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Studies in Tunicate Development. Part IV. Asexual Reproduction

N. J. Berrill

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IX—Studies in Tunicate Development

Part IV—Asexual Reproduction

By N. J. BERRILL

(*Department of Zoology, McGill University, Montreal*)

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I—INTRODUCTION

Budding in Tunicates has long been a subject of considerable interest and controversy. The earlier investigations were concerned for the most part with the morphology of the budding processes, and many classifications based on the nature of the organs and tissues were evolved. More recently the emphasis, as represented by the series of papers by Brien, has been applied to the more fundamental problems of morphogenesis. The point of view of the present paper lies between the two just mentioned. The general biology of asexual reproduction has been studied throughout a much wider range of animals than in previous accounts, while in addition the nature and nutrition of the tissues of the bud have been investigated in each case but without analysis of the process by which the undifferentiated bud tissue develops the organization of the adult.

Before any general discussion is attempted, a description of asexual reproduction will be given for each tunicate genus with that faculty. In each there is a concentration on three questions, the internal or external factors that induce this method of reproduction, the process by which the actual or physiological isolation of the parts from the whole is accomplished, and the nature of the tissues so isolated.

II—SOURCE OF MATERIAL

It so happens that the most complex of the various methods of budding in Tunicates were the first to be examined, and the relation of one to another remained obscure. In the present account are included reviews or reinvestigations of the types of budding already fairly well known, and at the same time descriptions of budding in a number of genera hitherto ignored. One great difficulty in the way of such investigations is the fact that asexual reproduction in its less specialized forms is, among Tunicates, a winter phenomenon and the colonies themselves are often unrecognizable. Some material in budding condition was obtained during summer months at the marine laboratories at Plymouth and Bermuda, but for the most part material was collected by Mr. G. A. Steven, of the Marine Laboratory at Plymouth; by Dr. J. F. G. WHEELER, of the Bermuda Biological Station; and by Dr. E. RICKETTS, of the Pacific Grove Biological Station, California, to whom acknowledgments are gratefully made. Additional and rare material was also examined through the courtesy of Miss ANNA B. HASTINGS, of the British Museum of Natural History.

III—MUTUAL INFLUENCE OF FORM OF COLONY AND ZOOID

The older classifications of the Tunicates were based primarily upon the presence or absence of the faculty of budding and upon the degree of fusion of the constituent zooids of a colony. This view was especially developed by HERDMAN and was generally held for a long while after the appearance of LAHILLE's monograph in 1890. LAHILLE introduced a scheme of classification that was based almost entirely upon the structure of the zooid, whether it formed one of a colony or was a solitary individual, and the faculty for budding and its variability was relegated very much to the background. Eventually, however, LAHILLE's conception predominated, and the process of budding in ascidians is not given great taxonomic value. Actually there are two questions involved. Budding may have been acquired or lost on one or many occasions; and the degree of separation of the constituent zooids of a colony may or may not be of much significance. The first of these is still a debated question, but the degree of fusion of zooids in a colony has long been recognized as being of little importance. JULIN, in 1892, observed a migration of mesenchyme cells through the epidermis to form test substance, while recently BRIEN (1930) observed a similar migration of cells to form new test material from stolonic vessels of *Clavelina*. The test, in fact, is an external collagen secreted by mesenchyme cells that have migrated through the epidermis and especially from vascular processes. At the same time such cells apparently are able to live and secrete tunicin only within a certain distance from the adjacent epidermis. Thus the degree of fusion of the zooids of a colony depends upon two factors, the distance apart of the whole zooids and the number and distribution of the stolonic vessels. Of these, the latter is the more important. As an example the various species of *Clavelina* may be cited. In *Clavelina lepadiformis*, *Clavelina huntsmani*, and others the stolonic vessels remain posterior to the zooids, and colonies consist of individuals each surrounded by test but united to adjacent zooids only by basal test. The zooids when growing rapidly are too far apart for test material to be secreted in sufficient quantity to embed them in a common mass. Mere contact of fully formed zooids is insufficient to produce fusion. In *Clavelina picta* the stolonic vessels are not only posterior but are also reflected anteriorly between the zooids, so that the intervening space is filled with mesenchyme cells and tunicin, and the zooids are embedded in a common test.

In the perophorids the vessels are all posterior, and the zooids, which are usually some distance apart, are united only by the slender stolons themselves. In the polystyelids such as *Symplegma* and *Botryllus*, the vessels are numerous and scattered so that the test envelops the whole colony.

In contrast with the above the confinement of the zooids of synoicids such as *Aplidium* or *Morchellium* within a common test is due not to the presence of intervening stolonic vessels, which are very poorly developed, but to the fact that the zooids all develop very close together and simultaneously secrete tunicin.

Minor variations in the spacial arrangement of the zooids or in the distribution of the vessels may accordingly result in marked changes in the general appearance of a colony.

Not only is the form of a colony greatly influenced by the nature of its constituent zooids, but also in many cases the zooids themselves have obviously been influenced by colonial requirements. The most obvious of these adaptations is to be found in those forms in which the zooids are arranged in definite systems or patterns. In these and in some others the atrial siphons open not independently to the exterior but into a common cloacal cavity, and the siphon may be reduced and modified to form an atrial languet.

Compound ascidians may briefly be grouped under three heads :

(*a*) clusters composed of zooids loosely united posteriorly, corresponding to HERDMAN's social ascidians, among which there is little modification to form a co-operative system ;

(*b*) sheet-like encrusting forms tending toward a two-dimensional state with a maximum of attachment ; in these the zooids may be extremely shortened antero-posteriorly, the number of stigmata rows being reduced to as few as three, and the oviduct serving as a uterus as the atrium becomes too small to contain more than one or two eggs ;

(*c*) pedunculate forms of various size and area of attachment, ranging from the relatively enormous masses of *Diazona* to the inconspicuous distomids. In *Diazona* the zooids are large and unmodified. In the synoicids and in *Tylobranchion* the colonies are both smaller and more pedunculate so that the surface is decreased much less than the volume. Apparently as an adaptation to such conditions, the zooids have become extended so that volume for volume they are relatively long and narrow, the posterior end of each zooid with the contained heart extending basally as a post-abdomen, in which are to be found the dislocated gonads.

Such differences between zooids naturally are of importance in connection with the process of budding. The most striking variations are shown in fig. 1, and it can be seen that, viewing the diazonid zooid as the least modified, there have been several departures of much significance. On the one hand are the extensions resulting in a post-abdomen containing heart and gonads. On the other are the extreme reductions in body-size, the expansion and extension of the basal stolon, the reflection of the abdomen along the left side of the pharynx, and the loss of the stolons.

The nature of the zooid will obviously limit and modify whatever faculty for budding that may exist.

IV—Budding in the Diazonidae

Species—Diazona violacea Savigny. Figs. 2 and 3

Source of material—Mewstone Ledge and Stoke Point, Plymouth.

Remarks—The colonies of *Diazona* may reach relatively enormous dimensions (40 × 40 × 15 cm), and there are many reasons for regarding the genus as one of the most primitive of ascidians. It is the only recorded oviparous ascidian with the faculty of budding.

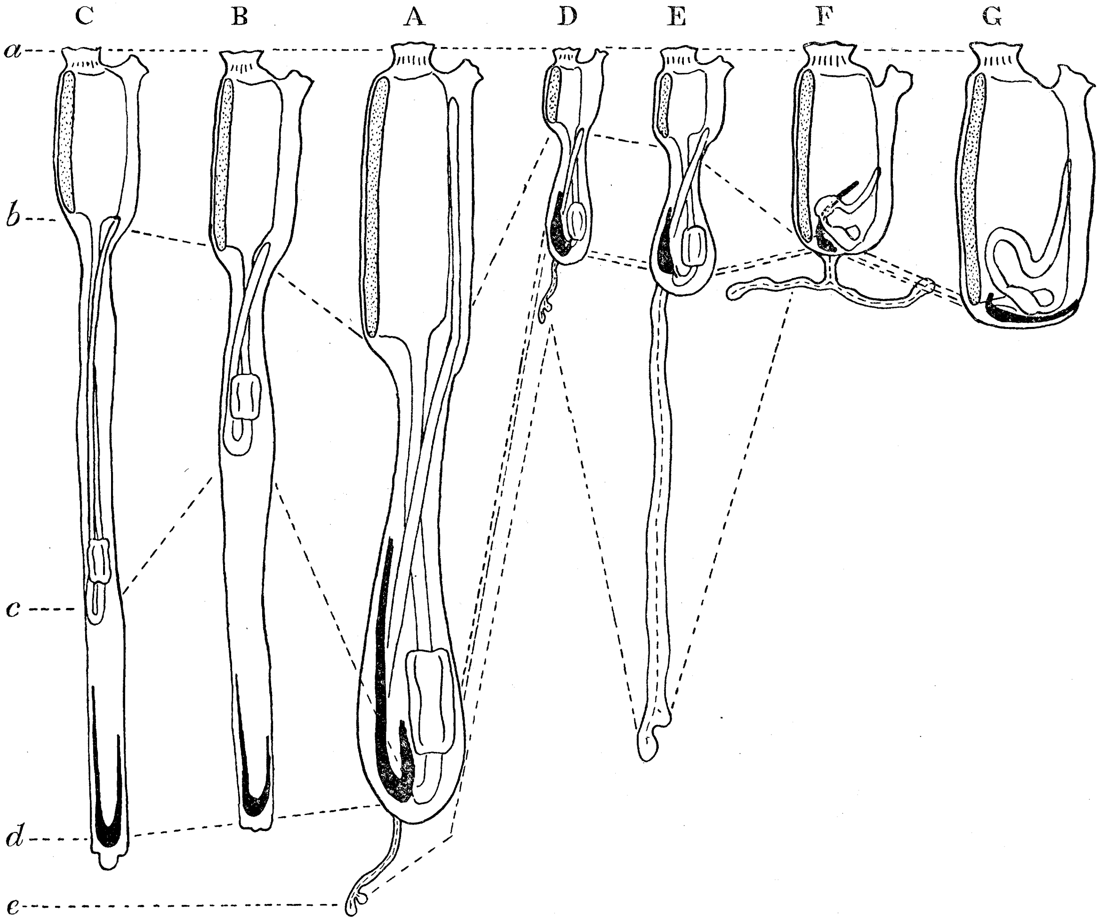

Fig. 1—Diagram to illustrate various modifications of zooids. A, primitive diazonid type ; B, synoicid ; C, *Euherdmania*, with post-abdomen between digestive tube and heart ; D, distomid dwarf ; E, distomid with hypertrophied ventral vessel ; F, perophorid with ventral vessel but with digestive tube and heart shifted forwards ; G, solitary ascidian without ventral vessel but with reflected viscera ; *a*, level of branchial siphon ; *b*, base of endostyle ; *c*, base of digestive loop ; *d*, base of heart ; *e*, tip of ventral stolonic vessel ; the heart is shown in black, the endostyle stippled

