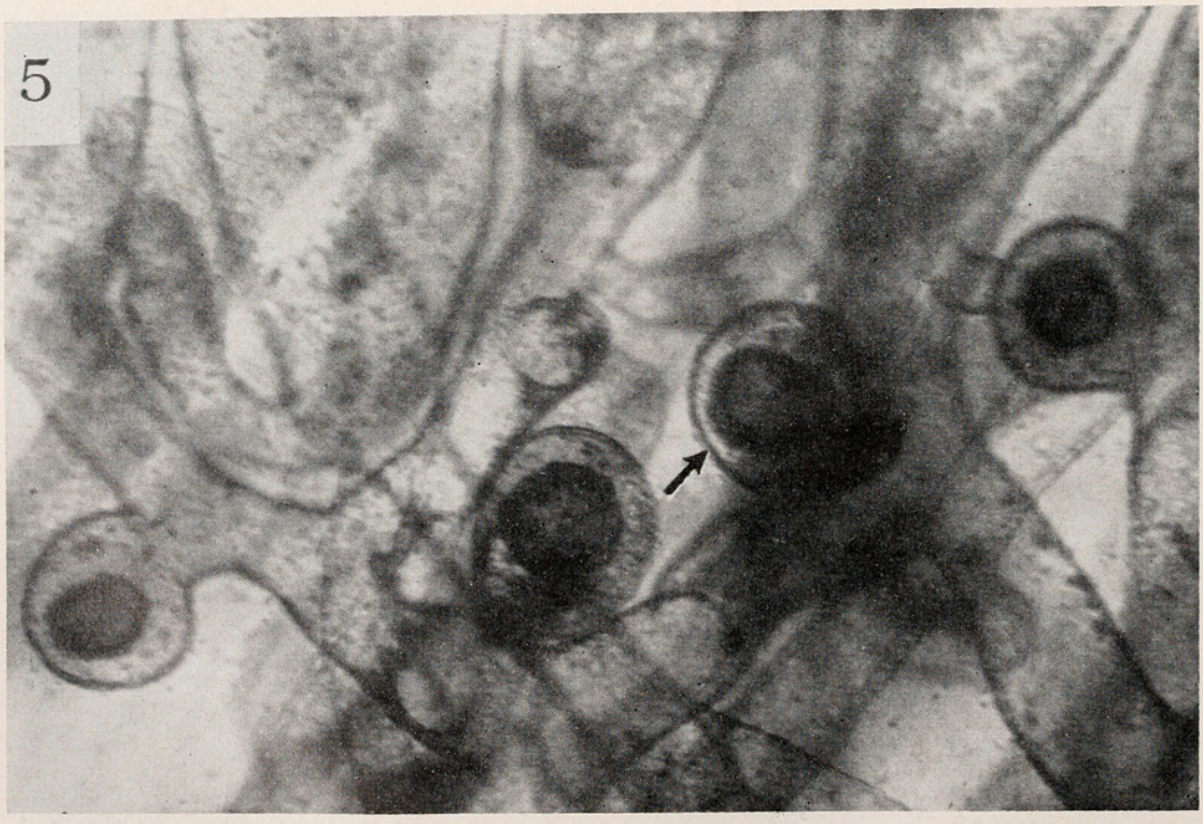
Thus, the blastogenesis in vascular buds is an exact replica of that in palleal buds.



