REGENERATION OF THE ENTEROPNEUST, SACCOGLOSSUS KOWALEVSKII

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Aside from the morphological similarities which they share with the chordates (Hyman, 1959), the Enteropneusta have developed regenerative methods common to both the invertebrates and the vertebrates. The regenerative capacity of the tongue worms has been noted in early reports by Spengel (1893) in *Glossobalanus minutus,* by Willey (1899) in *Ptychodera flava,* by Cori (1902) in *Balanoglossus clavigerus* and Kuwano (1902) in *Balanoglossus misakiensis.* The first thorough investigation of regeneration was done on the Mediterranean form, *Glossobalanus (Ptychodera) minutus* by Dawydoff (1902, 1907, 1909). More recently Rao (1955) has reinvestigated regeneration in *Ptychodera flava.* A form of asexual reproduction and subsequent regeneration of tail fragments in *Balanoglossus capensis* was reported by Gilchrist (1923). All of these forms are members of a highly evolved family, the Ptychoderidae (Hyman, 1959). The current form under study is *Saccoglossus (Dolichoglossus) kowalevskii,* a species common to the shores of the North Atlantic. The only previous reference to regeneration in the present genus or its family was noted by Assheton (1908) in *Saccoglossus serpenticus,* found along the coast of Scotland.

Dawydoff (1909) discovered that when the animal was cut up into many parts, regeneration occurred from the anterior end of each fragment but never from the posterior end. Secondly, regeneration resulted either as a bud formation originating from a direct proliferation of the immediate cephalic surface (epimorphosis) or from a remodeling of the anterior portion of the amputated animal (morphallaxis). More often it was a combination of these two methods.

The factors that direct this polarized regeneration are not known. Conceivably, the anterior cut surface may inhibit regeneration from a more posterior locus. Similarly, the type of structure removed or the level of amputation may control the nature of the regenerative process utilized. These queries are approached here with an investigation of the regenerative potential of *Saccoglossus kowalevskii.*

MATERIALS AND METHODS

Living specimens of *Saccoglossus kowalevskii* were provided by the Supply Department at the Marine Biological Laboratory in Woods Hole, Massachusetts. Additional animals were collected by the author on the mud flats at Cotuit in West Falmouth, Massachusetts.

Both pre- and post-operative animals were maintained in the laboratory as follows. Individual animals were placed in small dishes (3 × 1 inches) filled

with a mixture of clean sand and a small amount of organic material. The dishes were then submerged in two-inch-deep enameled pans filled with sea water. A constant flow of fresh sea water entered the pans and flowed over the submerged dishes, which were kept at a depth of one to two inches.

(The animals were difficult to keep in the laboratory except under ideal conditions. Many of the animals reported as "not recovered" in the results died from inadequate environments. They cannot survive long on glass in either standing or running sea water, nor with an excess of organic material from their natural environs.)

Since the trunk portion of the animal is rather fragile, the animals were removed for examination by first washing away the loose sand with a gentle stream of sea water. When the casting containing the animal was exposed, it was carefully lifted out and the animal dissected from its tube.

Prior to preservation, the animals were placed in a dish of clean sea water for several hours to allow removal of the digestive contents. All mucus and adherent sand were carefully stripped from the body. Anesthesia was produced by adding 85% alcohol drop-wise to the dish until the specimens were fully relaxed. Fixation was in Stockard's solution or in 10% formalin in sea water. The animals were sectioned at 10 micra and stained with Delafield's hematoxylin and Eosin-Azure II.

General Procedure. In each series, the animals were amputated transversely, with successively larger pieces being removed in succeeding experiments, beginning at the anterior end. This provided a single amputation surface on each of two animal fragments, a posterior surface on the more cranial portion and an anterior surface on the more caudal portion. The amputated animal was then returned to its dish and periodically observed for signs of anterior regeneration. The amputated cranial portions were also studied for any indication of posterior regeneration. Representative animals were fixed daily for two weeks and then at weekly intervals.