The colonies are massive, semi-pedunculate, and grow in several fathoms of water on exposed current-swept rock and coarse shell gravel. The zooids, fig. 2 B,

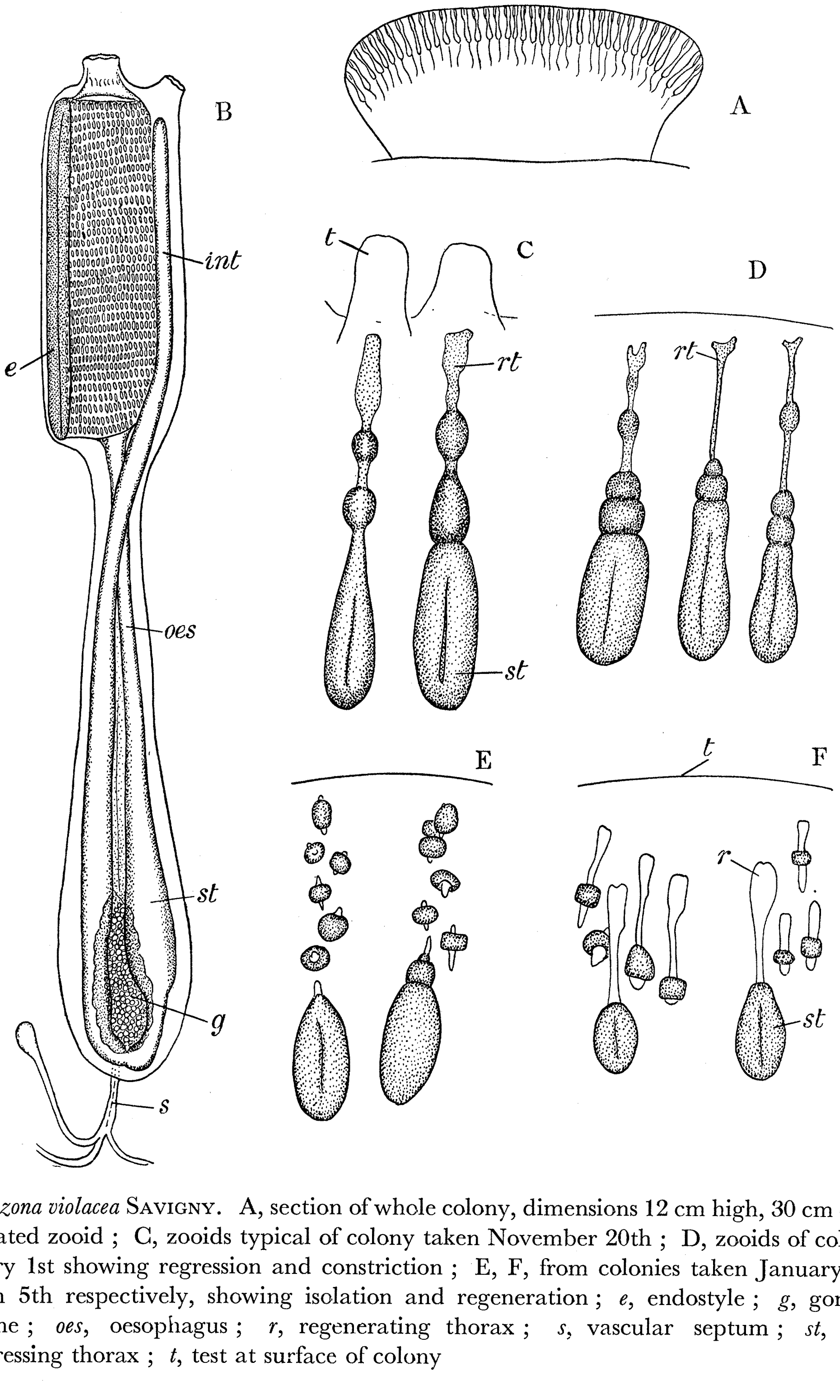

FIG 2—*Diazona violacea* SAVIGNY. A, section of whole colony, dimensions 12 cm high, 30 cm diameter; B, isolated zooid; C, zooids typical of colony taken November 20th; D, zooids of colony taken January 1st showing regression and constriction; E, F, from colonies taken January 30th and March 5th respectively, showing isolation and regeneration; *e*, endostyle; *g*, gonads; *int*, intestine; *oes*, oesophagus; *r*, regenerating thorax; *s*, vascular septum; *st*, stomach; *rt*, regressing thorax; *t*, test at surface of colony

are primitive inasmuch as the abdomen lies posteriorly to the pharynx, and the heart and gonads have not been displaced into a post-abdomen.

Reproductive cycle—The sexual breeding season at Plymouth lasts for not more than six weeks, but may commence at any time from early July to mid-September, depending on the average temperature of the preceding months. The period of formation and isolation of buds immediately follows the sexual breeding season, while during the greater part of the winter and early spring the buds are reorganized into new zooids that become active usually in April or May. Thus spring is a period of growth, summer one of maturation and sexual activity, autumn and early winter of asexual reproduction, winter of bud development, and the whole cycle is associated very definitely with the seasonal environmental changes.

Formation of buds—Closely following upon the breeding season, the thoracic parts of each zooid undergo regression. The siphons close, the thorax as a whole condenses and there is considerable autolysis. In fact, the whole of the thorax anterior to the oesophagus eventually disappears, although certain cells accumulate reserve substances during the period of autolysis and migrate posteriorly. There is thus a storage of the anterior part of the zooid in the form of trophocytes in the posterior part. During and subsequent to this posterior migration of trophocytes a series of transverse constrictions appear in the region of the oesophagus of each zooid. The constrictions appear first at the anterior end so that there is a definite antero-posterior time-lag. In the region of the stomach the zooids are apparently too bulky for successful constriction and the final appearance is that of a string of beads representing the oesophagus and a single mass the stomach region of the zooid.

The constrictions themselves are the result of epidermal activity alone. The tissues and organs internal to the epidermis remain passive and are cut through as the latter constricts. Epidermal cells are normally cubical or even squamous; but in the constricting area they become columnar, a change in shape that may in itself produce a constriction. At any rate, the nature of the epidermal cells lining the groove is one that indicates marked activity, and there is no doubt that the constricting force resides in this layer.

In such manner the middle and posterior parts of the zooids of *Diazona* are fragmented and the subsequent development of new zooids is one of regeneration and to a certain extent of reorganization.

Development of buds—The development of a bud consequently varies somewhat according to its original position relative to the whole. The bud mass does not become directly reorganized into a new zooid but regenerates the missing parts as in isolated annelidan segments. The large posterior abdominal bud regenerates only anteriorly to form a new oesophagus and thorax. The smaller and more anterior buds regenerate both a new thorax and a new abdomen, the relative lengths of the two regenerates depending on the original position of the bud.

The above phenomena can be seen in fig. 3, and at the beginning of development the bud is usually packed tightly with large trophocytes; these tend to disappear

