

# REGENERATION IN PEROPHORA VIRIDIS<sup>1</sup>

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The potency of the Ascidians to regenerate and their susceptibility to environmental agents have long been known. Driesch (1902) found that small isolated portions of *Clavellina* stolon could form complete individuals. Huxley (1921) studied dedifferentiation in *Perophora* and later (1926) dedifferentiation in *Clavellina*. Berrill and Cohen (1936) studied the regeneration of isolated pieces of *Clavellina* stolon with respect to the polarity of the parent zooid.

*Perophora*, a colonial Ascidian, because of its relative structural simplicity and the uniformity of its growth pattern, seemed to offer excellent material for regeneration experiments, especially with respect to the polarity relationships of isolated portions to the colony as a whole.

The experiments to be described were carried out with the following objectives: (a) To investigate the regeneration of isolated stolons; (b) to study the regeneration of stolon tips; (c) to test the regeneration of stolon material when expressed from the tunic; (d) to determine the effect of ligatures on isolated stolons; (e) to study the regeneration of stolon-zooid systems.

The author wishes to acknowledge the helpful guidance of Dr. Lester G. Barth during the course of this investigation.

## MATERIAL AND METHODS

The experiments were performed chiefly upon freshly collected *Perophora* colonies. Satisfactory stock material was also cultured by suspending "grape-like" clusters of colony by string in a tank of running sea water. In three or four days new stolon outgrowths, which could be handled conveniently, appeared. The experimental animals were kept in separate syracuse dishes which were immersed in a large glass aquarium through which filtered sea water constantly flowed.

A *Perophora* colony consists of a system of zooids appearing at fairly regular intervals from a branched stolon (Fig. 1, A). The zooids show a size seriation, becoming progressively smaller toward the free end of the colony. This seriation is due to the mode of origin of the zooid, for as the stolon grows in length buds appear near the free end and develop into mature zooids as the colony grows. Thus, the size seriation is an index of the age of the zooids, the largest zooids always being the oldest. The free growing end of the stolon in the experiments to be described will be called the distal end, as opposed to the attached end which is called proximal.

As described by Deviney (1934), the stolon of *Perophora* is cylindrical, its

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wall being composed essentially of three layers. It consists of an outer tunic with scattered cells plus intercellular substance, a middle epithelio-ectodermal layer which is quite thin, and an inner discontinuous thin layer of mesenchyme. The mesenchymal layer lines the stolon cavity through which blood flows; this cavity is divided vertically by a single-layered septum. The septum disappears near the distal end of the stolon, allowing continuity of blood flow between the right and left halves of the cavity.

The operations and observations were made under a binocular microscope. All cuts were made by means of a pair of iridectomy scissors. Photographs were taken with an Argus camera, attached to a compound microscope and drawings were made from these photographs.

## RESULTS AND DISCUSSION

### *A. Regeneration of isolated stolons*

In these experiments the portion of stolon lying between two zooids was excised by two cuts and observed for regeneration (Fig. 1, B). The excised stolons varied in length from 2.5–6.0 millimeters. Either distal or proximal ends of the excised stolon were stained with Nile blue sulphate suspended in agar, for purposes of orientation. The results are listed in Table I.

