Determination of Polarity and Bilateral Asymmetry in Palleal and Vascular Buds of the Ascidian *Botryllus schlosseri*

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Reversal of the bilateral asymmetry of the zooids was induced in a series of colonies of *Botryllus schlosseri*. Palleal buds from colonies with normal or reversed bilateral asymmetry were isolated in the early stages from the parental zooids and cultured in the vascularized tunic of the same colony or of another colony with opposite asymmetry. Vascular budding was induced in colonies with either type of asymmetry.

The bud polarity was shown to depend on the vascularization; the test vessel entering the isolated palleal bud always causes the entrance point to become the posterior end of the developing zooid. On the contrary, the bilateral asymmetric type is predetermined in the bud primordium; the isolated palleal buds develop the type of asymmetry of their parents, even when grafted in the test of a colony with opposite asymmetry. Since the same was also true of the vascular buds, it is concluded that the information for the kind of bilateral asymmetry to be developed is conveyed by the epidermal envelope of the bud. The epidermis of the parental zooids influences the palleal buds, whereas the wall of the test vessels, epidermal extrusions of the zooids, influences the vascular buds.

INTRODUCTION

A full account of the morphological expression of polarity and bilateral asymmetry in the development of the palleal bud of *Botryllus schlosseri* has been given by Izzard (1973). Both these features coincide with those of the parental zooid, to which the bud remains attached, suggesting that they are impressed on the bud by the parent itself. However, experimental evidence for this conclusion is rather scanty. Moreover nothing is known about the determination of these characters in vascular buds originating in the tunic after the removal of the zooids which therefore should be ruled out as direct causative agents.

The present research is an attempt to fill these gaps in our knowledge. It was first suggested by our interest in the mechanism by which the reversed bilateral asymmetry, once established in the zooid, is thereafter transmitted to the buds of all successive generations (Sabbadin, 1958; 1960).

Two experimental approaches were adopted: 1) Palleal buds in the early stages were allowed to develop in the vascularized tunic after removal of the parental zooids which had either normal or reversed bilateral asymmetry, or the buds were interchanged between colonies of opposite asymmetry; 2) vascular budding was induced in two sets of colonies with opposite bilateral asymmetry, the two series of vascular buds then being compared for the kind of asymmetry expressed.

A preliminary report of this work has been presented elsewhere (Sabbadin *et al.*, 1971).

MATERIALS AND METHODS

Starting with metamorphosing larvae attached to glass slides, colonies were obtained and reared in aquaria with aerated, periodically renewed sea water maintained at a constant temperature of 18°C. They were fed with unicellular algae.

The colony (Fig. 1) consists of three coexisting generations of zooids: The adults and two successive generations of buds. The stages in the life cycle of the three generations are so correlated that when the adults regress their place is taken by the older buds, while the younger ones give rise to a new generation (Berrill, 1941). The

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bud originates bilaterally by an extrusion of the two mantle layers (atrial epithelium and epidermis) of the parental zooid, which form a vesicle that then undergoes organogenesis. The bud is connected to the parental zooid through an epidermic stalk that conveys the blood. The sequential series of epidermic stalks persists after the regression of the adults of each generation, forming a vascular network. This transverses the tunic and opens at the periphery into a collecting marginal vessel completely surrounding the colony and from which a number of peduncled ampullae branch out. The zooids have their anterior ends facing the marginal vessel to which the adults and the older buds are joined by radial vessels (Fig. 1).

We may therefore envisage two elements in the colony: The zooids, which are continually renewed, and the persisting colonial matrix with its vascular system.

The colonial matrix, owing to the contraction of the vascular ampullae maintaining an autonomous circulation, may survive for weeks in the absence of zooids and, moreover, it can reconstitute the entire colony by a process of vascular budding (Milkman and Byrne, 1961; Milkman, 1967).