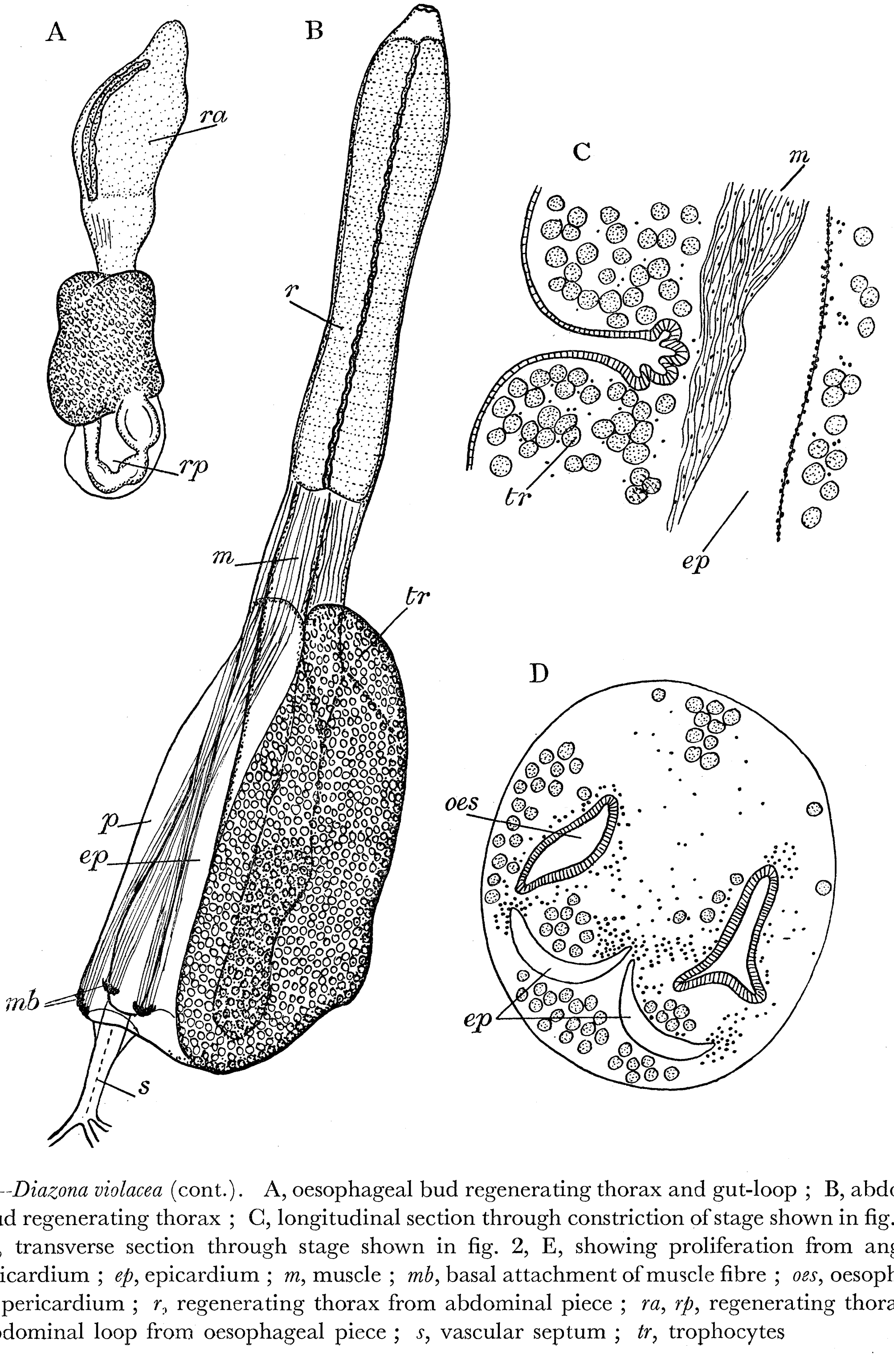

FIG. 3—*Diazona violacea* (cont.). A, oesophageal bud regenerating thorax and gut-loop ; B, abdominal bud regenerating thorax ; C, longitudinal section through constriction of stage shown in fig. 2, D ; D, transverse section through stage shown in fig. 2, E, showing proliferation from angles of epicardium ; *ep*, epicardium ; *m*, muscle ; *mb*, basal attachment of muscle fibre ; *oes*, oesophagus ; *p*, pericardium ; *r*, regenerating thorax from abdominal piece ; *ra*, *rp*, regenerating thorax and abdominal loop from oesophageal piece ; *s*, vascular septum ; *tr*, trophocytes

as the process of regeneration progresses and their substance is needed. The cells that actually build up the new tissues are, however, not the trophocytes themselves but minute cells that arise from the epicardial lining. In particular, they arise from the two lateral corners of the epicardium, fig. 3 D.

Species—Tylobranchion speciosum HERDMAN. Fig. 4

Source of material—Type colony in British Museum, from the Challenger Expedition near Kerguelen Islands.

Remarks—The type colony alone is in good condition and available for examination; and the mere fact that the majority of zooids within the colony are distended with developing embryos precludes any possibility of obtaining more than a fragmentary view of the process of budding, for sexual and asexual modes of reproduction are not mutually compatible. There is some evidence, however, that the colony in question was taken near the end of the sexual breeding season, with a few zooids entering the asexual phase.

The zooids are smaller and the colony more pedunculate than *Diazona* and the relationship with the synoicids even more pronounced. In fact, the zooids resemble those of the synoicids in the presence of a true post-abdomen containing heart and gonads.

Formation of buds—The great majority of zooids in the colony are breeding individuals, but a few are evidently in the throes of budding. As in *Diazona*, there is a disintegration of the anterior part of the zooid and a concentration of food-laden cells posteriorly. The dissolution is not confined to the branchial region, but involves the whole of the gut so that only the post-abdomen survives. Only a few instances were found where the post-abdomen had apparently constricted into two parts, most showing evidence of commencing regeneration without having divided, but there is every likelihood that colonies taken at a later period of the year would show a larger proportion of zooids in fission or even in multiple constriction.

V—BUDDING IN THE SYNOICIDAE

Species—Morchellium argus MILNE-EDWARDS. Fig. 5
Sidnyum elegans GIARD

Source of material—Tidal waters near Plymouth during August and September.

Remarks—The two species are typical of the family as a whole and the difference in their mode of budding is insignificant. Compared with *Diazona* the colonies are very small, much smaller even than *Tylobranchion*. While placed in a different order from these two genera, the zooids show a general similarity to those of *Tylobranchion* that far outweighs the difference in branchial structure used to separate

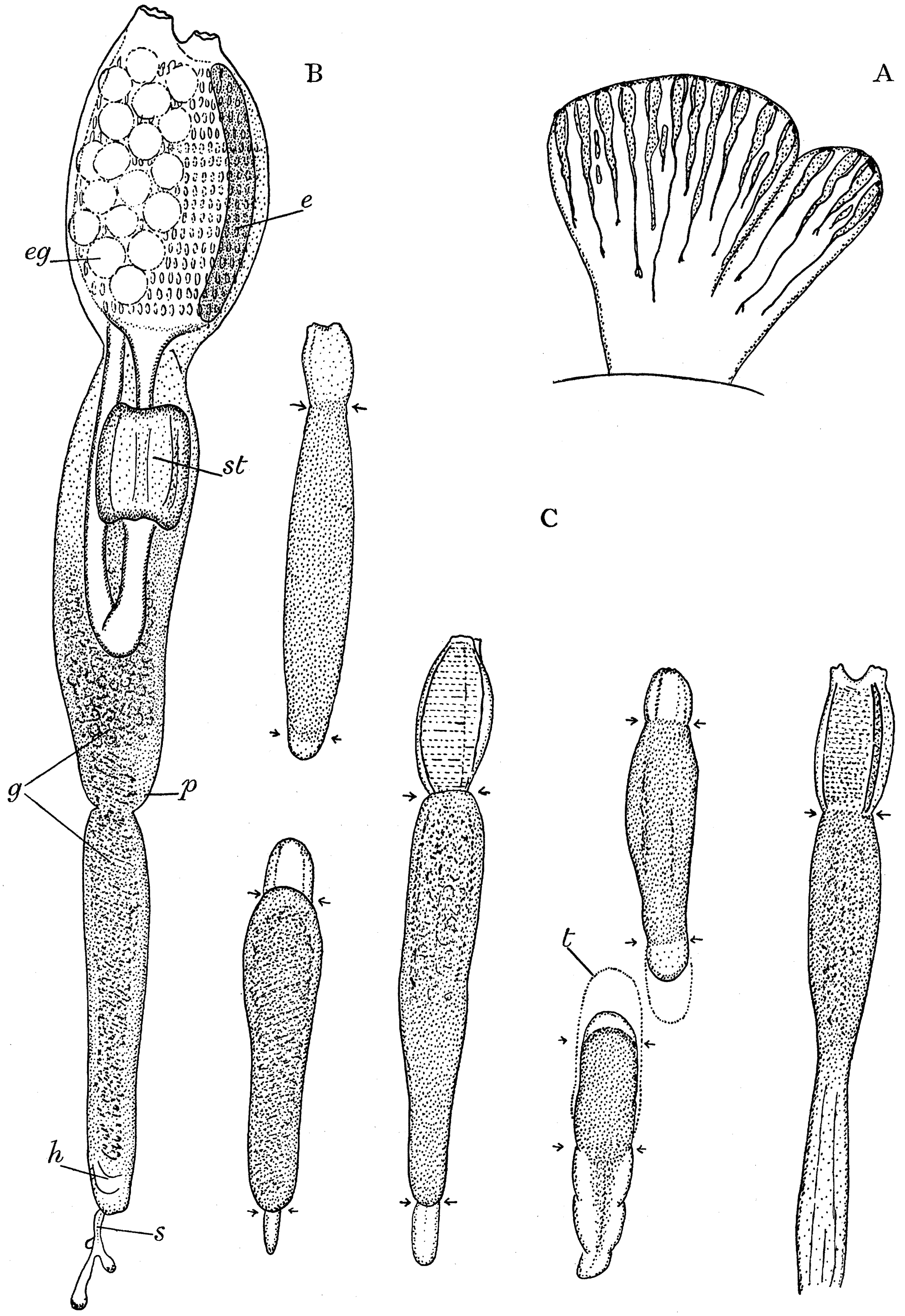

FIG 4—*Tylobranchion speciosum* HERDMAN. A, section of colony, 6 cm high ; B, zooid of colony showing general organization ; C, post-abdominal fragments exhibiting regeneration and drawn at respective levels beneath surface of colony as compared with a normal zooid B. An arrow marks the junction of old and regenerated tissue ; *e*, endostyle ; *eg*, embryo ; *g*, gonads ; *h*, heart ; *p*, post-abdomen ; *s*, vascular septum ; *st*, stomach ; *t*, newly secreted test

them. Each zooid has a long post-abdomen containing the heart and gonads, and, as in *Tylobranchion*, it is this region that is primarily concerned with the process of budding.

Reproductive cycle—In *Diazona* the sexual and asexual reproductive phases coincide so closely with the ascending and descending environmental temperature that it is difficult to ascertain whether the temperature or an internal condition is the stimulus for the change of phases. In the two synoicid species investigated there is no such difficulty, for the sexual breeding season is over and the asexual phase well under way, while the environmental temperature is still short of its summer maximum. There is little doubt that the necessary condition for the onset of budding on a large scale is the state of exhaustion associated with the end of the breeding season. Sexual reproduction in these forms begins in May and ends in August, while asexual reproduction begins in August or early September.

Formation of buds—As in *Diazona* and *Tylobranchion*, the anterior part of each zooid degenerates and the posterior region becomes packed with resulting trophocytes. In *Diazona* where there is no post-abdomen the trophocytes of necessity accumulate in the oesophageal and abdominal regions, but in the synoicids *Morchellium* and *Sidnyum* the trophocytes migrate posteriorly into the post-abdomen, and all the zooid anterior to this part degenerates. In each case, however, there is the same development and migration of trophocytes to the posterior region of the zooids, the main difference being the displacement of the posterior extremity from the abdomen in the case of the synoicids, fig. 5.