TABLE I

*Location of regenerants in excised stolons of Perophora*

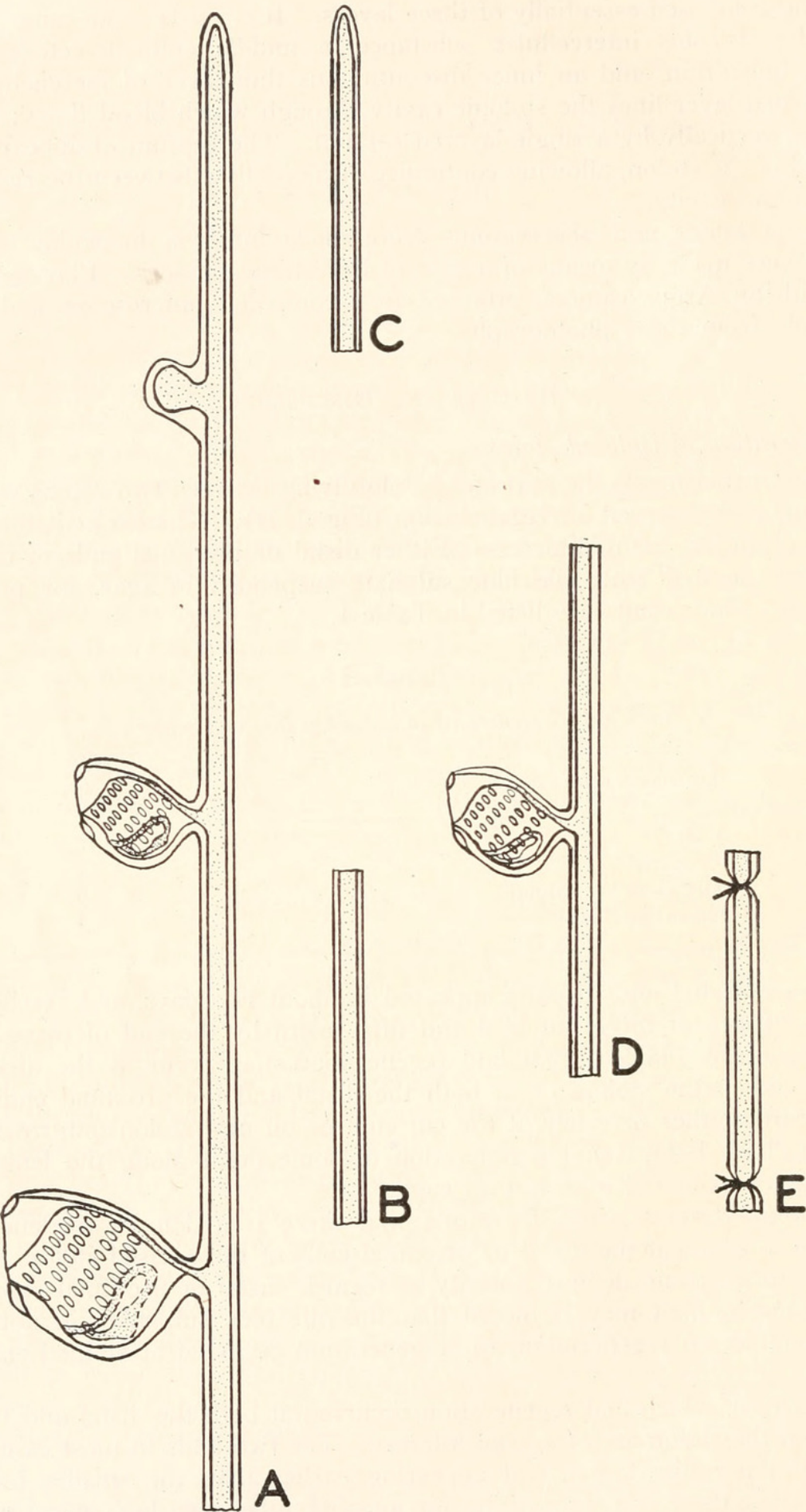
Location of regenerated zooid	No. of specimens regenerated
Distal	18
Proximal	11
Distal and proximal	9
On original stolon	4

The regenerated buds usually appeared in about two days, and reached an approximate length of three hundred and fifty micra by the end of three days. It can be seen from Table I that bud regeneration may occur at the distal or the proximal end of the stolon, or at both the distal and the proximal ends. These buds appeared either precisely at the cut end, or on new stolon outgrowths at the cut end (Pl. I, 1–4). Bud regeneration at some point along the length of the original stolon occurred in only four cases.