In order to induce vascular budding, all the zooids, both adults and buds, were removed with dissecting needles from 139 colonies. In this way a preparation composed of the peripheral part of the tunic with the marginal vessel and the system of ampullae intact was obtained (Fig. 2). The extirpation of zooids resulted in a slowing down of the circulation with stasis of clotted hemocytes in the vessels. When vascu-

lar buds developed (Figs. 5 and 6), their heart beats revived the circulation and the clots dissolved. We obtained 97 vascular buds; 49 of them became adult zooids distributed in 40 surviving colonies.

Which tissues were involved in vascular bud formation could not be established by *in vivo* observation. We have been dealing with vascular buds in the strict sense in that the epidermis of the two-layered bud vesicle was always an extrusion of the wall of a vascular element, a vessel or an ampulla, to which the bud remained connected by a stalk (Fig. 5). It is reasonable to believe that the inner layer was derived from an aggregation of undifferentiated hemocytes, as demonstrated by Oka and Watanabe (1957; 1959) in *Botryllus primigenus* and in *Botrylloides violaceum*.

The colonial matrix was also used as a culture medium for palleal buds in the early stages.

The thickening of the atrial epithelium (1), which represents the palleal bud primordium, its arching into a prominent hemisphere (2) and its later development into an almost closed vesicle covered by epidermis (3) were classified by Sabbadin (1955) as stage 1,2, and 3, respectively. At a temperature of 18°C the stages succeed each other in the following sequence of 24-hr periods: 1, 1+, 2, 2+ and 3; stage 1+ indicates the initial arching; stage 2+ the skewing of the hemisphere from its lateral orientation towards the anterior end of the parent, together with an increase in area which foreshadows the subsequent closure. Since the bud on the right side is more advanced than that on the left, its stage 2+ corresponds to stage 2 of the

FIG. 1. A young colony of *B. schlosseri* consisting of a single system of adult zooids (z) and two successive generations of buds (b1, b2), seen from the ventral side. Normal type of bilateral asymmetry: Heart (h) on the right and intestinal tract (i) on the left. Marginal vessel (mv) with radiating ampullae (a) and radial vessels (rv) in the tunic. × 20.

FIG. 2. Colonial matrix prepared for vascular budding or ingrafting of palleal buds. × 16.

FIG. 3. Reversed type of bilateral asymmetry in the zooids (compare with Fig. 1). × 20.