RESULTS

1. *Amputation through the proboscis*

The first regenerative challenge resulted from a transverse amputation of the proboscis at a point halfway back from the tip of the animal. Near the proximal end of the proboscis four coelomic pockets surround a median group of organs, the buccal diverticulum (stomochord), which is supported by a proboscis skeleton and encased by the glomerulus and heart vesicle. Since the amputation was anterior to these organs, only the epidermis, connective tissue and musculature of the proboscis were affected. (See Fig. 1, operation A.) Of 21 operated animals, 14 regenerated the lost portion of the proboscis within seven to nine days post-amputation. Examples of the amputated fragments and regenerates were fixed at one, two and three weeks. In some cases the junction of the new regenerate and the stump was clear because of a slight telescoping between the host animal and the regenerate. (See Fig. 2.) Several of the new regenerates exhibited various degrees of bifurcation at the tip. The degree of separation ranged from slight dichotomy to almost complete separation of the proboscis regenerate, as seen in Figure 3. In all of these cases, regeneration of the amputated proboscis

occurred as a local proliferation of the tissues at the cut surface (epimorphosis) and resulted in a rapid replacement of the portions removed.

Occasionally, the amputated stump regressed instead of regenerating. Regression was noted in two cases. In one specimen examined histologically after 24 days, the original proboscis had disappeared and regression had continued to the collar. The azure-colored collar epidermis had closed the opening into the branchial chamber except for a small slit. Laterally, at the junction of the collar epidermis and the purple-staining trunk epidermis, a small proboscis formed as a bud from the trunk epidermis. (See Fig. 4.) The core of the outgrowth was filled with loose muscle fibers, connective tissues and coelomocytes.
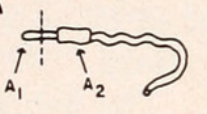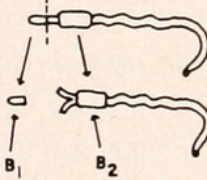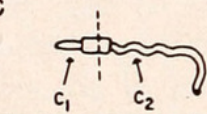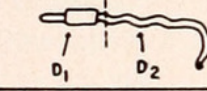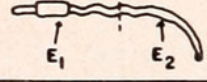
TYPE OF OPERATION	NUMBER OPERATED	ANTERIOR PIECE NUMBER OF:		POSTERIOR PIECE NUMBER OF:		REGENERATION	
		SURVIVORS	REGEN.	SURVIVORS	REGEN.	WITH REGRESSION	WITHOUT REGRESSION
A A_1 A_2	21	13	0	16	16	2	14
B B_1 B_2	12	11	0	7	5	3	2
C C_1 C_2	58	48	0	9	3	2	1
D D_1 D_2	35	29	0	4	2 ?	2 ?	0
E E_1 E_2	36	32	22	27	0	0	22

FIGURE 1. Comparison of regeneration originating from the anterior or posterior pieces of transected *Saccoglossus kowalevskii* after amputation at succeeding levels.

This one example indicated that in rare instances, at least, the proboscis did not regenerate directly but rather underwent a more extensive remodeling of the existing tissues that remained in the amputated animal (morphallaxis.) The exact parameters for this type of response are not known at this time.

The amputated anterior halves of the proboscises remained alive on the surface of the sand for several weeks. These isolated fragments stayed active, moved about by ciliary action, and reacted to mechanical stimulation. This response was similar to the irritability to mechanical and photic stimulation of enteropneusts reported by Bullock (1940) and Hess (1937) in similar isolated fragments. Shortly after amputation, the proboscis fragments were able to burrow into the

sand, but later they lost this response. After three weeks, the pieces tended to decrease in size and developed rugate folds in the epidermis along the lateral surfaces. Multi-branched processes of solid epidermis often developed on the caudal surfaces of the proboscis fragments. However, in no instance was there any other evidence of regenerative activity in these isolated portions of the proboscis. This portion of the animal was apparently incapable of posterior regeneration or of reorganization into new individuals.