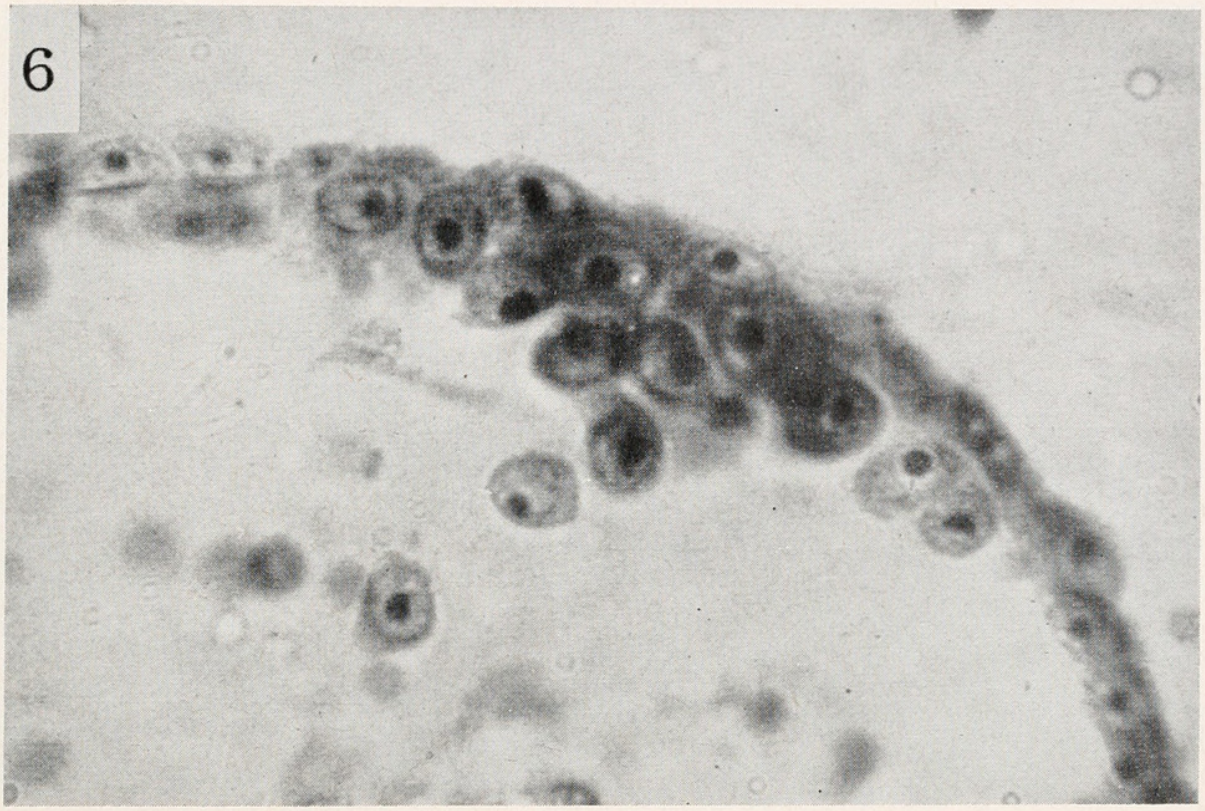


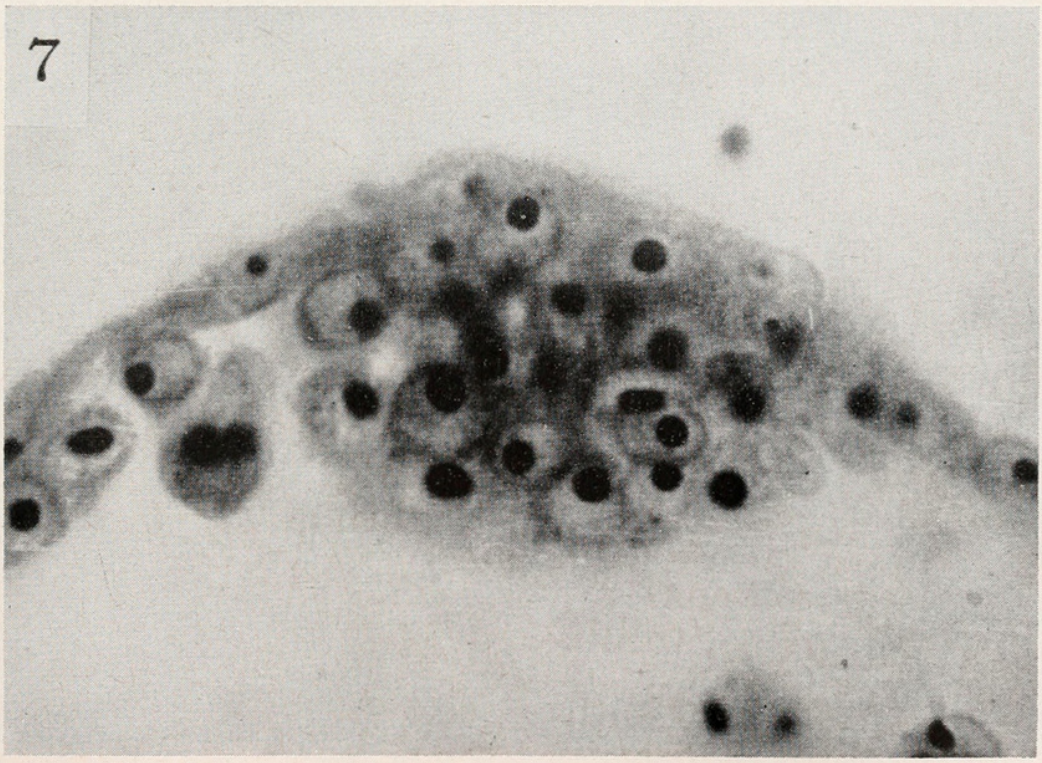
FIGURES 2–3. Development of the vascular buds. Figure 2 shows ampullae just before the appearance of vascular buds. × 120.