FIG. 4. Greater blastogenic potential on the right side of the zooids; one of the two adult zooids (z) lacks the sinistral bud; in the other the bud is atrophied (ab1); dextral buds (b1) normal. × 28.
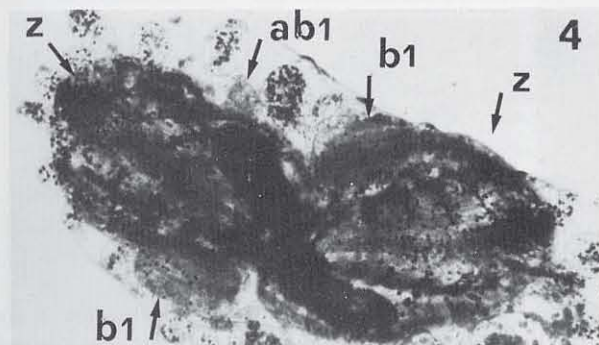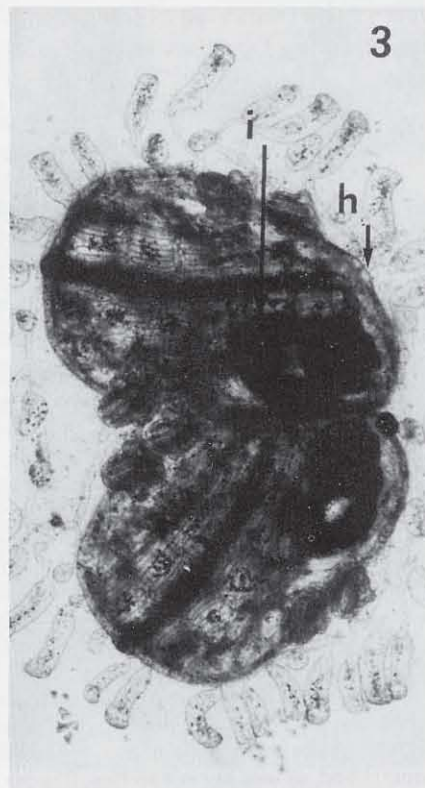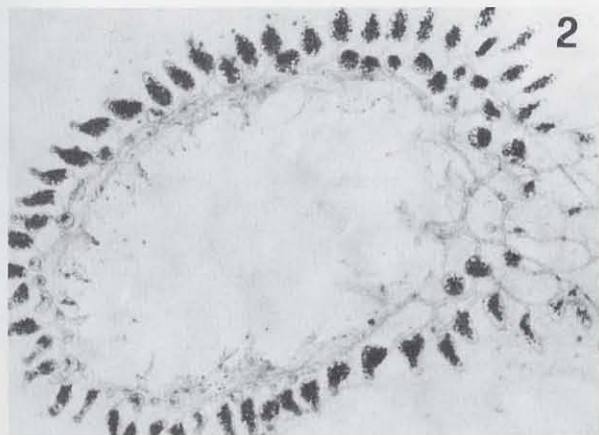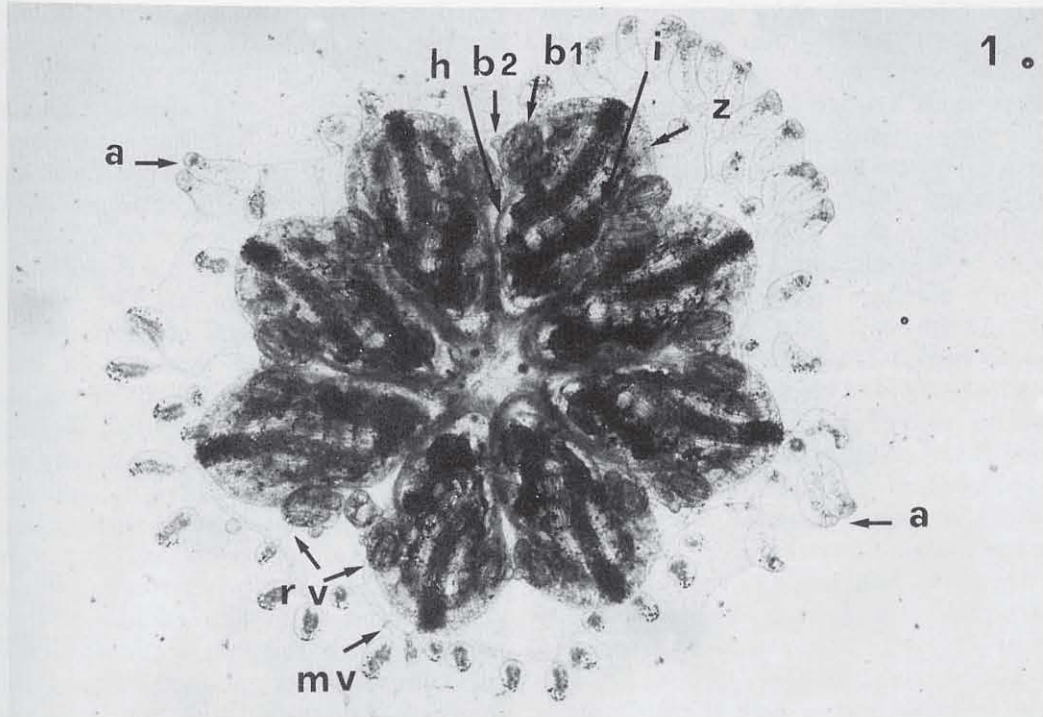
sinistral bud, and, when the latter reaches stage 3, the dextral bud has already expanded along the antero-posterior axis of the parent, a stage which we call 3+. At this stage both polarity and bilateral asymmetry are morphologically recognizable (Izzard, 1973).