2. *Amputation through the proboscis and sagittal splitting*

In an attempt to determine the cause of the dichotomous regeneration of the proboscis, a series of 12 animals were amputated frontally through the proboscis and then the remaining stump was split sagitally to the anterior edge of the collar with iridectomy scissors. (See Fig. 1, operation B.) After five days, two animals had regressed, one to the collar region and the other to the branchial chamber. Five animals regenerated the proboscis in part. In three animals, one-half of the proboscis regenerated distally while the stump of the remaining half fused with the regenerate at the base.

Curiously, in a fourth case, the regenerated half arose from the lateral surface of the amputation stump, forming a lateral sprout from the main axis.

In two of the asymmetrical regenerates, the original organs of the proboscis had regressed. This apparently depended upon the degree of injury from the experimental incision. A new circular-shaped glomerulus, heart vesicle, and a stubby buccal diverticulum formed that extended from the proboscis stalk into the extreme proximal end of the proboscis. (See Fig. 5.) These new organs appeared to develop from the coelomocytes of the proboscis and stalk, but the exact cell precursors could not be determined from these few examples. However, each of the structures was surrounded with a very heavy non-cellular lamellated membrane, originating directly from the body of the proboscis skeleton.

In one other example, both halves of the proboscis fused and the two regenerated together. Five other animals were not recovered.

The above results suggested that the dichotomy in the previous series was the result of regeneration from two separate loci, which sometimes fused and at other times failed to do so.

3. *Amputation through the collar*

Each animal of this group was amputated transversely through the middle of the collar, as indicated in Figure 1, operation C. The latter half of the animal, consisting of the posterior collar region and the attached trunk, was retained, as well as the proboscis and anterior half of the collar.