Constrictions appear as in *Diazona*, but posteriorly, transverse epidermal, to the abdomen. This process, according to Brien (1925), may occur occasionally in functional summer zooids in *Aplidium zostericola*, but it occurs on a large scale only after the breeding season has ended. The division of the post-abdomen in *Sidnyum* may begin while the zooid is fully active, although the complete multiple division occurs only after the dissolution of the rest of the zooid. In *Morchellium* the process of regression is virtually completed before strobilization starts, but a string of buds is always formed differing from those of *Diazona* only so far as they have been formed from the post-abdomen rather than the abdominal and oesophageal regions. The trophocyte accumulation and the epidermal constriction are the same.

Development of buds—The development of the buds of *Aplidium* has been investigated in full detail by Brien (1925-7).

In these as in the buds of *Morchellium* and *Sidnyum*, there is no part of the digestive tract, and the whole of that structure has to be formed anew. Otherwise the buds contain much the same tissue as do those of *Diazona*. The new epidermis is always formed from the old, but the remainder of the new tissues arises from the cells of the epicardium, the trophocytes maintaining a nutrient medium. In *Diazona*, there is a process of true regeneration, the bud mass eventually occupying the same relative position in the new whole as it did in the old. In the synoicids the absence

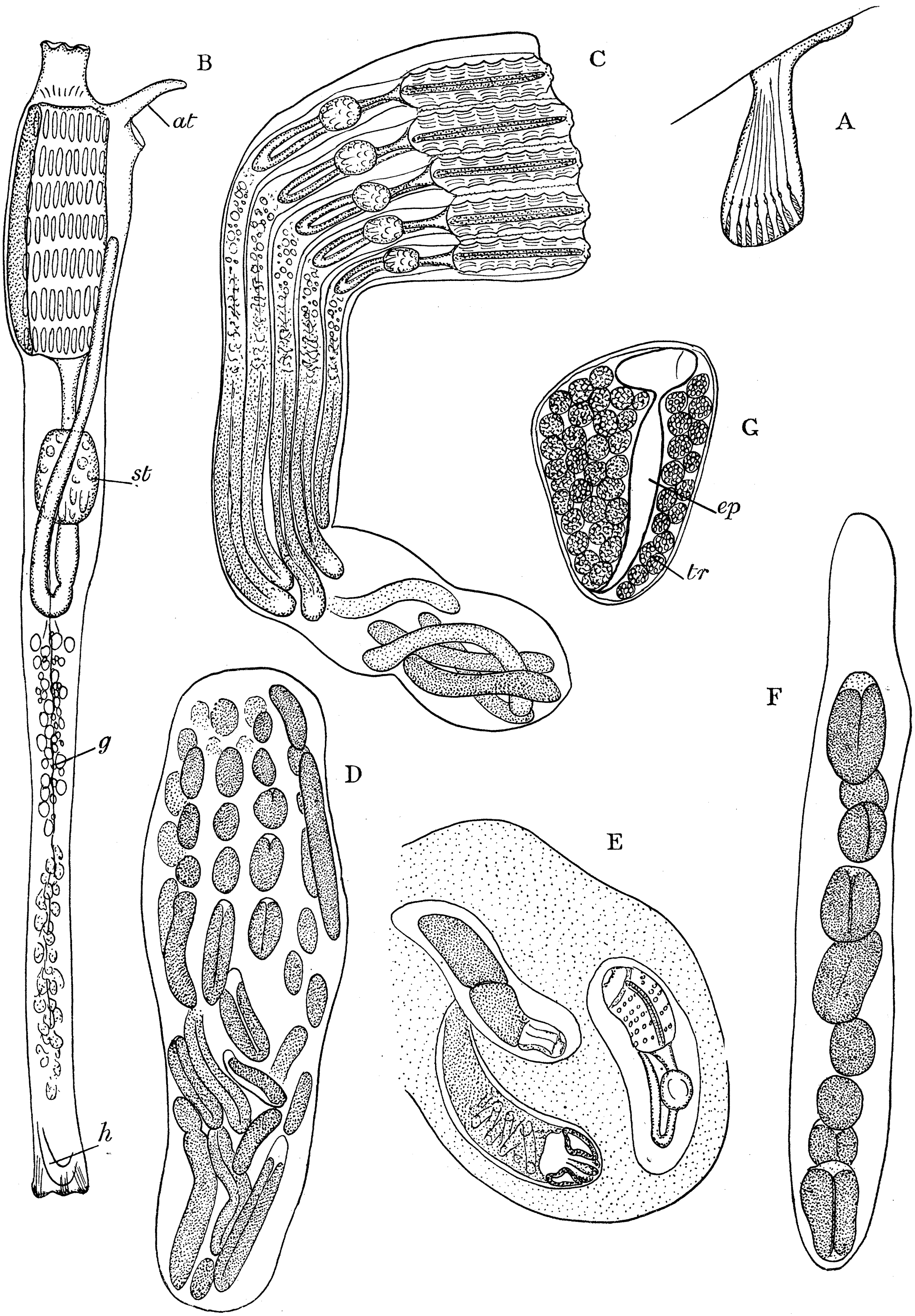

FIG. 5—*Morchellium argus* MILNE-EDWARDS. A, section of colony, 3 cm. long; B, isolated zooid showing post-abdomen and modified atrial siphon; C, young colony beginning to constrict basally; D, part of older colony with completed constriction and bud formation; E, three basal buds undergoing reorganization; F, constricted oozoid; G, longitudinal section of young bud showing epicardium and trophocytes; *at*, atrial languet; *ep*, epicardium; *g*, gonads; *h*, heart, *st*, stomach; *tr*, trophocytes

of any part of the alimentary canal apparently leaves the bud more plastic, and in place of typical regeneration there is reorganization, the new zooid forming within the mass even before there is any significant outgrowth.

Species—Euherdmania claviformis Ritter. Fig. 6

Source of material—Pacific Grove Laboratory, California.

Remarks—*Euherdmania* has been described only by Ritter (1903), who was unable to glean any information concerning its method of budding. It is a genus usually placed in a family of its own, but in general structure it is very similar to the synoicids, although the zooids and stolons are attached only by their bases and are so heavily encrusted with sand that the necessary observations are difficult to make.

Reproductive cycle—The sexual breeding season is during the summer months, asexual reproduction being a winter phenomenon and the spring a period of growth of immature zooids.

Formation of buds—Only a partial picture has been obtained of this process. Colonies collected in November had a large percentage of zooids that had lost or absorbed the thorax and were beginning to regenerate new branchial structures, fig. 6. It is quite possible that a colony may persist through a winter without any change other than the renewal of the thorax. In some colonies taken in December, however, only the post-abdominal parts of the zooid persisted, and these parts were divided into a considerable number of small elongate masses similar in appearance to those of *Morchellium* and *Sidnyum*. Their arrangement and dimensions give the impression that there has been a median longitudinal division of the post-abdomen

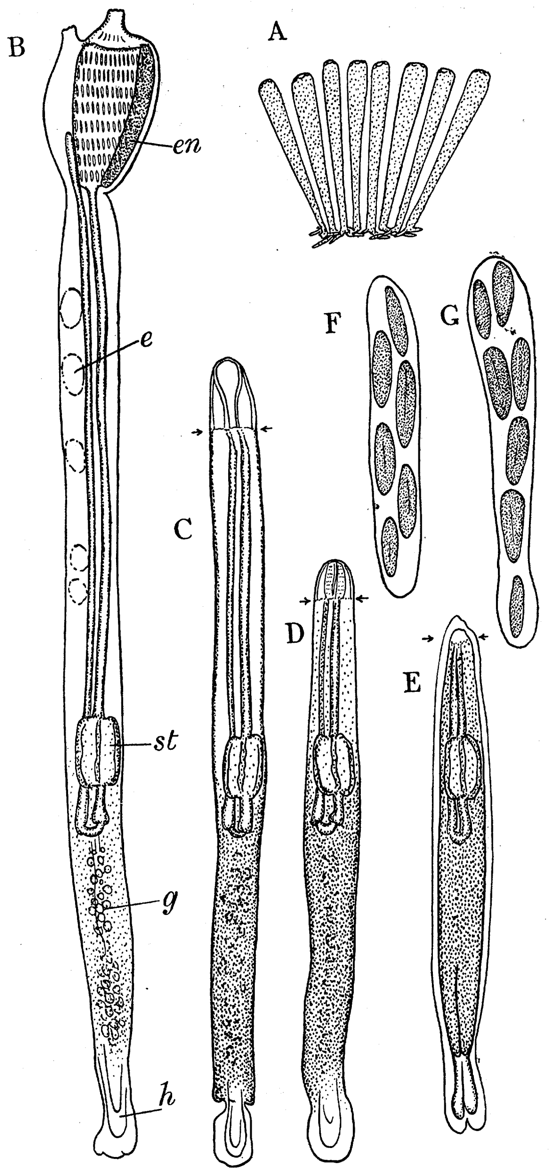

Fig. 6—*Euherdmania claviformis* Ritter. A, diagram of colony, 2 to 3 cm high; B, normal zooid of summer colony; C, D, E, zooids of a colony taken in November showing anterior regeneration following regression of the thorax and oesophageal region, and an accumulation of trophocytes in the post-abdomen; F, G, zooids of a colony taken in December showing only masses isolated from the post-abdomen; *e*, embryo; *en*, endostyle; *g*, gonads; *h*, heart; *st*, stomach

in addition to the transverse strobilization. No colonies were found in which development of the buds had commenced, and the observations recorded above need confirmation and extension. In general, however, the mode of budding is similar to that of the synoicids.

VI—Budding in the Distomidae

Species—Archidistoma aggregata Garstang. Fig. 7

Source of material—Dukes Rock, Plymouth, and the Salstone, Salcombe.

Remarks—Discovered and described by Garstang (1891), but has not yet been redescribed, and there are no records of the method of budding. Two colonies only have since been discovered, during August, when the breeding season was at its height, and only a few zooids showed evidence of asexual reproduction.

Formation of buds—There is no post-abdomen so that the synoicid type of budding is precluded. At the same time the stolonic blood vessel is small and insignificant and most unlikely to form a budding stolon. A few zooids were discovered, two of which are figured, that indicate a process of budding similar to that of *Diazona*, even though there has been an extreme reduction in body-size from that genus, fig. 7.