Buds at stages 1 through 3+ were isolated by a semicircular cut of the parental mantle at the stages 1 and 1+ and by cutting the connecting stalk for the subsequent stages. The operation was preceded by the removal of the adults and concluded by the removal of the parental buds. In a majority of cases the isolated buds were left in place (Figs. 7 and 10), whereas in other cases they were grafted onto the periphery of the marginal vessel (Fig. 8) by inserting them into a hole made in the tunic of the same colony or in that of another colony from which all the zooids had been removed. Various numbers of buds per colony were isolated or grafted. All of them were of the same side of the parental buds, thus assuring that they were all at the same stage.

The buds which survived and developed are listed in Table 1.

The vascularization was a necessary condition for bud survival. The buds left in place were sequentially vascularized by one and then another collateral of the marginal vessel (Fig. 9); the same was true for most of the buds grafted peripherally, some of which, however, were vascularized by extensions of the ampullae or extensions of their stalks. The first vessel to arrive

acted as affluent, the second as effluent, the two connecting the bud with the general circulation.

In both the above experiments, vascular budding and bud isolation or grafting, colonies with normal and reversed bilateral asymmetry were used. In the normal bilateral asymmetry the digestive tract is located to the left of the midline and the heart to the right (Fig. 1). The colonies with reversed bilateral asymmetry are characterized by the inversion of *situs viscerum et cordis* (Fig. 3). Inversion was first obtained (Sabbadin, 1956) working with young colonies consisting of only one adult zooid with the bud on the right side developed and that on the left atrophied. The removal of the dextral bud close to the time of the change of generation caused the atrophied sinistral bud to resume development; however, the congestion of the bud with the resorption products of the adult sometimes disturbed the course of organogenesis with the possible sequela being the inversion of asymmetry. In the present

TABLE 1

NUMBER OF PALLEAL BUDS ISOLATED AT VARIOUS STAGES FROM THE PARENTS WHICH SUBSEQUENTLY DEVELOPED INTO ADULT ZOOIDS IN A COLONIAL MATRIX

Buds	Stage at isolation					
	1	1+	2	2+	3	3+
Isolated in place	18	16	47	44	56	15
Transplanted	1	1	3	11	7	4

FIG. 5. Vascular buds (vb) originating from test vessels. × 150.

FIG. 6. Developing vascular buds. × 15.

FIG. 7. Palleal buds (pb) at the vesicle stage isolated in place in the tunic. × 17.

FIG. 8. Palleal buds (pb) grafted into the tunic peripheral to the marginal vessels. × 32.

FIG. 9. Vascularization of an isolated palleal bud (pb). × 150.

FIG. 10. Developing isolated palleal buds. Some of them in regression (rb). × 15.

FIG. 11. Orientation of the antero-posterior axis (ap) in an isolated palleal bud. The posterior end of the bud turned toward the affluent vessel (av). × 36.