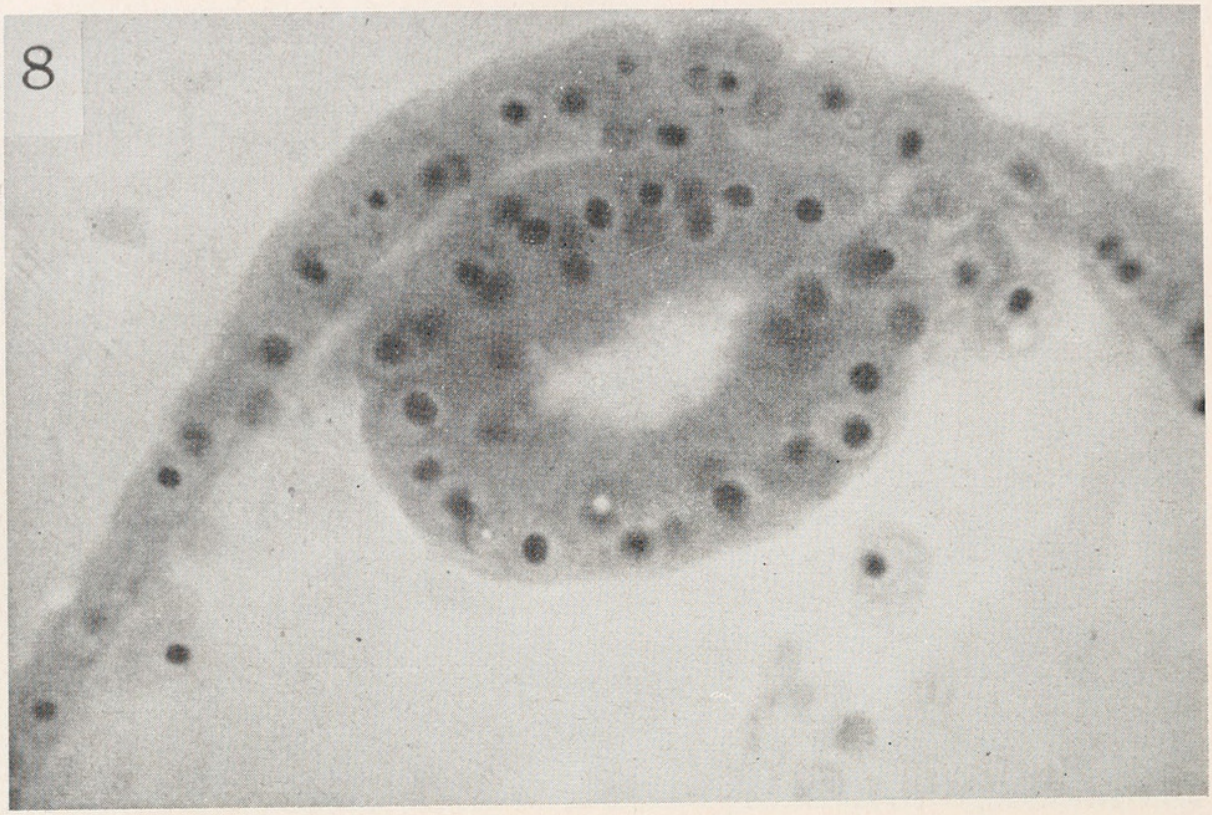


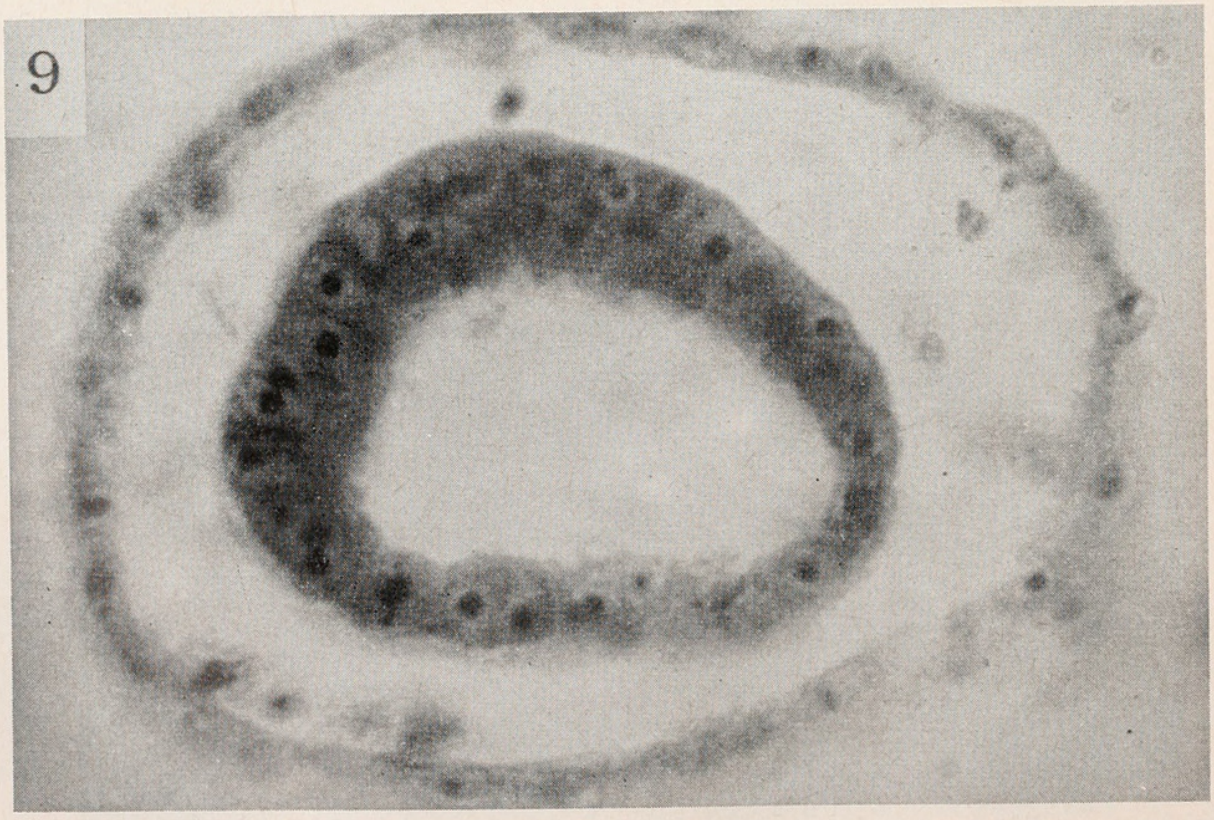
FIGURES 4–5. Development of the vascular buds (continued). × 120.





FIGURES 6–7. Development of the vascular buds; in sections. × 1200.





FIGURES 8–9. Development of the vascular buds; in sections (continued). Fig. 8, × 1200; Fig. 9, × 1000

Nature of the blood-cells

The blood-cells from which the inner vesicle originates are small, round cells. They show a strong affinity for thionin; further, they are more strongly stained by such vital stains as methylene blue (1/10,000 aq. sol.) or neutral red (1/10,000 aq. sol.) than other blood elements.

In sections, it is seen that the cells have a hyaline cytoplasm; their nucleus is filled with a dense network of chromatin and lacks a nucleolus. The total picture suggests that they are cells of a primitive nature. They are the lymphocytes in the terminology of Sabbadin (1955).

Time of appearance

The appearance of the vascular buds is limited to a certain phase in the life cycle of the colony. They appear only during a short period (about 10 hours), extending from later phase B to early phase C. At that time, the colony is at the maximum of its activity and, accordingly, the growth of the ampullae is also very active.

In other phases, no buds are formed even where they are expected to appear. In phase D we sometimes find small buds; however, they are not new buds but older ones that have appeared in early phase C and are now on the way to dissolution.

Site of appearance

In *Botryllus* colonies, numerous blood vessels traverse the test and terminate in contractile ampullae at the periphery of the colony; a flow of blood is maintained by them independently of heart action. The buds appear at the base of ampullae at a distance of about 0.6–0.7 mm. from the tip (Fig. 10).