These experiments show, therefore, that there is a definite tendency for regeneration to occur at the distal or proximal ends of the excised stolon. Further, there appears to be no definite polarity as regards distal or proximal regeneration. In this connection, it may be noted that the Nile blue sulphate did not have an inhibitory effect on regeneration, for regeneration occurred at stained ends of the stolons.

The cases in which bud regeneration occurred at both the distal and the proximal ends of the stolon are of special interest. The two buds in most cases showed a size difference, the larger bud appearing earlier than the smaller bud. This may mean that there is a competition for metabolite between buds when more than







one is involved in regeneration. The smaller bud is less successful in this competition and develops more slowly. In one case, the smaller bud was eventually resorbed at the expense of the larger bud. That a competition between two regenerating regions exists is further evidenced by the fact that when bud formation occurred at only one end, there was frequently a preliminary stolon growth at the other end of the excised stolon. This stolon growth was eventually resorbed and there was a general drawing of the original stolon materials towards the developing bud (Pl. I, 2, 4).

### *B. Regeneration of stolon tips*

The portions of stolon used in this experiment consisted of distal pieces. The stolon was isolated by a cut made just distal to the first visible bud (Fig. 1, C). Of eleven such stolon pieces, varying in length from 1.7 to 4.8 millimeters, all formed buds in one to two days. These buds appeared in approximately the same position on the stolon as do new buds in normal colony formation (Pl. I, 5). No buds were observed at the proximal end of the isolated stolon, though in five cases small stolon outgrowths did appear. In two of these five cases the proximal growth was later resorbed at the expense of distal growth.

There is thus a difference in the mode of regeneration of isolated stolon tips as compared with that of stolon pieces isolated from between two zooids. In the former, the growing distal tip is probably more active in development at the time of the cut; after the cut is made, this lead is maintained. In the latter case, both ends are cut and their development may proceed on a more nearly equal basis as regards competition.

### *C. Regeneration of stolon material expressed from the tunic*

Wilson (1923) attempted to test the regenerative power of mesenchyme cells which he pressed out of the stolon. He obtained clumps of cells which stuck together, but which died within a day. Since, as shown by Deviney (1934), in the budding of *Perophora viridis*, the unspecialized cells of the septum and of the blood stream are the formative elements, it seemed likely that with more favorable conditions the stolon material might be kept alive, and its ability to regenerate tested.

The technique employed was as follows: The portion of stolon lying between two zooids (Fig. 1, B) was removed from the colony. Holding the tunic at one end with jeweler's forceps, a glass needle was passed gently over the tunic and the inner stolon material was expressed from the opposite end. It was found that the inner stolon material could be removed as a unit in this manner. The expressed stolon material was placed in syracuse dishes and maintained in running, filtered sea water.

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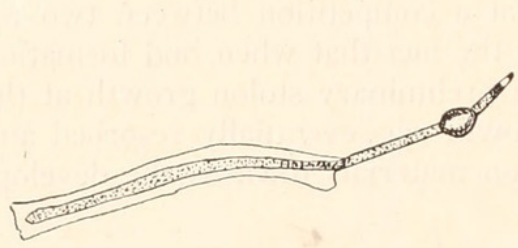
#### EXPLANATION OF FIGURE

Diagram to show the types of isolations which were made from the *Perophora* colony.

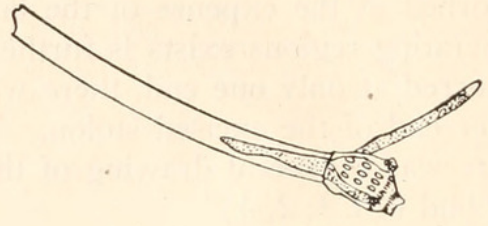
- A. *Perophora* colony.
- B. Stolon isolation.
- C. Stolon tip isolation.
- D. Stolon-zooid system.
- E. Stolon ligatured at both ends.