FIG. 12. The orientation of the dorso-ventral axis (dv) of the younger buds compared with that of the parental bud, as seen in cross section. The position of the heart primordium (hp) marks the developing bilateral asymmetry. × 190.
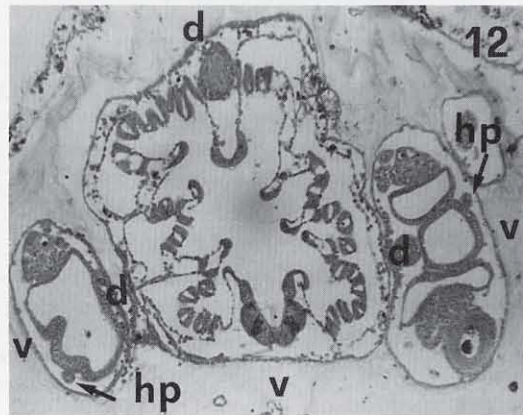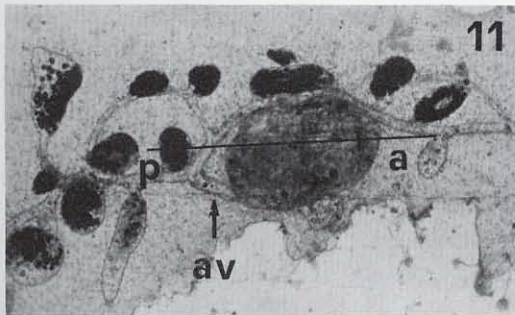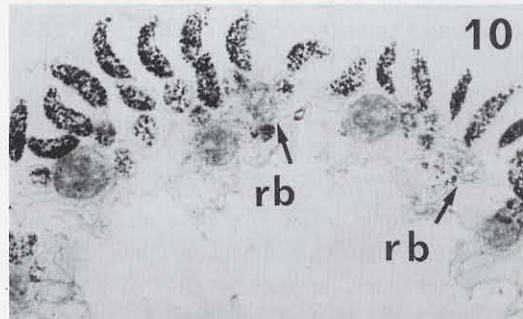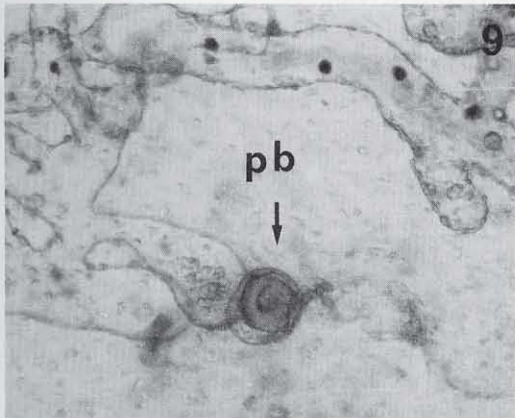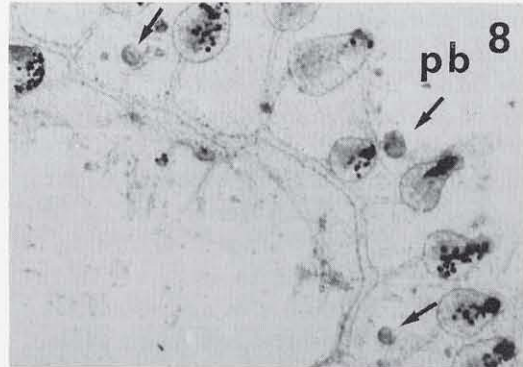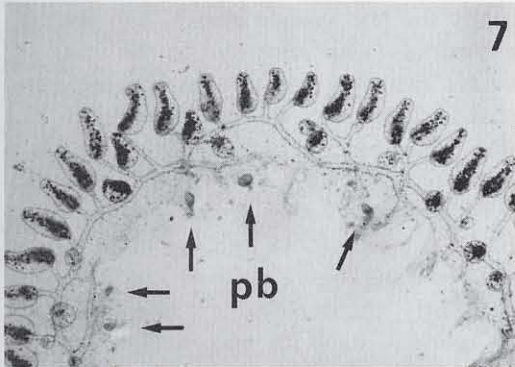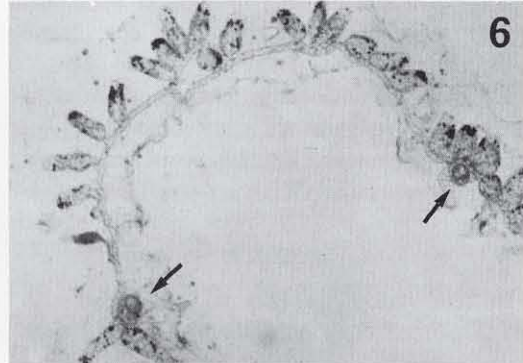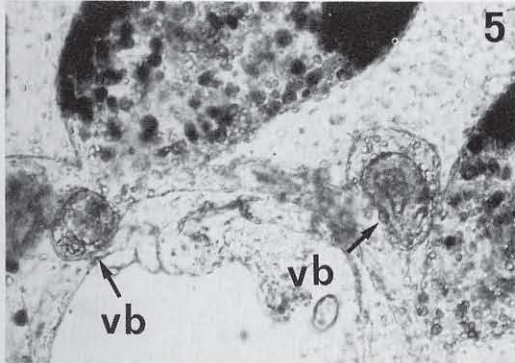
research, colonies with reversed bilateral asymmetry were obtained by the same basic procedure.