The amputated animal was less capable of withstanding the operation than the anterior portion that was removed. This may have been due to the trauma of operation or possibly it was a result of mucus-collection, which had a tendency to strangle the animal unless it was removed. Out of a total of 58 operated animals, only nine amputated animals were recovered. The large number of regenerative failures from the C_2 pieces was thus, in large part, due to the very high rate of mortality among the amputees. Regression of the remaining collar was quite common and usually began within two to three days after amputation. Al-
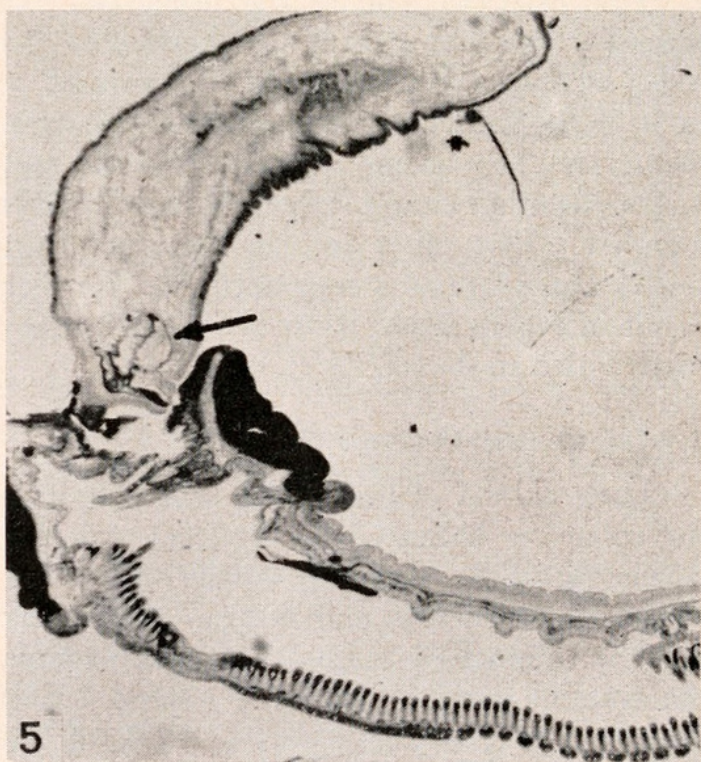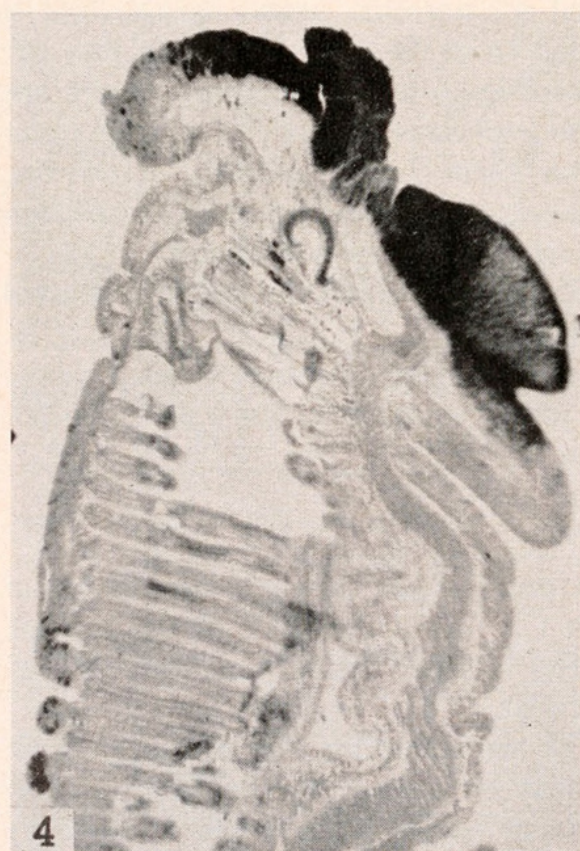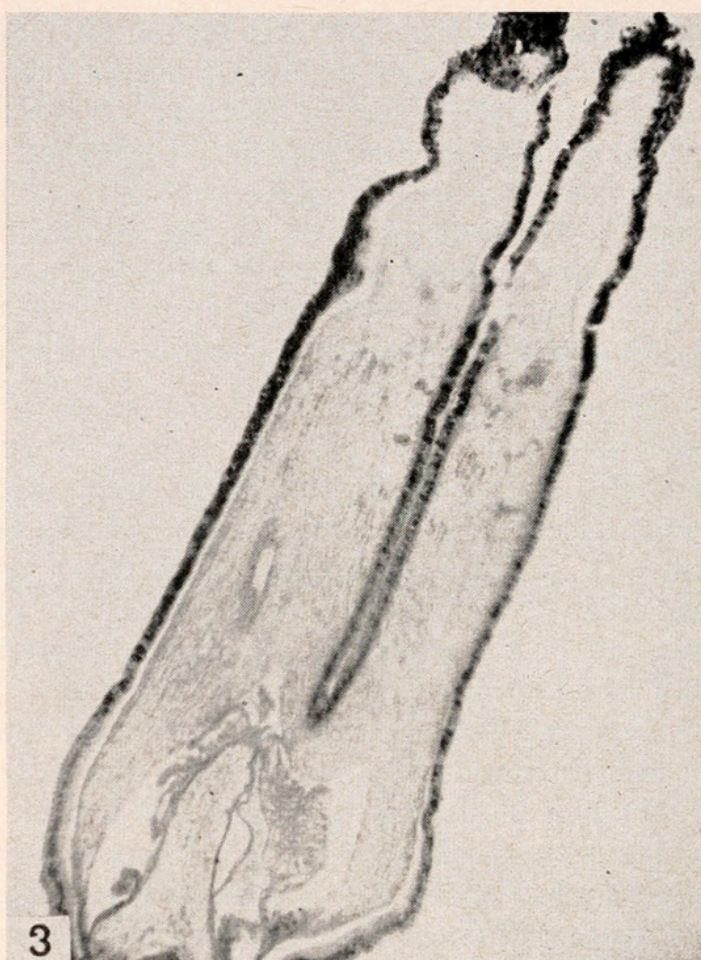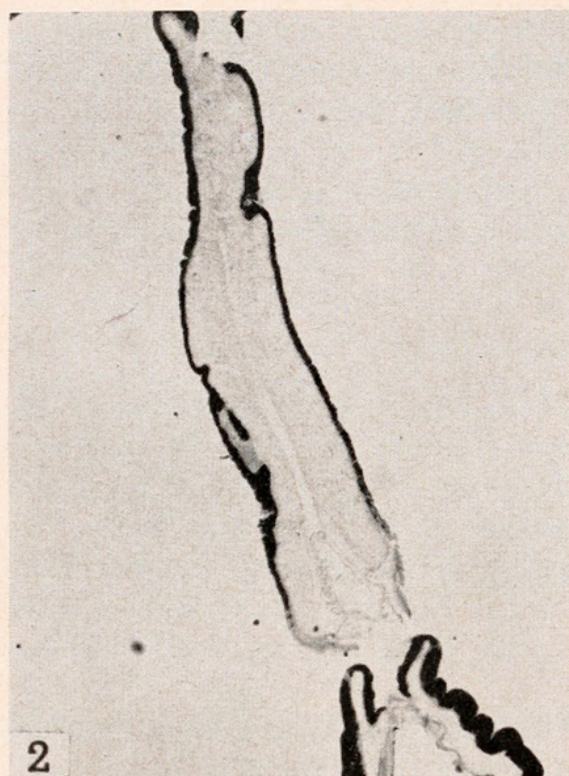
FIGURE 2.   Regeneration of the distal end of the proboscis after transverse amputation halfway back from the tip.   Replacement of tissue results as a direct proliferation of the amputation surface.   Fixed at seven days post-amputation.