The thoracic region is absorbed, large trophocytes accumulate in the oesophageal and abdominal regions, a series of constrictions divide the posterior part into three or four masses, the most posterior mass containing the heart and stomach, the others sections of the oesophagus and intestine.

This number would account for the fact that the zooids of a colony are usually raised from the general surface in closely united clusters of three or four.

Sections of the strobilating zooids show that there is an active proliferation of epicardial cells as in *Diazona* and synoicids, and also that the epidermal constrictions are to be associated with a change from a cubical to a columnar type of epithelium. The inner tissues are merely passively cut through.

Species—Eudistoma olivacea Van Name. Fig. 8, A–K

Eudistoma lobatum Ritter. Fig. 8, L–M

Source of material—The winter colonies of *Eudistoma olivacea* from Bermuda. Those of *Eudistoma lobatum* from Pacific Grove, California.

Remarks—The zooids of *Eudistoma* are remarkably similar to those of *Archidistoma*. In each, there are but three rows of stigmata, a hypertrophied oesophageal region, no post-abdomen, a small stolonic vessel, and an unmodified atrial siphon. The *Eudistoma* zooids are completely embedded in a common test, those of *Archidistoma* extend freely from the surface of the colony for more than half their length.

Reproductive cycle—The alternation between sexual and asexual phases of reproduction is the same as in *Diazona* and synoicids, the breeding season being a summer, budding a winter, activity.

Formation of buds—Following the breeding season, the zooids always undergo a marked contraction and condensation and become relatively inactive. There is,

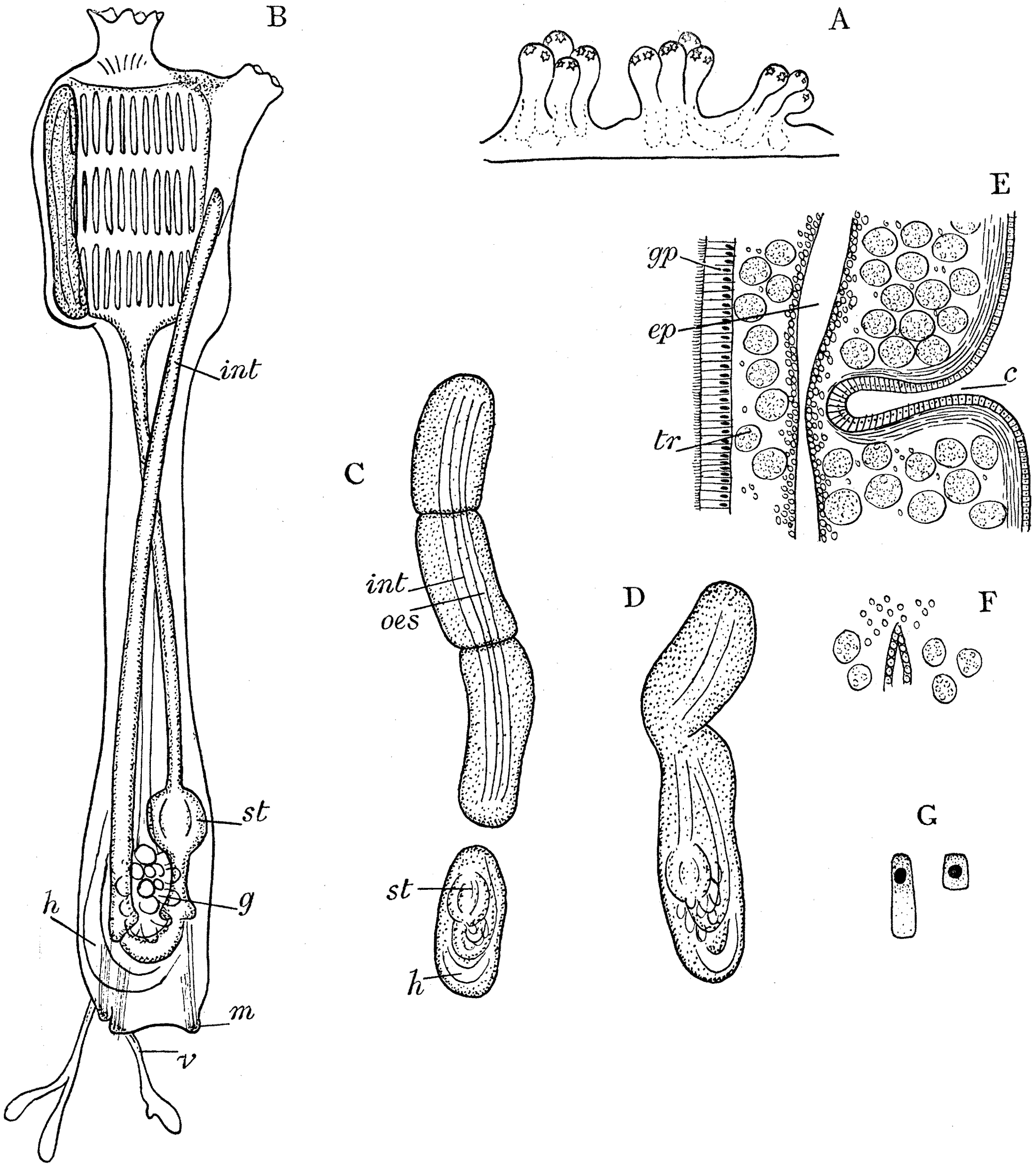

Fig. 7—*Archidistoma aggregata* Garstang. A, part of colony, 5 mm high; B, mature zooid of same; C, D, zooids with regressed thorax and undergoing constriction to form buds; E, vertical section through a constriction showing epidermal activity and the inactive inner tissues; F, upper angle of epicardium showing cell proliferation; G, enlarged epidermal cell in constriction area and a normal epidermal cell; *c*, constriction; *ep*, epicardium; *g*, gonad; *gp*, digestive epithelium; *h*, heart; *int*, intestine; *oes*, oesophagus; *m*, insertion of longitudinal muscles; *tr*, trophocytes; *v*, vascular stolon with septum

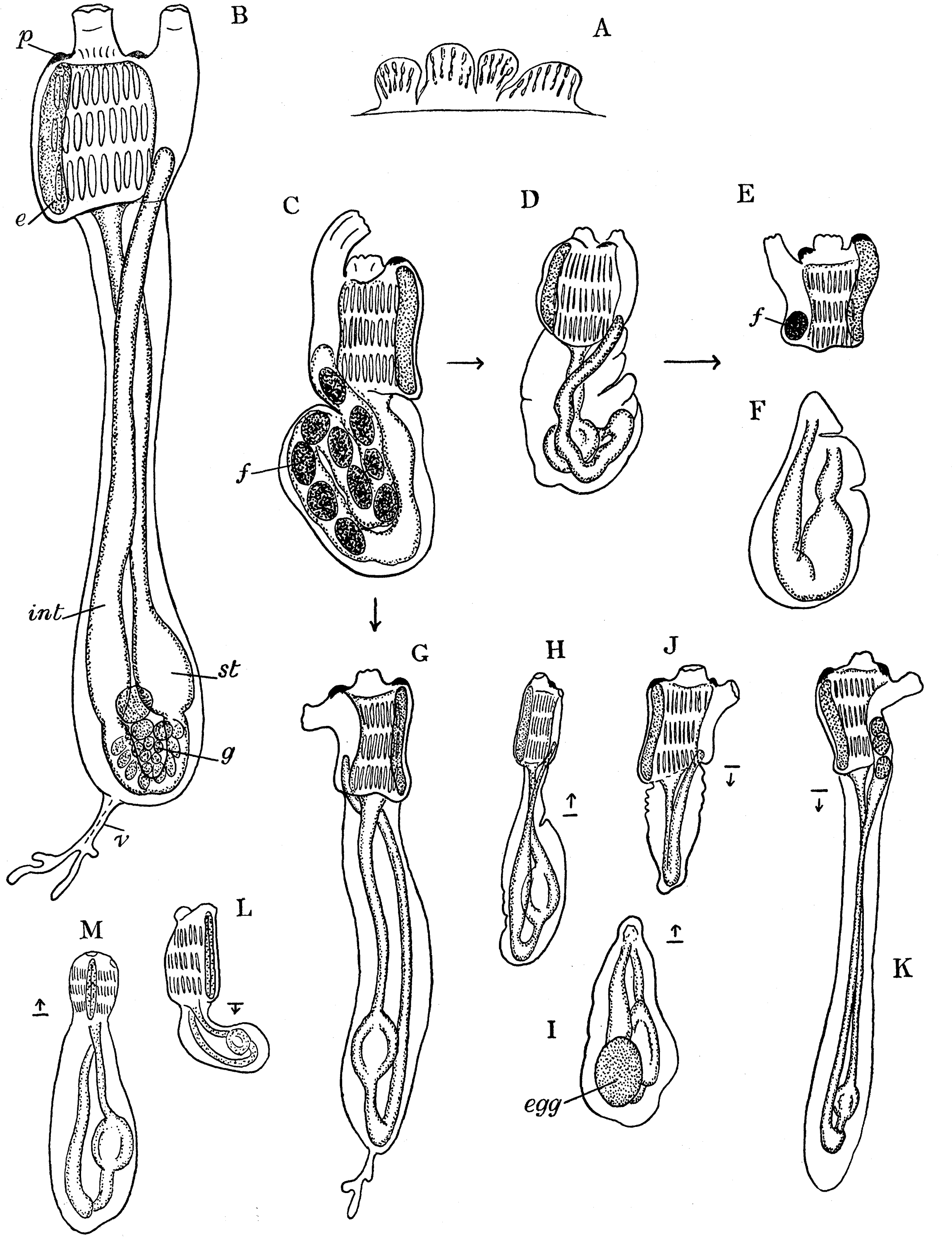

FIG. 8—*Eudistoma olivacea* VAN NAME. A, section of colony, 1 cm high; B, mature summer zooid of same; C–F, zooids from colony collected in November; C, D, two stages in condensation; E, F, zooid constricted at junction of thorax and oesophagus; G–K, zooids from colony collected in January; G, rejuvenated zooid resulting from extension of condensed zooid of type shown in C; H, I, two stages of anterior regeneration from zooid fragment similar to F; J, K, posterior regeneration from thorax E; L, M, posterior and anterior regeneration from constricted zooid of *Eudistoma lobatum* RITTER; *e*, endostyle; *f*, faecal pellet; *g*, gonad; *int*, intestine; *p*, pigment; *st*, somach; *v*, vacsular stolon with septum

however, no regression of the thorax and no marked accumulation of trophocytes. The contraction affects particularly the oesophageal region, and the intestine and oesophagus may become coiled and crowded together. At the same time, a series of epidermal grooves or constrictions appear between the thorax and stomach, but there is no strobilization as in *Diazona* or *Archidistoma*. Frequently there is no division of the zooid at all and it eventually elongates again, presumably having undergone a certain reorganization of its tissues. On the other hand, many zooids divide into two parts. The most anterior constriction, immediately below the thorax, cuts through the inner tissues, the longitudinal muscles of these and other ascidians being weakest at this point. This process is immediately followed by regeneration, the isolated thorax growing a new abdomen, the isolated abdomen a new thorax. The above observations are as valid for *Eudistoma lobatum* as they are for *Eudistoma olivacea* on which they were first made.