Daily observations of the experimental material were done with a Zeiss dissecting microscope; rough sketches were made and pictures were taken with a Zeiss Tessovar.

RESULTS

In the study by Izzard (1973) on the development of polarity and bilateral asymmetry in the palleal bud the following points were established: a) At the hemisphere stage (stage 2) the sagittal plane of the future zooids is defined by the major axis of the bud primordium and the axis of its arching into a hemisphere; b) later, when the hemisphere skews in the sagittal plane towards the anterior end of the parental bud (early stage 3 according to Izzard, corresponding to stage 2+ in the present report) both the antero-posterior and the dorso-ventral axes become evident (the anterior end of the bud eventually becoming the anterior end of the zooid and the side facing the parent becoming the future dorsal side); c) at the vesicle stage (late stage 3 according to Izzard, stage 3 in this report) the bud is still bilaterally symmetrical. The bilateral asymmetry becomes evident when the bud expands in the antero-posterior direction (stage 4 in Izzard's report, stage 3+ in the present one), its right posterior corner being more acute and projecting further posteriorly than the left one.

This sequence of facts implies a sequence of steps in the expression of polarity and bilateral asymmetry. However, as stated by Izzard, when and to what extent the axes of symmetry and the bilateral asymmetry are determined is a question far from being answered, especially because of the possible continuing influence of the parent to which the bud remains attached.

Isolation of buds at the critical stages and manipulation of vascular budding were the tools we chose in order to study the problem experimentally.

Determination of Antero-Posterior and Dorso-Ventral Axes

The vesicle stage, stage 3, is quickly attained by the buds isolated from the parents at stages 1 and 2. The isolated vesicle shows no fixed bilaterality; without exception its antero-posterior axis becomes oriented in the direction of the affluent vessel, its entrance marking the posterior end of the axis (Fig. 11). For those buds isolated in place at stages 2+ through 3+, this would mean a reversal of the axis. Actually the bud vesicle attached to the parent turns its anterior end to the marginal vessel, which gives rise to the collateral that is directed to the isolated vesicle. But it is more reasonable to say that the axis is actually reformed by the ensuing vascularization, the direction of the arriving vessel being unpredictable and, at least for the grafted buds, being quite at random. Consequently, the dorsoventral axis will orient or reorient itself in accordance with the antero-posterior one.

For the buds attached to the parents, as well as for vascular buds, the axiation also coincides with the vascularization and occurs in the same way, the blood arriving through the connecting stalk being directed from the future posterior to the future anterior end. At the early stages the dorso-ventral axis of the palleal bud does not correspond to that of the parental zooid but lies perpendicular to it in a horizontal plane, the future dorsal side of the bud turned to the parent (Fig. 12). The blood entering the dextral or sinistral vesicles through their future left or right side, respectively, passes anteriorly and thence posteriorly, leaving the bud through the opposite side after crossing the future midventral line. The differential vascularization of the future ventral and dorsal sides is perhaps the polarizing factor of the dorsoventral axis.