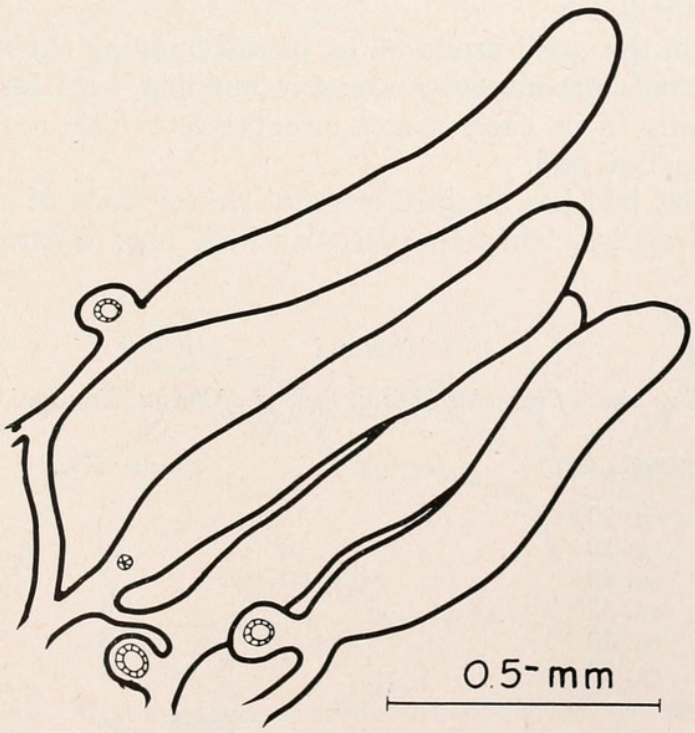


FIGURE 10. Vascular buds at the end of budding period. Note the various sizes.

On each ampulla, only one bud is formed at a time.

Not a single case has been recorded in which the buds are formed from the side-wall of the ordinary vessels.

So far as is known, the appearance of the vascular buds is restricted to the most actively growing edges of the colony where the ampullae are especially numerous and active.

Degeneration of the buds

At the end of the budding period (early phase C) we often find tens and hundreds of newly formed buds, but not all of them continue to develop. Only those which surpass a certain size can develop and form perfect zooids, while the remaining ones undergo involution without even approximately reaching the stage when the two vertical folds appear. For example, in a case out of 200 newly formed buds, only 70 developed into perfect ascidiozooids.

Table I shows an example of different sizes of the buds in a colony at the end of the budding period.

Of these 36 buds, those larger than 40 μ continued to develop (20 buds, or 56%), while those under 35 μ soon began involution and disappeared completely (16 buds, or 44%).

Relation between palleal and vascular budding

As is shown in Table II, the palleal bud appears in phase A. The vascular bud appears, as has already been pointed out, at the end of phase B, and attains stage 3 in phase C. It grows rapidly and becomes stage 5′ (a stage a little behind 5) in phase D and stage 6′ (a stage a little behind 6) in phase A of the following cycle. In phase B the vascular bud attains the stage 8, and from this moment on it develops exactly synchronously with the corresponding palleal bud. It also dies synchronously with the latter.

In brief, the life of the zooid produced by palleal budding extends over 12 days, while that of the zooids produced by vascular budding lasts for ten days. The development, especially in its early stages, progresses a little more rapidly in the vascular than in the palleal bud.

When the vascular bud has attained stage 6′ we see buds of stage 1 appearing on its peribranchial walls. Thus the vascular bud, once formed, propagates by palleal budding.

TABLE I

Various sizes of vascular buds at the end of the budding period

Diameter (in μ)	Number of buds
ca. 70	3
ca. 50	10
ca. 40	7
ca. 35	4
ca. 30	8
ca. 20	4
Total	36

TABLE II

Relation between palleal and vascular budding. Vascular buds are printed in bold-face type

Day	Cycle	Phase	Palleal budding	Vascular budding		
I		A	1			
II		B	3			
III	n	C	4	**3**		
IV		D	5	**5′**		
V		A	1–6	1–**6′**		
VI		B	3–8	3–8		
VII	n 1	C	4–8	4–8	**3**	
VIII		D	5–9	5–9	**5′**	
IX		A	1–6–9	1–6–9	1–**6′**	
X		B	3–8–9	3–8–9	3–8	
XI	n 2	C	4–8–9	4–8–9	4–8	**3**
XII		D	5–9–11	5–9–11	5–9	**5′**

Each vertical column represents the development of the same individual.