PLATE I



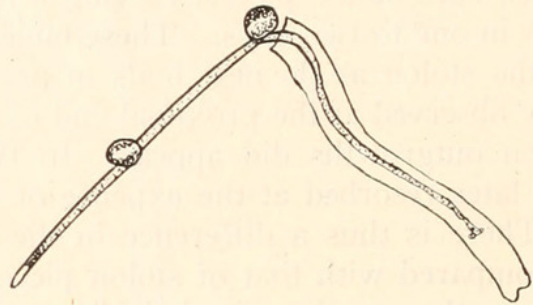
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2



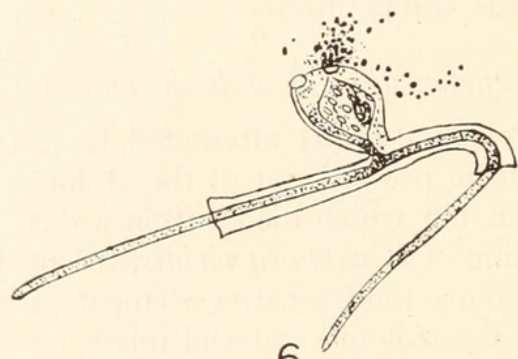
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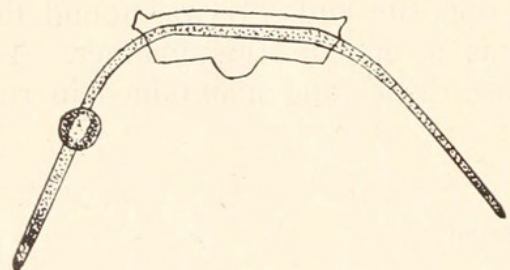
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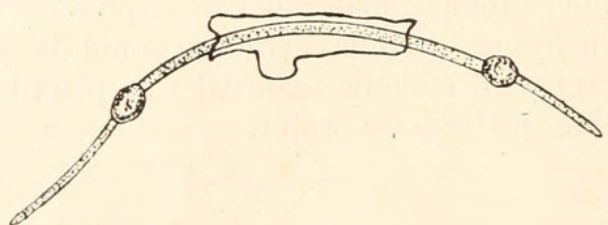
5



6



7



8



Of a total of nineteen such cases, nine showed stolon growth and bud formation after three days, while ten showed stolon growth but no bud formation. A new tunic was observed around the stolon materials at this time. The point of origin of new buds showed marked variation.

Since the expressed material contained the unspecialized cells of the septum and of the blood stream, it is not surprising that under favorable environmental conditions the material could survive and form buds. Wilson (1923) assumed that the material expressed from the tunic consisted of mesenchyme cells. However, Deviney (1934) has shown, as cited earlier in this paper, that there is a thin epithelio-ectodermal layer surrounding the stolon cavity. It is possible that cells of the epithelio-ectodermal layer were included with the expressed material in these experiments, so that it may not be concluded that a particular cell type was solely responsible for bud formation. However, it is suggested that, by this method, particular cell types may be isolated, and their ability to regenerate studied.

#### *D. Ligature of isolated stolons*

The thread ligature as a means of isolation was first employed by Morgan (1902), and later by Peebles (1931) and Barth (1938 a), on *Tubularia*. In *Tubularia* the perisarc remains intact after the ligature, but the coenosarc is severed, thus isolating the stem on each side of the ligature. The thread ligature acts in a similar way in *Perophora*. The tougher tunic is not cut, but the inner stolon tube is effectually severed by the ligature.

Twenty-five stolon pieces, each of which was isolated from between two zooids, were ligatured at each end by means of fine cotton threads (Fig. 1, E). Twenty-two specimens consisting of similarly isolated stolon pieces, but without any ligatures, were used as controls. The results of the experiment are summarized in Table II. The ligature hindered stolon growth and bud formation, while typical stolon growth and bud formation occurred in the controls. It may be that in the one instance that regeneration occurred in the ligatured stolon, it was because the ligature was so tight that it cut through the tunic.