Species—Distaplia (*Holozoa*) *rosea* Della Valle. Fig. 9
D. magnilarva Della Valle and *D. bermudensis* Van Name

Source of material—Colonies of *Distaplia rosea* and *D. magnilarva* were collected during summer months at Plymouth, colonies of *Distaplia bermudensis*, also during the summer, at Bermuda.

Remarks—The zooids of *Distaplia* resemble those of *Eudistoma* and *Archidistoma*, except in three particulars. The atrial siphon is modified to open into a common cloacal cavity, the oviduct, which serves as a uterus in *Eudistoma*, here protrudes in a double loop to form a brood pouch, while the stolonic vessel is long and hypertrophied.

Reproductive cycle—There is a regular alternation as in the preceding forms between phases of sexual and asexual reproduction, but there is no approximate synchronization with summer and winter. The two phases follow each other in fairly rapid succession, so that there may be several or many alternations in the course of a year. There is, however, a certain amount of overlapping between the two phases. After the brood sac is filled with developing embryos, the sac becomes detached as the rest of the zooid degenerates. The embryos continue to develop because the sac is surrounded by blood vessels in the test.

Formation of buds—As the mature zooids degenerate and the brood sac becomes isolated, two small masses of tissue develop and survive in the region of the oesophagus. These masses are composed of very numerous and very small cells and arise near the outer wall of the oesophagus. As far as could be determined, they arise from the anterior horns of the epicardia, as described by Julin (1892), and not from the oesophageal wall itself as generally believed. In the tadpole the buds may be said to be of endodermal origin since they are derived from the pharyngeal floor between endostyle and oesophagus. In reality, however, it

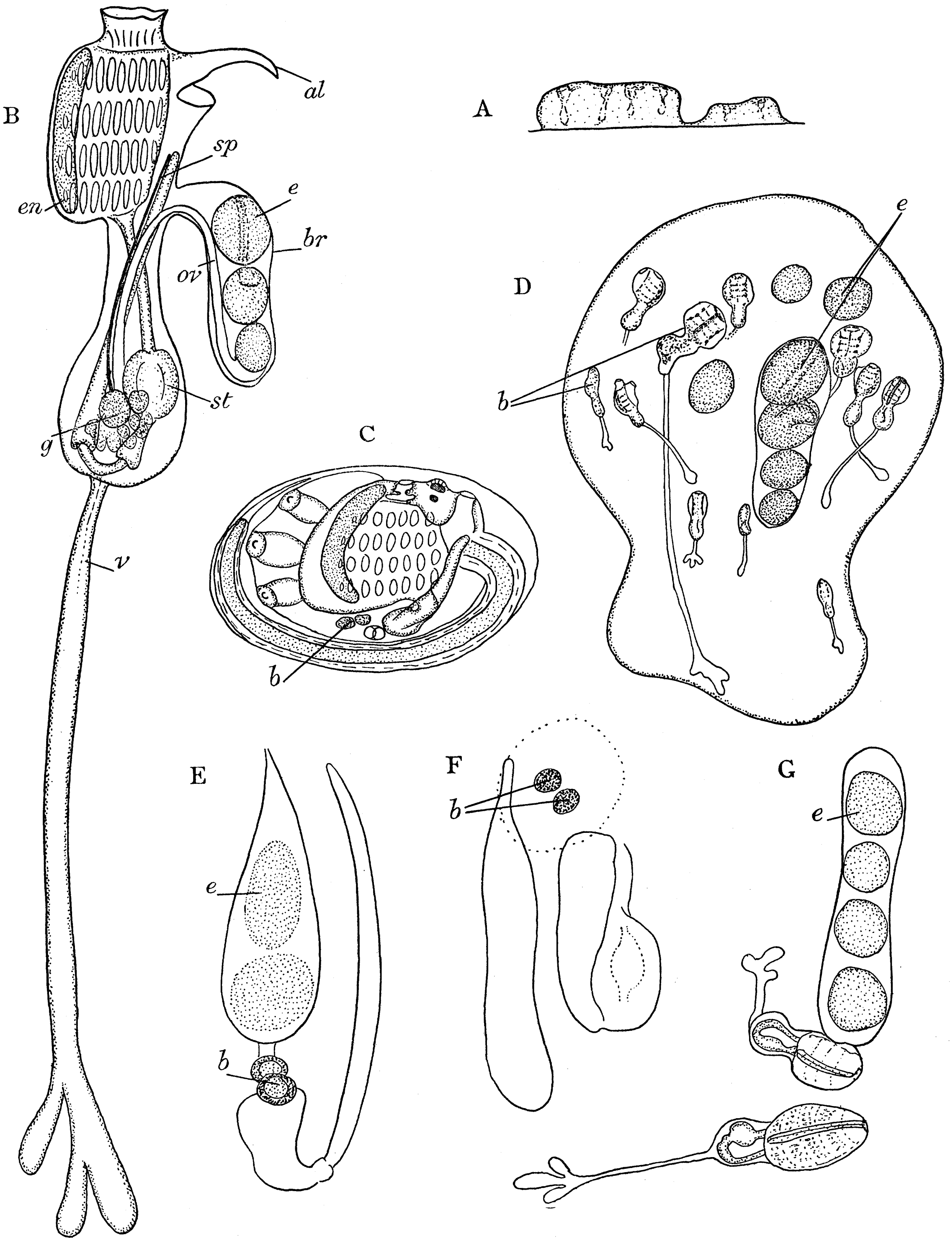

FIG. 9—*Distaplia rosea* DELLA VALLE. A, section of colony, 5 mm high; B, mature zooid of same, showing hypertrophied ventral vessel, oviducal brood pouch, and atrial languet; C, tadpole larva with two buds; D, small colony at close of sexual breeding period and beginning of bud development; E, F, two late stages in autolysis of zooid and isolation of two bud masses; G, two buds developing at base of surviving brood pouch, the rest of the parent zooid having completely disappeared; *al*, atrial languet; *b*, buds; *br*, brood pouch; *e*, embryos; *en*, endostyle; *g*, gonad; *ov*, oviduct; *sp*, sperm duct; *st*, stomach; *v*, hypertrophied vascular stolon with septum

becomes clear that they arise from the epicardia as those structures are themselves developing from the floor to the pharynx.

After the zooid has completely degenerated the two buds survive and lie just beneath the isolated brood pouch. By the time the contained embryos have developed and escaped the buds have become immature zooids. Growth is rapid, but the energy and raw material is derived from the stolonic vessels surviving from the preceding generation of zooids. The distintegration and autolysis of the old zooids must necessarily produce a rich nutrient medium for the young buds.

In the tadpole or young post-larva one or two buds, depending on the species in question, are constricted from the junction of the oesophagus and branchial sac. The oozoid may live actively for a day or as long as two or three weeks, again depending upon the species, but eventually autolysis occurs, and not until then do the buds develop into new zooids. Larval and adult budding is therefore essentially the same, fig. 9.

Species—*Colella thomsoni* Herdman. Fig. 10, A–C
Colella concreta Herdman. Fig. 10, D–F

Source of material—British Museum.

Remarks—*Colella* (*Sycozoa*) is an Australasian genus and little is known concerning its reproductive cycle. The colonies are usually stalked and the zooids in some species are polymorphic. The distal ends of the colonies are swollen and contain zooids at various stages of sexual maturity, while in the stalk, or at the junction of the stalk and head, young buds may be found at the same time.

The zooids are somewhat like those of *Distaplia*, in spite of a much greater number of rows of stigmata. The ventral stolonic vessel is wide and long and the embryos burst from a temporary brood pouch into the surrounding test. Unlike those of *Distaplia*, the stolonic vessels of *Colella* are fertile.

Formation of buds—Caullery had shown (1909) that in *Colella pedunculata* budding is essentially a process of strobilization on the part of stolonic vessels in the stalk, apparently from their anterior ends after separation from the zooids, as shown in fig. 10 G. In the species here described, *C. thomsoni* and *C. concreta*, the process, while obviously related, shows certain differences. In *Colella concreta* the stolons become separated from the zooids and, by a number of transverse constrictions, buds are divided off the posterior ends, although there are indications that the same may occur from the anterior ends and even that the whole of the stolon may become involved, as apparently occurs in *C. pedunculata*. In *Colella thomsoni* budding seems to be confined to the junction of the stalk and head of the colony, the greater part of the stalk remaining sterile and containing an anastomosis of stolonic vessels. From the anterior surface of the network buds are formed wherever there is sufficient space, the buds developing not as isolated units but in permanent connection with the ventral network.