In the growing edges of a colony, the test vessels grow out continuously. The distal end of the ampulla grows out and forms the new tip, while its basal part is continuously transformed into the ordinary vessel. This means that the vascular bud, though first formed at the base of the ampulla, is gradually removed from the latter. By the time the bud has attained stage 4–8, it is already at some distance from the ampulla. At the same time we see that a new vascular bud is being formed at the base of the ampulla (Fig. 11). Thus an ampulla forms only one bud at a time, yet it can produce many consecutively.

As the vascular bud is formed a little later than the corresponding palleal one, it is at first smaller than the latter. At the end of development, however, no difference in size is perceptible between the two.

The full-grown zooids formed by vascular budding are the same as those produced by palleal budding in almost every respect, even in the number of tentacles

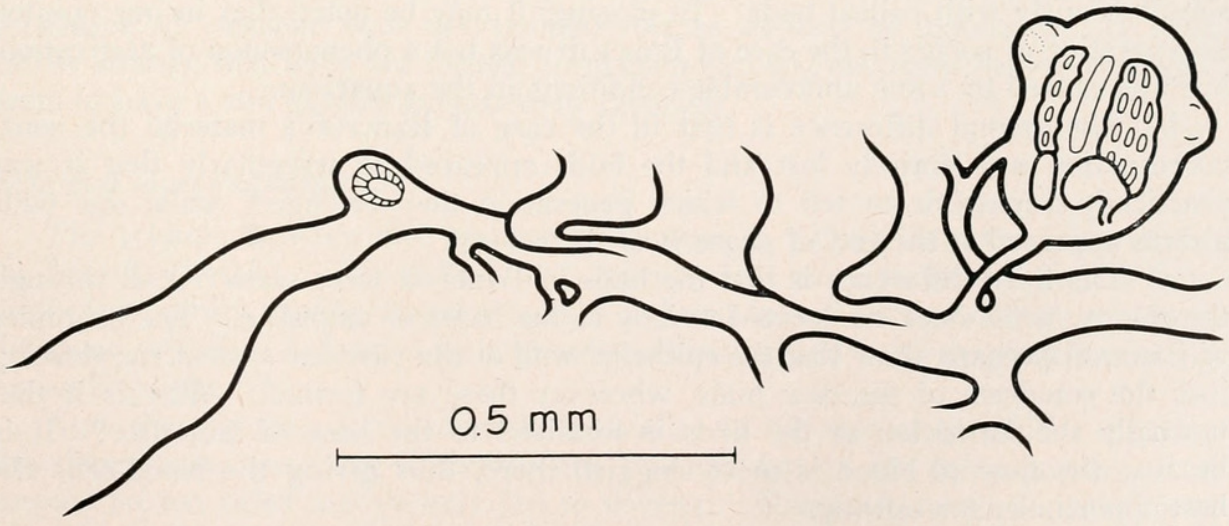


FIGURE 11. Two buds formed consecutively on the same ampulla.

or rows of stigmata. The only difference is that in vascular buds no gonads are formed. In palleal buds, the gonads segregate as a mass from the lateral walls of the bud at an extremely precocious period. Such a segregation has never been observed in vascular buds.

Discussion

Critical review of the earlier data

With this fully established vascular budding at hand, a critical review of the earlier data concerning budding in botryllids will be attempted.

Some of the earliest descriptions of the stolonial budding in *Botryllus,* such as those of Savigny or Milne-Edward, might have been founded on erroneous observations, but what Herdman observed in *Sarcobotrylloides wyvillii* and Bancroft in *Botrylloides gascoi* was in all probability vascular budding analogous to that described here. Some passages in Giard's paper (1872, p. 573) also make it probable that he really observed vascular budding in *Botryllus.*

According to W. H. Herdman (1886; pp. 59, 90), the buds were formed from three sources: large cells which became ova, blood corpuscles, and the wall of blood vessels. In the case of our buds, large cells which were to become ova could not be detected in the mass of cells.