Barth (1938 b) has shown that the availability of oxygen is an important factor in controlling the rate of regeneration. It is suggested that in *Perophora*

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#### EXPLANATION OF PLATE I

##### FIGURES 1-8 ( $\times 12$ )

1-4. Types of regenerants obtained after isolation of stolons removed from between two zooids. 1. Bud formation on a stolon outgrowth from the distal end of the stolon, four days after isolation. 2. Zooid formation at the distal end of a stolon, six days after isolation. 3. Bud formation at both ends of a stolon, after five days. 4. Stolon growth and bud formation at the proximal end of an isolated stolon, after five days.

5. Bud formation on an isolated stolon tip, after two days.

6-8. Types of growth and regeneration obtained after isolation of stolon-zooid systems. 6. Growth of the zooid and stolon growth at both ends of the stolon, after 6 days. 7. Resorption of the zooid, stolon growth at both ends of the stolon, and bud formation on the proximal stolon outgrowth, after 6 days. 8. Resorption of the zooid, stolon growth at both ends of the stolon, and bud formation on both stolon outgrowths, after 6 days.



TABLE II

*The effect of thread ligature on stolon and bud regeneration*

	Time in days	No. of specimens	Bud regeneration with stolon growth	No bud regeneration	
				Stolon growth	No stolon growth
Ligatured	5	25	1	3	21
Control	5	22	11	4	7

there is a lowering of the availability of oxygen at the cut ends brought about by the thread ligatures, thus inhibiting regeneration.

### *E. Regeneration of stolon-zooid systems*

By isolating a normal zooid along with a short length of stolon from the *Perophora* colony, Huxley (1921; 1934) demonstrated a differential susceptibility of the stolon-zooid system. When the stolon-zooid system is starved under normal conditions, the stolon is resorbed by the zooid. When the stolon-zooid system is placed in dilute toxic solutions, the zooid is resorbed by the stolon. This may be interpreted as meaning that under normal circumstances there is a competition between zooid and stolon, the zooid being dominant because of a higher metabolic rate. Under moderately unfavorable conditions, the metabolically more active zooid is more severely effected, and the stolon assumes the dominant role. Thus, there is a delicate equilibrium between zooid and stolon. That region which is more active grows at the expense of the other region.

Since running sea water seems to offer the most favorable environment for survival of *Perophora* material, it was thought advisable to isolate stolon-zooid systems (Fig. 1, D) and place them in running filtered sea water. Presumably, under these conditions, food is available; and it was felt that further information might be obtained on the competitive relations of stolon to zooid. Each specimen was kept in a separate syracuse dish and immersed in the container through which the sea water flowed.

For the thirty-four stolon-zooid systems isolated, the results fell into three classes:

(a) The original zooid was maintained, and actually increased in size in many cases. In addition, there was stolon growth at either or both cut ends, and buds formed on these outgrowths. This occurred in thirteen cases (Pl. I, 6).

(b) The original zooid was resorbed, there was stolon growth at either or both ends, and bud formation occurred on these outgrowths. There were twenty such cases (Pl. I, 7, 8).

(c) The zooid grew at the expense of the stolon, the latter being resorbed. This occurred in only one of the thirty-four cases.

From these experiments it would appear that under the most favorable conditions, i.e. running sea water plus healthy stolon and zooid, the entire stolon-zooid system may grow. The stolon grows and may form buds, and the original zooid may maintain itself and even increase in volume. Evidence of feeding in healthy



zooids under these environmental conditions has been noted (Pl. I, 6). Rather than assume a competition between stolon and zooid, from the experiment as run in this fashion, it would seem more plausible to assume that a competition occurs between three growing regions, namely the zooid and the two cut ends of the stolon. Under the most favorable conditions, when all three regions are developing equally rapidly, all continue to grow. When one gets ahead in development, it may develop at the expense of one or both of the others.

That there is this competition is demonstrated in the cases where stolon growth occurred at only one end, for in eight of these cases, it first occurred at both ends, but was later followed by stolon resorption at one end, and further stolon growth at the other end. Of the three growth zones in the isolated stolon-zooid system, the zooid appears to be most sensitive, having been resorbed in twenty of the thirty-four cases.