FIGURE 3.   Occasional bifurcation seen in regenerates after transverse amputation of the proboscis.   The buccal diverticulum, heart and glomerulus are present in the proximal portion

most all of the operated animals regressed into the branchial region of the gonadal area within five to six days post-amputation. After regression, the animals either became stabilized or degenerated completely.

Of the animals recovered, three were found in different stages of regeneration. One had completely regenerated the amputated half of the collar and a full proboscis at 26 days post-amputation. This animal had not regressed prior to regeneration. (See Fig. 6.) Two other specimens, fixed at six days post-amputation, showed initial bud or blastema formation. The latter two cases were animals which had regressed prior to the beginning of regeneration.

The isolated pieces of proboscis and attached collar showed a gradual reduction of the attached collar between five and seven days post-amputation. Collar portions were still found 14 days after amputation but at the end of 18 days, almost all of the fragments had regressed collar parts with the denuded skeleton projecting out of the proboscis. Highly differentiated structures within the proboscis or proboscis stalk, such as heart, glomerulus, and buccal diverticulum, were retained for as long as 15 days. However, these structures disappeared from the proximal end of the proboscis, leaving only the muscle and epidermal portions of the proboscis intact. Although small buds of epidermis often appeared on the posterior surface, there was no indication of posterior regeneration. These isolated proboscises remained active and survived on the surface of the sand up to four weeks, and then gradually shrank and disintegrated.
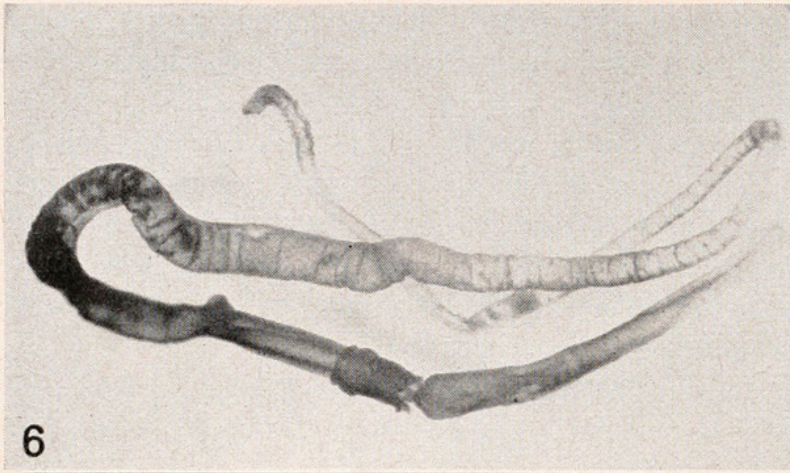
4. Amputation posterior to the collar