The buds which were found in *Botrylloides gascoi* during aestivation, and which were entirely independent of parent zooids, are in our opinion certainly vascular buds, and not palleal buds developed from the zooids of the original colony and later carried into the yellow lobe, as was assumed by Bancroft. Bancroft met with the so-called yellow lobe only once, so he had no idea of its significance at the time when this was developing. He says (1903b, p. 152), "It is to be hoped that some future investigator will preserve and section the early stages of the development of the yellow lobe before the degeneration of the rest of the colony, and discover why in this case the buds separate from the parent zooids at such an early stage." These buds in all probability were formed *in situ* from blood cells.

There are some differences between our buds and those of Bancroft.

a) The buds described by Bancroft were formed after all the zooids had died away. Our vascular buds, on the contrary, are formed in an active colony simultaneously with palleal buds. In passing, it may be noted that in our opinion degeneration of zooids in the case of Bancroft was not a phenomenon of aestivation, but was caused by some unfavorable conditions in the aquarium.

b) The second difference is that in the case of Bancroft's material the usual coordination was entirely lost and the buds appeared so irregularly that it was practically impossible to tell to which generation they belonged, while our buds always appeared at the end of phase B of the colony.

c) The third difference is that the buds of Bancroft were scattered all through the colony, while ours are located strictly at the bases of ampullae. The examples of Bancroft perhaps show that the epithelial wall of the vascular system can develop into the ectoderm of the new buds, wherever these are formed. Why is it that normally the formation of the buds is localized to the base of ampullae? It is because the flow of blood is most sluggish there, thus giving the blood-cells the best opportunity for settling.

Vascular budding in the system of budding of ascidians

Whatever the type of budding may be, the initial stage of blastogenesis is a double-walled vesicle. The outer wall, called ectoblast, forms the epidermis, while the inner wall, called endoblast, forms the rest of the new zooid. The ectoblast invariably derives from the epidermis of the parent zooid, while the endoblast is variable in origin. According to this origin, the budding of ascidians is divided into three main groups: ectodermic, endodermic and mesoblastic. Vascular budding as described here, together with stolonial budding, belongs to the last group. Of all known cases of stolonial budding, that of *Perophora* comes nearest to the vascular budding of *Botryllus*. In *Perophora* the endoblast is formed from the mesenchymatous septum of the stolon.

Blood-cells in relation to budding

Generally, blood-cells are considered to have nothing to do with bud formation, Berrill (1951), for example, in a survey on regeneration and budding in ascidians states: (p. 468): "Trophocytes, 'cell-packets,' and the various blood cells, other than those lymphotic cells which at times arise from septal mesenchyme or the epicardial epithelium, apparently play no part in any morphogenetic or histogenetic processes, except as victuallers." Neither epicardium nor mesenchymatous septum does exist in *Botryllus*. But, since the mesenchymatous septum in other forms, notably in *Clavelina*, is known to gain or lose cells from or to the haemocoele as lymphochytes, the blood-cells of *Botryllus* forming new buds may possibly be conceived as mesenchymatous septum dissolved into its cellular elements and circulating with the blood stream.

According to the recent investigation of Sabbadin (1955), the blood-cells of *Botryllus* are of dual origin. One evolutionary series begins with a hemoblast. It has a vascular nucleus with a small amount of chromatin and cytoplasm rich in RNA-proteins. The other series begins with a lymphocyte. This is half the size of a hemoblast and is formed from the latter by division. Its nucleus is filled with a dense network of chromatin and lacks a nucleolus. Both series have their own leucocytes and vacuolated cells.

As is clear from the foregoing descriptions, the blood-cells partaking in the formation of vascular buds are lymphocytes as defined by Sabbadin. It seems rather strange that buds are formed from lymphocytes, not from hemoblasts which seem to have a still greater evolutionary capacity.

Size and morphogenesis

The relation between size and morphogenesis in palleal buds has been studied by Berrill (1941b). The palleal bud appears first as a thickened disc of atrial epithelium, which rapidly transforms into a closed sphere surrounded by epidermis. The new organism is formed by a process of folding and local evaginations of this expanding sphere.

Now there is seen a considerable variability in the size of the initial disc, and this determines, according to Berrill, the size and fate of the future zooid. Buds formed on too small a scale may fail to develop. Relatively small bud rudiments give rise to small zooids without gonads and with about six rows of stigmata, while

large rudiments form a greater number of rows of stigmata and produce testes and ripening ova; between these two there are many intermediate forms. In general, discs and spheres produced by early generations are relatively small compared with those produced by later generations. According to Berrill this accounts for the fact that juvenile colonies are asexual, somewhat older colonies have well-developed testes but no ova, and only at the last do both testes and ova appear.