The vertical orientation of the dorso-ventral axis is attained rather late by an outward and opposite rotation of the dextral and sinistral buds. It may be that the

presence of the parent in some way governs this rotation. Actually, in the vascular buds, as well as in the isolated or transplanted palleal buds, an anomalous orientation of the axis was sometimes observed: In some cases it was horizontal, in other cases reversed, the bud turning its dorsal side to the substrate, or in some intermediate position attained. The reversed condition usually prevented the bud from opening its siphons, and it underwent a premature resorption; interestingly enough, its own buds developed with the dorso-ventral axis correctly oriented. No significant difference in the frequency of anomalously oriented buds was recognizable which would have reflected either dependence on the treatment or dependence on the stage.

It seems safe to conclude that both antero-posterior and dorso-ventral axes are not intrinsically impressed on the bud primordium but are imposed on the developing bud by environmental factors and that in the first developmental steps they can be restructured if the external factors change.

Determination of Bilateral Asymmetry

Three prominent features are evident in the bilateral asymmetry of the zooids of *B. schlosseri:* The *situs viscerum et cordis;* the greater blastogenic potential of the right side (Fig. 4); the greater gonadogenic potential of the left side. All three are reversed with reversal of bilateral asymmetry. The different blastogenic and gonado-

genic potentials of the two body sides might well fulfill an adaptive role in the colonial life cycle. The lower viability of the sinistral buds might allow the colony to survive with a minimum growth, yet retaining both sexual and asexual reproductive potential even under unfavorable conditions.

The influence of the parental zooid on the type of bilateral asymmetry that develops in its buds is quite evident, since the reversed asymmetry induced in the zooid is transmitted to the buds in all successive generations. Previous experiments of parabiosis between colonies of opposite bilateral asymmetry had excluded a humoral influence transmitted through the circulation or the tunic (Sabbadin, 1960). The present experiments, involving both isolation in place or grafting of palleal buds prior to or just at the time of expression of bilateral asymmetry, and observation of vascular budding were intended as a new approach for elucidating the mechanisms responsible.

Table 2 indicates the fate of vascular buds and of the palleal buds listed in Table 1. It will be noted that the palleal buds cultured in the colonial matrix always developed the same kind of bilateral asymmetry characteristic of the parental zooid regardless of the stage at which they were separated from it, even when they were grafted into colonies of opposite asymmetry. In the latter case, the colonial matrix; while determining the bilaterality

TABLE 2

NORMAL OR REVERSED BILATERAL ASYMMETRY EXPRESSED BY PALLEAL BUDS ISOLATED IN PLACE OR TRANSPLANTED AND BY VASCULAR BUDS

Buds	Asymmetry of the parental colonies	Buds developed (No.)	Asymmetry of buds	
			Normal (No.)	Reversed (No.)
Isolated	Normal	106	106	—
	Reversed	82	—	82
Transplanted	Donor and recipient reversed	22	—	22
	Donor normal, recipient reversed	5	5	—
Vascular	Normal	34	33	1
	Reversed	15	—	15

of the bud by vascularization, did not affect its presumptive asymmetry. We conclude that the type of bilateral asymmetry to be developed is impressed on the bud primordium.

The second important point is that the vascular buds developed the same kind of bilateral asymmetry as the zooids of the colony in the matrix of which the buds were generated. This means that the colonial matrix, like the zooids, is endowed with the capacity to impart information to the buds that will determine their eventual asymmetry. The only case in which bilateral asymmetry did not correspond to that expected was not surprising; it was a case of reversal of the expected normal asymmetry which, as we know, can be induced in the course of development by circulatory stasis.