In the fourth experiment, the animals were amputated at a position posterior to the collar so that a small portion of the branchial region was included. (See Fig. 1, operation D.) Mortality here was again very high, possibly due to both mucus constriction and the inability of the trunk region to burrow. Out of 35 operated animals, only four survived. Two of these showed no sign of regeneration and had regressed to the hepatic region at the end of nine days. The remaining two had also regressed to the posterior branchial region and were fixed at seven days. Outwardly, they had developed small, yellow fleshy bulbs at the anterior end. Each bulb consisted of a median diverticulum which formed from the mucosal lining of the old branchial region. (See Fig. 7.) However, it was very doubtful that these epidermal blebs represented anything more than local metaplasia, and they were probably not true regenerates. As shown in the next section, amputation through the identical area (the post-branchial region) often produced similar small epidermal bulbs. Histological examination of the latter growths, at three weeks post-amputation, never indicated that they differentiated further.

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of the proboscis. Regeneration here occurs as a direct proliferation from the double amputation surfaces.

FIGURE 4. Formation of a new proboscis through morphallaxis after regression into the collar region. The proboscis is forming at the junction of the collar and trunk epidermis. Fixed at 24 days post-amputation.
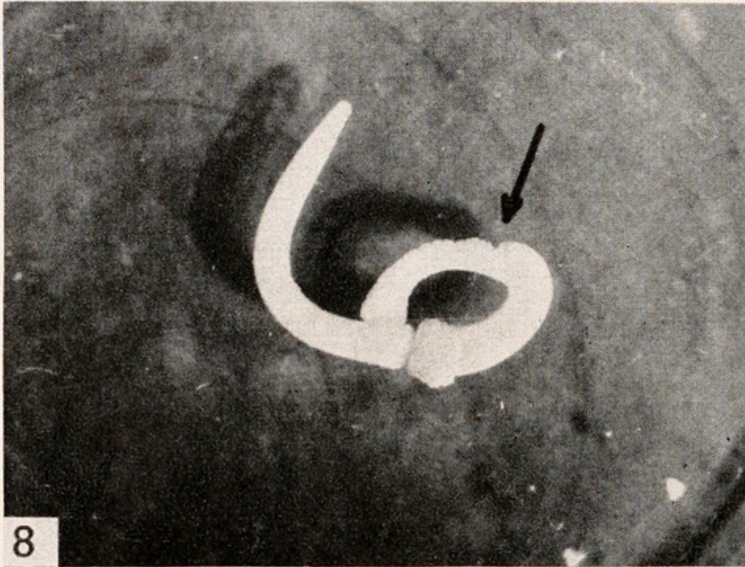
FIGURE 5. An asymmetrical regenerate after amputation and sagittal splitting of the proboscis stump. A new glomerulus, heart vesicle and buccal diverticulum, indicated by the arrow, are beginning to form in the proximal end of the proboscis.

FIGURE 6. Complete regeneration of the proboscis and anterior half of the collar after amputation through the collar. This animal had not regressed prior to regeneration but had regenerated directly from existing primordia. Fixed and cleared at 26 days post-amputation.