This explanation may be considered, then, as an extension of Huxley's ideas on equilibrium and competition, rather than as a negation of his fundamental assumptions.

#### GENERAL DISCUSSION

The results of these experiments are interesting in view of those obtained with *Tubularia*. Both *Tubularia* (Goldin and Barth, 1941) and *Perophora* have relatively unspecialized cells which have the ability, under the proper environmental conditions, to reconstitute the whole individual. In the normal colony formation of *Perophora*, buds do not normally form on the stolon lying between two zooids. In *Tubularia*, similarly, a hydranth generally does not form between the distal hydranth and the proximal end of the stem. A cut in either *Perophora* or *Tubularia* provides an environmental stimulus which initiates the process of regeneration at the point of the injury. Barth (1938 b) has suggested "that the stimulus for regeneration is the exposure of tissue to high oxygen." Goldin (1942 a; 1942 b) has shown that both oxygen and hydrogen ion concentration influence the rate of regeneration of *Tubularia*. Increased oxygen stimulates regeneration, while increased hydrogen ion concentration has an inhibitory effect on regeneration. That in *Perophora*, too, similar mechanisms are involved, is supported by the following evidences: In the isolation of stolons excised from between two zooids, and of stolon-zooid systems, regeneration occurs at the cut ends or on stolon outgrowths at the cut ends. In *Perophora* regeneration is hindered when both ends of the stolon are ligatured, this inhibition probably being correlated with the lowered availability of oxygen and increased concentration of acid metabolites brought about by the thread ligature. Finally, when stolon material is expressed from the tunic, providing ready diffusion of oxygen and metabolites, regeneration may occur at any point along the axis of the expressed material.

It has been shown in *Tubularia* that there is a definite polarity present in the stem. After isolation, distal levels exercise dominance over more proximal levels and the distal end regenerates more rapidly. In *Perophora*, stolons isolated from between two zooids show no evidence of original polarity relationships in regeneration. Regeneration may occur at either end or both ends, with equal frequency. In *Perophora*, proximal regeneration unaccompanied by distal regeneration represents a reversal of polarity, and is in marked contrast to the situation in *Tubularia*.



Despite this failure to maintain the original distal-proximal polarity, however, once regeneration has begun, a new competitive physiological axis is set up without any relation to the original distal-proximal orientation. This competition has been manifested in the following way: In isolated stolons regenerating new stolon material at both ends, one end may often cause resorption of the stolon growth at the other end. A similar situation may occur when buds form at both ends of the isolated stolon, the larger bud eventually causing resorption of the smaller bud. In isolated stolon-zooid systems there exist three active regions, namely, two cut ends, and a zooid. More rapid growth at any one of these may progress at the expense of other regions. It is interesting to note that under optimum conditions, competition is eliminated and growth occurs at all of these regions.

That factors other than oxygen availability and diffusion of metabolites are involved in the regeneration of *Perophora* stolons is demonstrated by experiments in which distal stolon tips are isolated. In these stolon tips there is probably greater oxygen availability and diffusion at the cut proximal end. Despite this fact, regeneration and growth occurs at the distal end.

It is suggested that, as with *Tubularia*, *Perophora* offers a good biological system for studying, further, problems of regeneration, polarity, and differentiation.

#### SUMMARY

1. Stolons of *Perophora*, isolated from between two zooids, regenerate at the cut ends but do not necessarily maintain their original distal-proximal polarity relationships.
2. Stolon tips, after isolation, maintain their distal-proximal polarity, the distal end displaying dominance.
3. The inner stolon material, when expressed from the tunic, may grow and regenerate buds at any point along its axis.
4. Regeneration is hindered at ligatured ends of stolons.
5. When stolon-zooid systems are isolated and maintained under optimum conditions, the entire stolon-zooid system may grow.
6. The theoretical implications of the results of these experiments are discussed.

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