DISCUSSION

The statement by Berrill (1941) that "both the antero-posterior axis and the left-right axis coincide with those of the parental zooid and must exist from the beginning" is not entirely tenable in the light of our experiments. In the bud primordium, isolated within the colonial matrix, the antero-posterior axis is governed, both in origin and polarization, by the orientation of the vessel of the colonial matrix that supplies the isolated bud. In the buds isolated from the parents after the expression of symmetry, this feature is reestablished in dependence on the direction from which the vessel enters. On the basis of these results, it seems safe to assume that symmetry has the same origin in the vascular buds and in the palleal buds attached to the parental zooids. Actually, the polarization of the antero-posterior axis coincides with vascularization. Differential vascularization of the future ventral and dorsal sides might be the polarizing factor for the dorso-ventral axis. In the beginning this axis in the palleal buds does not coincide with that of the parent but lies perpendicular to it in a

horizontal plane. It will attain the vertical position only later through an outward rotation of the bud. This rotation seems to be controlled by the relationship of the bud with the parent: In the vascular and in the isolated palleal buds the dorso-ventral axis is sometimes inclined or even reversed with respect to the normal orientation relative to the substrate, and such zooids with reversed axis produce normally oriented buds. Therefore with respect to bilateral symmetry, we agree with the statement by Izzard (1973) that "the developmental pattern transmitted from parent to bud is highly regulative and not mosaic in form."

On the contrary, the bilateral asymmetry is rigorously predetermined in the bud primordium. Both in the normal and in the reversed condition, even in buds developed from isolated primordia, the asymmetry always corresponds to that of the parental zooid. This is also true of the vascular buds produced by the colonial matrix. These buds develop with asymmetry corresponding to that of the removed zooids. Clearly there is not a predetermination of the territories that will produce asymmetrical structures. There is a predetermination of the position that the asymmetric characters will occupy with respect to the median plane, in conformity with the disposition they exhibit in the zooids of the preceding generation. This is an informational type of predetermination: Once the system of coordinates which defines the bilateral symmetry has been laid down, the asymmetric structures will occupy a fixed position with respect to it. This condition does not differ from that which has long been known for the developing egg of amphibians: The experimental change or reversal of bilateral symmetry has little influence, if any, on the bilateral asymmetry (discussion in Huxley and De Beer, 1934).

A question arises about the mode in which the information is transmitted. Of the two components of the palleal bud, the atrial epithelium and the epidermis, only the first is involved in the formation of

organs other than epidermis. It is lacking in the vascular buds, its substitute being a cluster of blood cells, not a structured tissue. Since with regard to the bilateral asymmetry the vascular buds behave like the palleal buds, we are forced to assume that the information for the type of bilateral asymmetry to be developed is transmitted by the epidermal component, which in the vascular buds is represented by the wall of the vessels of the colonial matrix. These are actually epidermal extrusions of the zooids. It is noteworthy that palleal buds exchanged between colonies of opposite bilateral asymmetry develop the same asymmetry as their parents, in spite of the new environment.

A controlling function of the wall of the vessels in vascular bud formation has been postulated by Berrill (1961) and by Milkman (1967). According to Berrill it would act as the necessary inductor for the aggregation of free blood cells to form a tissue with inherent organization, able to differentiate into the various organs of the zooid. According to Milkman the wall of the vessel ought to be responsible for the pigmentation pattern of the zooid which always fits the genotype of the parental colony even if the latter has been fused for a considerable period with another colony of a different genotype; in no case did the circulating pigment cells give somatic recombinants.

In order to test our hypothesis, we are presently attempting experimental exchange of the epidermal and endoblastic layers between buds generated by zooids of opposite bilateral asymmetry.

There is another difficult question yet to be answered: The nature of the information and the way in which it is encoded and how it is subsequently expressed. The recent ideas on spatial pattern formation put forwards by Goodwin and Cohen (1969), Lawrence (1970), Webster (1971), and Wolpert (1971) are enlighting, but even a simple attempt to apply them to this particular biological system would demand a body of knowledge concerning its development which is beyond our present capabilities.

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