FIGURE 7. Sagittal view of an amputated trunk which had regressed into the posterior branchial region. The arrow indicates typical epidermal outgrowths that form on the anterior surface but never differentiate further. Fixed at seven days post-amputation.

FIGURE 8. An animal which illustrates posterior regeneration originating from an intact proboscis, collar and branchio-genital region. This young, immature animal had formed a new post branchial trunk at seven days post-amputation. Arrow indicated the level of amputation.

The amputated anterior portion, consisting of proboscis, collar and a fragment of the branchial area, was self-sustaining from five to fifteen days. The newly created caudal opening remained open and, with the original mouth opening, provided double apertures into the collar region.

No proliferation was ever found on any of the anterior pieces. The branchial chamber and collar generally persisted for a week but then began to regress thereafter. Regression usually continued until only the proboscis remained. In some cases, however, the collar and trunk were still intact at the end of three and one half weeks.

5. *Amputation posterior to the branchial region*

The last and most posterior cut was made just behind the last gill opening. The point of amputation was determined by the terminal tip of the external gill

openings that taper to a point in the midline between the lateral gonads. (See Fig. 1, operation E.) Only completely intact animals were used, and both anterior and posterior pieces were kept. The anterior portion, consisting of proboscis, collar and branchio-genital region, always burrowed into the sand immediately, while the latter portion never burrowed.

At the end of three days, the anterior wound area of the posterior piece (hepatic area and caudal intestine) had healed. Of 36 operated animals, 27 pieces were still intact at the end of three days. The remainder had deteriorated. In the survivors, usually the gonadal ridges turned over to fuse with the paired, median ventral ridges, which completely sealed off the opening into the intestine. Occasionally, single or multiple transparent epidermal vesicles appeared on the healed surface, but these small blister-like growths never grew larger nor did they show further differentiation. Most of the pieces survived for seven to eight days, and at the end of one week some of these degenerated, generally starting in the hepatic region. The more anterior gonadal and posterior intestinal fragments that resulted always survived longer. In some animals, a new opening appeared in the lateral wall just behind the amputation site. Almost invariably, this was followed by an inversion of the complete trunk region behind the new opening. Thus, the entire mucosal lining was outermost while the epidermal covering now served as a lining. The posterior pieces persisted for ten days, gradually fragmenting thereafter until only long segments of the post-hepatic region remained. None of the posterior pieces had regenerated after three weeks.

For the first time in these experiments, the anterior portion, composed of proboscis, collar and branchio-genital regions, showed signs of posterior regeneration after three days post-amputation. Regeneration was first detected in the younger, immature animals but regeneration also took place in the mature animals.

As a group, the younger, immature animals had a greater growth rate than the older, mature ones. At the end of one week, each animal had developed a new post-branchial region equal in length to the intact branchio-genital region. The mean length of the regenerates at this time was 1.1 cm. (See Fig. 8.) In two animals, an entire new post-branchial trunk, equal to the animal's original length, had formed. Regional differentiation of the new trunk regenerates into gonadal, hepatic and caudal areas followed after outgrowth of the trunk. In one example, differentiation of the gonadal and hepatic regions was completed at the end of eight days.

The sexually mature animals regenerated at a slower rate than the younger forms, averaging 0.5 cm. of new growth in one week. At the end of one month, the mean length of the regenerates measured 1.6 cm. A second important difference between the immature and sexually mature animals was in the organs immediately replaced. Existing differentiated structures at the amputation surface usually accompanied the trunk outgrowths. Thus, in the short regenerates of two to three days, new gonadal tissue accompanied the regenerating trunk wall. In the females, the ovaries contained fully formed eggs, easily seen through the body wall.

Posterior regeneration was observed in 22 of 32 surviving animals. The remaining animals regressed in a manner identical to that noted in the preceding operations.