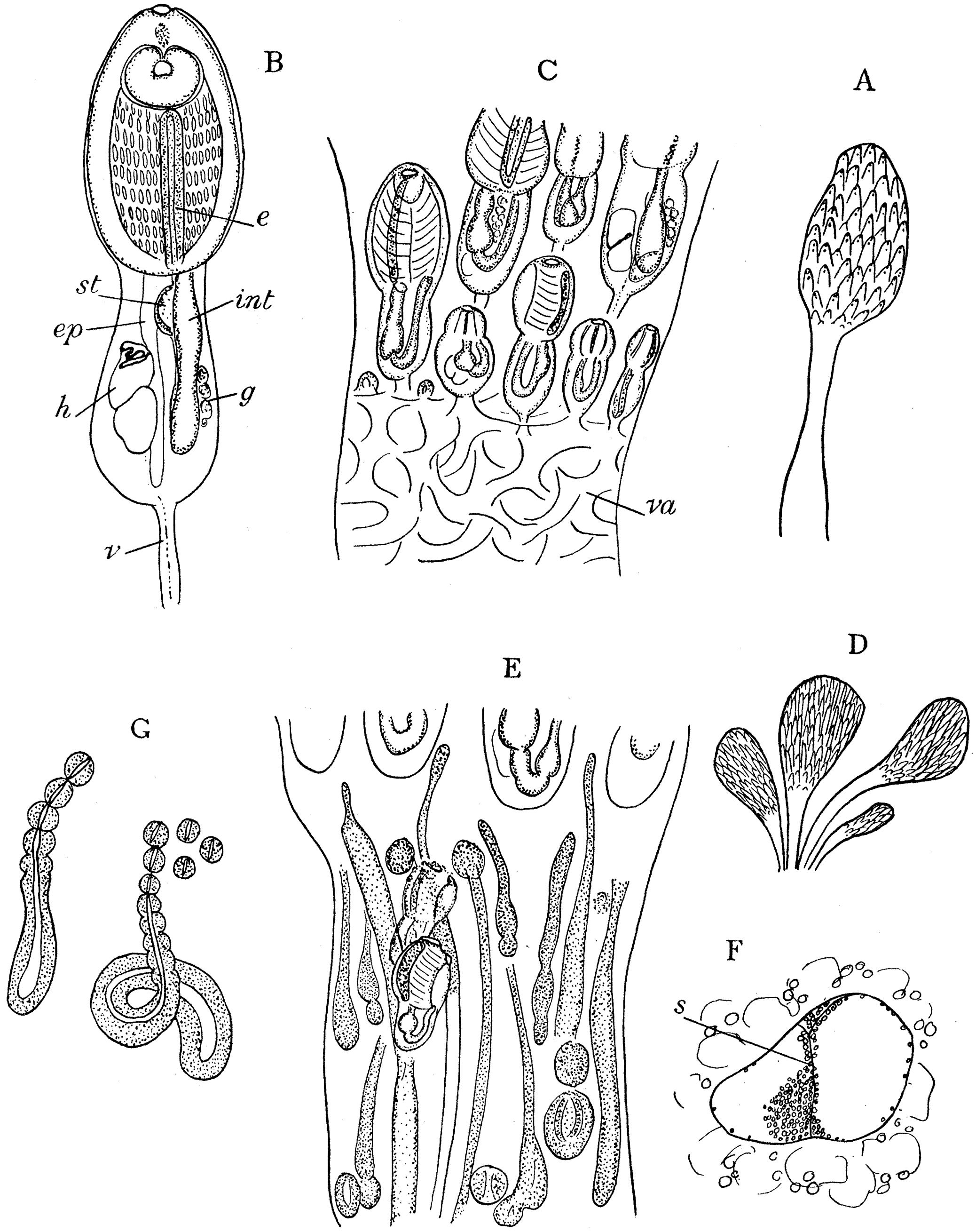

FIG. 10—*Colella thomsoni and C. concreta* HERDMAN. A, colony of *C. thomsoni* ; B, median view of mature zooid ; C, vertical section through junction of stalk and head showing origin of buds from dorsal ends of anastomizing ventral vessels ; D, colony of *C. concreta* ; E, vertical section through junction of stalk and head showing formation of buds from isolated ventral vessels ; F, cross-section of end of ventral vessel showing cell proliferation from vascular septum ; G, constricting ventral vessels of *C. pedunculata* after CAULLERY ; *e*, endostyle ; *ep*, epicardium ; *g*, gonad ; *h*, heart ; *int*, intestine ; *st*, stomach ; *v*, vascular stolon with septum ; *s*, septum ; *va*, anastomosis of stolonic vessels

The differences between the several species are thus quite marked, but there remains the possibility that if the complete reproductive cycles were fully known the differences might become less significant.

A cross-section through the budding end of a stolonic vessel, fig. 10 F, shows that the mesenchyme vascular septum is present and that it is from this septum that the cell proliferation necessary for bud development takes place. The septum in *Colella* thus replaces the epicardial lining of other forms as a source of totipotent cells.

VII—Budding in the Didemnidae

The didemnids form sheet-like encrusting colonies containing comparatively minute zooids, and have long been notorious for the apparent complexity of their method of budding. The test of most genera is so densely packed with spicules that they obscure the zooids and their activities, but in the genus *Diplosoma* the spicules are virtually absent and in consequence this form has been the subject of most of the investigations within this family. Even within this one genus, however, there seems to be considerable variation with regard to the process of budding.

According to Pizon (1905) buds are produced from the anterior ends of the epicardia in the region of the oesophagus. These are merely small masses of cells proliferated from the epicardial lining. The process begins during the embryonic period in *Diplosoma*, in the post-larval period in most other didemnids, and in *Diplosoma* results in a tadpole larva with a single tail but with two branchial sacs, intestines, and nervous systems. Budding continues, again from the proliferation of small cell masses from the epicardia, to form a new oesophagus and branchial sac which joins the oesophagus of the parent, while an evagination from the rectum forms a new rectum in connection with the old. Then either a third bud forms a new stomach and intestine and the organism splits so that the old thorax is attached to the new stomach and intestine and the new with the old, or the old thorax degenerates. The process is thus exceedingly complex, and it is hoped that a reinvestigation will clarify matters to some extent.

Species—Diplosoma listerianum var gelatinosum Milne-Edwards. Fig. 11

Source of material—Collected during summer months at Plymouth.

Remarks—The zooids of *Diplosoma* and other didemnids, but for the complexity introduced by their peculiar methods of budding, are very similar to those of *Distaplia* and the distomids.

Reproductive cycle—Sexual and asexual phases tend to alternate with one another as in other forms, but each phase occurs frequently and is of relatively short duration, and during summer months there may be considerable overlapping.

Formation of buds—Two centres of proliferating cells appear about midway down the oesophagus in close contact with its outer wall, and one anterior to the other.

The cells seem not to be oesophageal cells themselves, nor to arise from the basement membrane cells of the oesophagus, but to be a proliferation from the anterior horns of the two epicardial chambers. Each mass fuses with the outer surface of the oesophagus, and they are separated by a short interval of unmodified oesophageal epithelium. Fig. 11 D illustrates the junction and their complete separation from

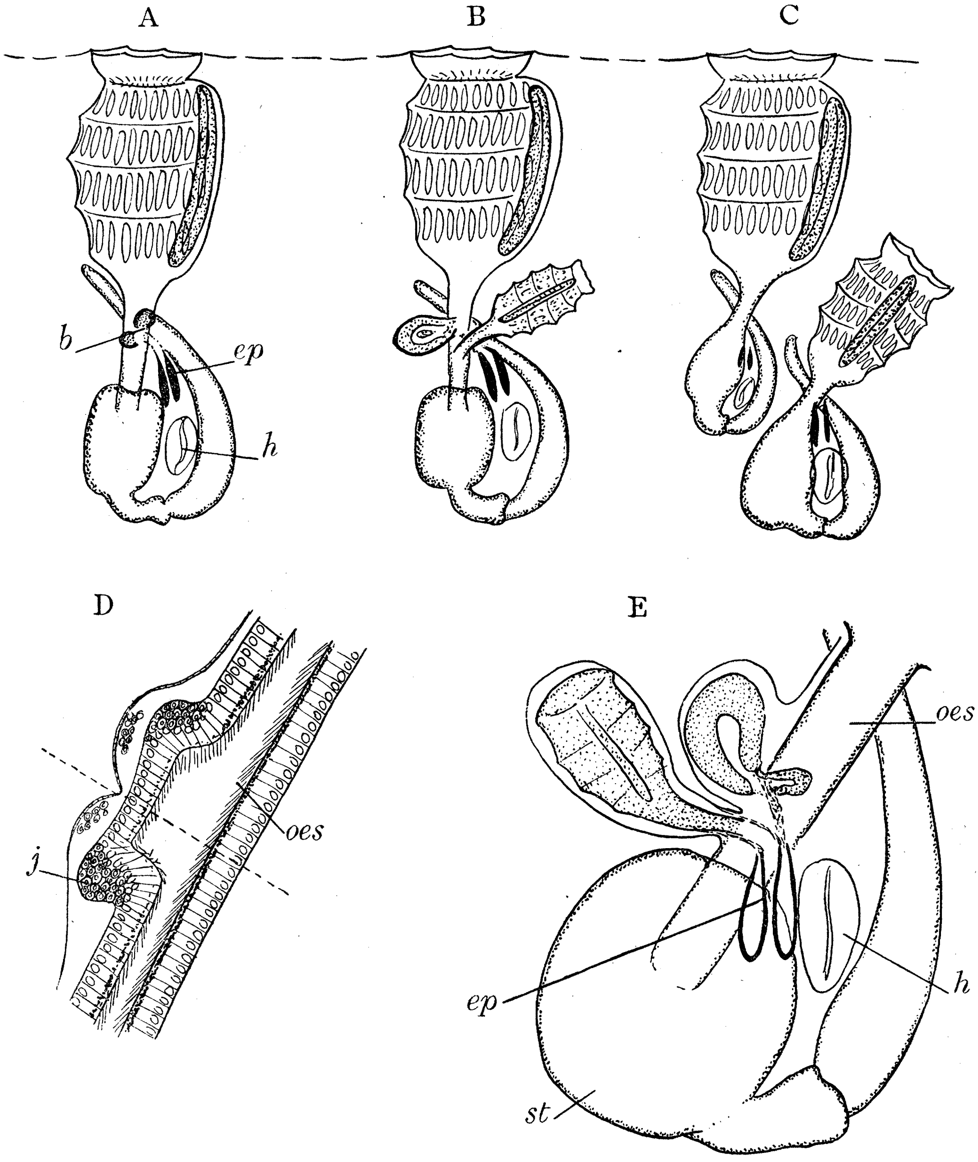

FIG. 11—*Diplosoma listerianum* MILNE-EDWARDS. A, zooid with two cell masses proliferated from anterior horns of the epicardia (shown in black); B, fusion of masses with oesophagus and subsequent regenerative growth to form a thorax and gut loop; C, separation of double individual so that old thorax possesses new gut, and new thorax old gut; D, section through junction of buds with oesophagus; E, reconstruction of early stage in bud development showing connections with oesophagus and epicardia; *b*, buds; *ep*, epicardia; *h*, heart; *j*, junction of bud and oesophagus; *oes*, oesophagus; *st*, stomach

one another is evident. Each mass as it enlarges is covered with an expanding sheet of epidermis, so that, as in the development of the buds of other forms, epidermal proliferation is of importance. Each of the two epicardial horns is concerned in the formation of each bud mass.