The same explanation does not apply to the case of vascular buds. As has been demonstrated, the period during which the vascular buds appear is relatively short, extending from later phase B to early phase C. At the end of this period, we see buds of various sizes, but only those which surpass a certain size continue to develop, all the remainder degenerating.

We have studied in sections the number of blood-cells taking part in the formation of buds and found that variation is rather slight. The initial number of blood-cells, therefore, cannot be the cause of different sizes. The cause of the small size of some buds is to be sought in the belatedness of their appearance. Thus, small buds seem to have as full developmental capacity as larger ones. Their degeneration must, therefore, be understood as a manifestation of regulation of the colony as a whole.

The absence of gonads in vascular buds is also not the sequence of small size, for the average size of vascular buds is of the same order as that of palleal buds, in which well-developed testes and ova appear.

Regulating mechanism

Of all colony-forming animals, *Botryllus* is perhaps the one in which the zooids are most perfectly coordinated. We still know too little of the nature of the regulating mechanism, yet the fact that such a mechanism is working can be inferred from the following facts:

a) In a colony, the zooids are exactly coordinated in budding and development.

b) When two pieces of related colonies at different developmental phases are fused together, this difference is invariably equalized. This was first demonstrated by Bancroft (1903a) and is now being extensively studied by one of us (*cf.* Watanabe, 1953).

c) The vascular buds are formed a little later than the corresponding palleal buds, but they are soon synchronized with the latter.

d) Vascular buds formed too late are forced to degenerate, thus being eliminated from the colony.

Vascular budding in relation to the growth of the colony

In *Botryllus primigenus*, palleal and vascular budding coexist in a colony. Maintenance and multiplication of zooids are brought about by palleal budding, while localized, rapid growth of the colony seeking a new substratum is effected exclusively by vascular budding.

The colony can continue its existence by vascular budding alone. Such a state is realized when a piece of a colony containing no zooids but ampullae is isolated, or when all the zooids are experimentally removed, or again when, as in the case of Bancroft's material, all the zooids have died, owing to some unfavorable external

conditions. According to Bancroft (1899, p. 451) an isolated piece devoid of zooids never regenerated a colony, and none of the ampullae in such a piece showed the least tendency towards budding. On this point, therefore, we are quite at variance with him.

Significance of our discovery for the general theory of budding in ascidians

Our discovery is of significance in the following points:

a) In Stolidobranchiata, to which the genus *Botryllus* belongs, palleal budding has been considered the only way of asexual reproduction. We now know that *Botryllus* propagates also by vascular budding. This indicates that budding of a rather primitive nature, as the vascular, has not completely disappeared even in such a highly specialized group as Stolidobranchiata.

b) It is generally admitted that each species is given a single mode of asexual reproduction. Now, *Botryllus* propagates by two entirely different kinds of propagation, one ectodermic-peribranchial and one mesoblastic-vascular.

c) The lymphocytes are capable themselves of organizing new individuals.

Is vascular budding peculiar to Botryllus primigenus?

If we consider that of all the genera of compound ascidians it is *Botryllus* that has been most extensively studied till now, it is almost inconceivable that so typical a budding as the vascular has escaped the eyes of previous investigators. It is possible, although not very probable, that this type of budding is peculiar to our Japanese species. We have observed the same budding also in *Botryllus communis,* another Japanese species, which, however, in our opinion is homospecific with *B. primigenus.*

Summary

1. In *Botryllus primigenus,* it has been found that, in addition to palleal budding, new buds are formed also from aggregations of blood-cells at the base of ampullae.

2. The blood-cells partaking in the formation of buds are lymphocytes as defined by Sabbadin.

3. The formation of buds is possible only at a certain phase in the developmental cycle of the colony.

4. Even then, the buds are formed, not on all ampullae, but only on those lying in the most vigorously growing edges of the colony.

5. Our discovery is of significance in the following three points:

a. The ability to form new buds from the vascular wall has not completely disappeared in Stolidobranchiata.

b. A species can propagate by two entirely different kinds of budding. In our case, one is ectodermic-palleal and one mesoblastic-vascular.

c. The lymphocytes are themselves capable of organizing new individuals.

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