DISCUSSION

The current experiments showed that the direction of axial regeneration varied, depending upon the level of amputation. In the anterior region of the body, regeneration never originated from a posterior amputation surface, but once the level of the branchial region was passed, regeneration was possible from a posterior amputation surface. Now, however, regeneration was not demonstrable from the anterior surfaces of lower trunk areas. These results both confirmed and differed with the results obtained by Dawydoff (1902, 1907, 1909) and Rao (1955). In Dawydoff's and Rao's experiments, the animals were generally cut into about four portions, the proboscis and collar, the branchial region, the hepatic region and the post-hepatic region. They found that anterior regeneration, where technically possible, occurred from the separate fragments, but they never reported regeneration from a posterior amputation surface in *Glossobalanus minutus* and *Ptychodera flava*. One difference in the present experiments on *Saccoglossus kowalevskii* was in the method of amputation. Each operation exposed only one cut surface on each piece, oriented either cranially or caudally. It was subsequently seen that posterior regeneration can occur from an anterior piece in the post-branchial area, a fact which diverges from other reports. It is conceivable that posteriorly-directed regeneration in the latter part of the animal was prevented by a more anterior dominant regeneration surface in the earlier experiments.

Two separate phenomena seemed related to the movement of the regeneration site posteriorly. The first, a tendency toward regression of the amputated parts, was lowest in the proboscis region. It became more common in the collar region and was found quite frequently in the trunk region. Regression always involved the loss and disappearance of structures in an orderly fashion from the wound, and should be distinguished from over-all deterioration of an animal fragment. From the present data, regression did not always lead to gradual but complete disappearance of the organism. In the first three experiments, regression was occasionally arrested by unknown parameters. Subsequently, the existing tissues were reorganized and regeneration followed.

A second observation was based on the type of regeneration involved, *i.e.*, epimorphosis or morphallaxis, and its relation to the level of amputation. When the proboscis was amputated, this almost always resulted in a quick and direct replacement of the lost portion through epimorphosis, the size of the regenerate approximating the original size of the amputated part. Transsection through the collar could result in either form of regeneration, with the rapid formation of a full-size regenerate or a small regenerate undergoing morphallaxis. When the amputation was made just behind the collar, regression always followed but regeneration was not demonstrable. In the post-branchial region, posterior regeneration took place again by the process of epimorphosis.

It appears that a certain degree of controlled regression immediately precedes regeneration whenever morphallaxis takes place. Continued regression results in no regeneration and gradual deterioration of the amputated stock. The factors that initiate and control regression are still unknown.

SUMMARY

1. Transverse cuts, which separated the animal into two parts, were made on *Saccoglossus kowalevskii*. When transsection occurred through the proboscis or

collar region, the posterior animal fragment was capable of regenerating lost parts anterior to the amputation surface but the anterior animal portion was not able to replace more posterior parts.

2. When amputation was performed behind the branchial region, anterior parts were never formed from the posterior animal portion. However, regeneration of more posterior missing parts was now possible.
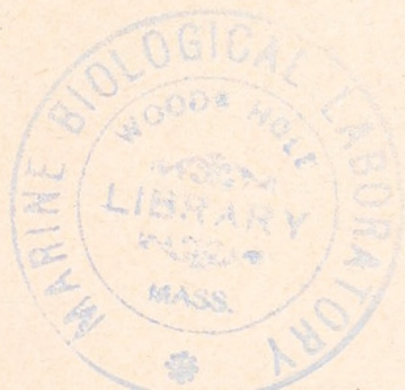
3. Regeneration occurred either from a direct proliferation of the tissues present at the amputation surface (epimorphosis) or through a remodeling of the anterior portion of the amputated fragment (morphallaxis).

4. As the locus of amputation was moved posteriorly, epimorphosis became less common and morphallaxis became the principal method of regeneration. Just behind the branchial region, successful posterior regeneration seemed to be accomplished exclusively through epimorphosis.

5. Immediately following amputation, regression often occured from the posterior fragments. If regeneration ensued, regression always preceded morphallaxis but did not precede epimorphosis.

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