Of the two masses, the anterior grows and develops into a new stomach, intestine, gonads, etc., in continuity with the old branchial sac and anterior half of the old oesophagus. The hinder part of the intestine and rectum is added, not by an evagination from the old rectum as described by Pizon for *Diplosoma spongiforme* and *Diplosoma listeri*, but by an additional proliferation from the anterior horns of the epicardia. The posterior bud mass also grows, but it develops into a new branchial sac attached to the hinder part of the old oesophagus by a new anterior oesophagus. While the original two masses of cells arise from the anterior horns of the epicardia, there seems to be a continuous proliferation of cells from this source throughout the whole period of bud development. Finally, the old oesophagus between the points of fusion with the two primary bud masses degenerates and two whole and separate individuals exist. One consists of the old branchial sac, old anterior half of the oesophagus, and new stomach, intestine, rectum, gonads, heart, etc. The other consists of a new branchial sac and anterior oesophagus and the old stomach, intestine, etc.

The whole process is only apparently complex. Assuming two masses of totipotent epicardial cells to be present along the sides of the oesophagus and some factor inducing a physical or physiological discontinuity between the anterior and posterior parts of the zooid at a region between the two masses, the rest follows. What happens is evidently a regeneration of the posterior organs by the anterior entity, and of anterior organs by the posterior entity, the epicardia merely supplying raw material in the form of plastic or totipotent cells throughout the process, just as in budding or regeneration in many other ascidians. There is, in fact, a close relationship between the budding process of *Diplosoma* and that of *Distaplia* and of *Eudistoma*. This will be discussed later.

The variations from the above account, apart from the detail of rectal duplication described by Pizon (1905), are readily explained. In certain cases only one mass of cells seems to be formed. At all events only one outgrowth occurs, namely, that to form a new thorax, and the equivalent of the anterior bud is absent. When this occurs the new thorax blends with the old abdomen, corresponding exactly with the development and connections of the posterior bud. The old thorax (branchial sac and anterior oesophagus) is thus left high and dry and not unexpectedly degenerates. Degeneration of a thorax and its replacement by epicardial cells is of common occurrence among ascidians, and the only striking feature about the present species is that the replacement occurs before the degeneration. The more typical example where two whole individuals are formed resembles the condition in *Eudistoma*, where the zooid tends to constrict into two parts at the level of the anterior horns of the epicardia, the anterior part regenerating a new posterior, and the posterior a new anterior part. The mysterious aspect of budding in *Diplosoma* thus become merely the factor that induces the regenerative processes of the anterior and posterior parts, while there is yet apparently a normal morphological continuity between them.

VIII—Budding in the Clavelinidae

Species—Clavelina lepadiformis O. F. Müller. Fig. 12

Source of material—Collected during August and September at Plymouth.

Remarks—*Clavelina lepadiformis* and the closely allied species *Cl. rissoana* have been the subject of much study with regard not only to budding, but also all aspects of adult and embryological structure. The zooids are relatively primitive since there is no post-abdomen and associated dislocation of heart and gonads. At the same time, in spite of the comparatively large size of the zooids, the heart and epicardium are similar to those of the distomids rather than to those of *Diazona* or of the synoicids. The stolonic vessels are large and extend as in *Distaplia* and *Colella*, and resemble those of the latter in particular as they are fertile.

Reproductive cycle—The sexual breeding season begins as a rule in early July and lasts for about two months. At the end of that period the adult zooids become somewhat degenerate and are sloughed off, leaving a basal mass of buds to develop during the winter to form a new colony. The spring is a period of growth of immature zooids and the whole cycle resembles that of *Diazona* except that budding does occur to a limited extent during the period of sexual maturity as well.

Formation of buds—During the closing period of the breeding season trophocytes accumulate in large numbers. According to Spek (1927), they arise in and migrate from the lining of the anterior regions of the alimentary canal. They are considered by this author to be the totipotent cells themselves, but Brien (1930) has shown that this is not so. In any event they pass into the body cavity and blood vessels, and, as in *Diazona* and the Synoicids, migrate to the posterior part of the zooids. In *Clavelina* the migration posteriorly is not checked by the abdominal loop region, and the trophocytes accumulate in *culs-de-sac* of the stolonic vessels (*see also* J. S. Huxley, 1926); but as long as these vessels remain in functional continuity with their respective zooids, development of buds is inhibited. Thus the stolonic ampullae packed with trophocytes do not actually become buds until the sexual zooids that gave rise to them degenerate or become broken off. As soon as that occurs the basal masses become active and develop into new zooids, fig. 12.

Development of buds—Some masses become detached even during the summer months and, at the relatively high temperatures then prevailing, development to form new individuals is a rapid process. A colony taken at that time may contain individuals at all stages of growth. During the winter the lower temperature of the water retards the development of buds greatly, so that the new colony as a whole does not become conspicuous until the following spring.

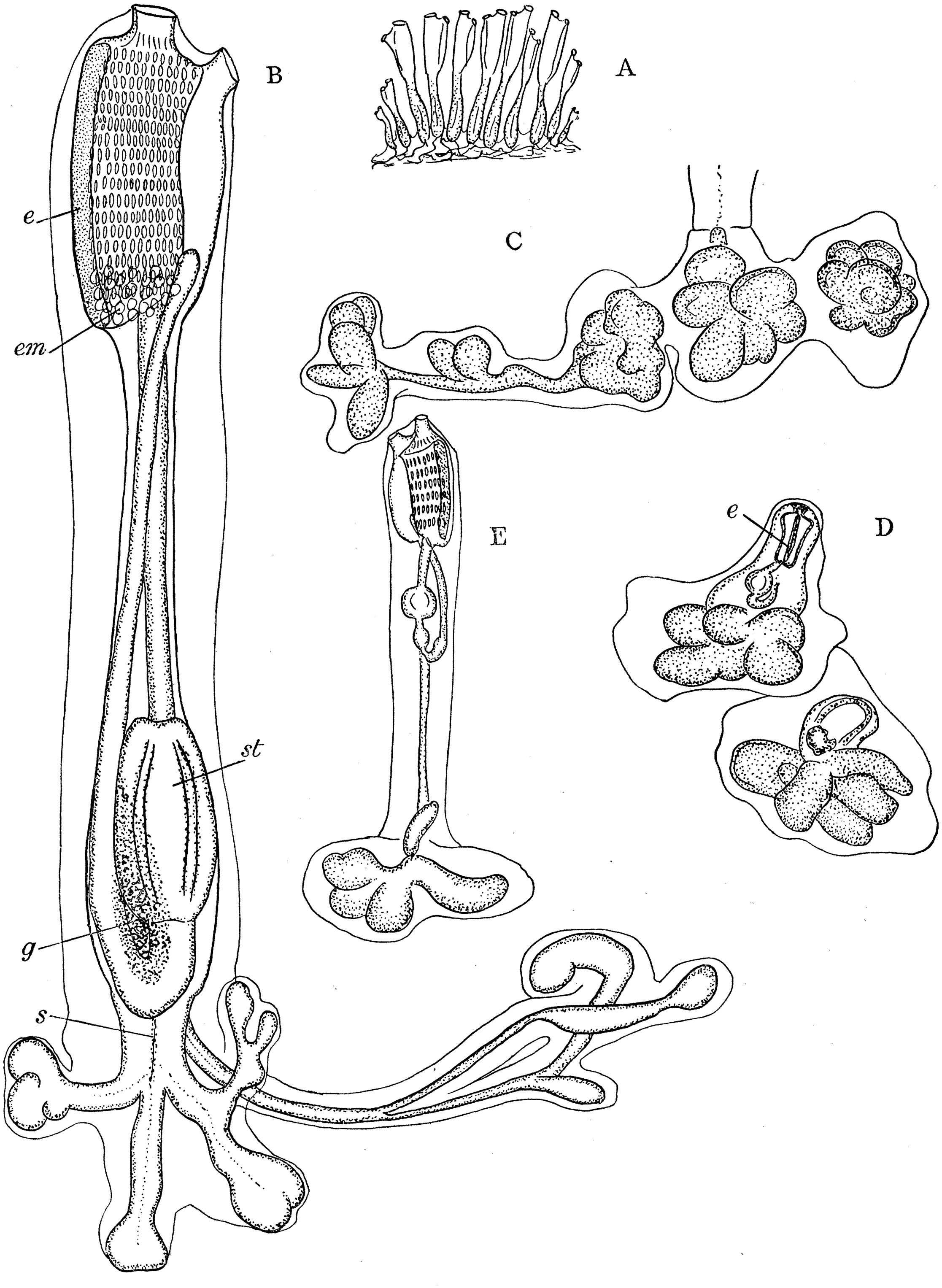

FIG. 12—*Clavelina lepadiformis* MÜLLER. A, colony; B, mature zooid showing hypertrophied ventral vessel; C, constriction of ventral vessel following degeneration of zooid to form bud masses; D, development of new zooids from single lobe of each of two isolated parts of ventral vessel; E, later development of bud; *e*, endostyle; *em*, embryos; *g*, gonads; *st*, stomach; *s*, septum of vascular stolon

Each isolated mass may consist of several connected lobes, but only one lobe ever develops to form a new individual, the rest being subordinated and serving as nutrient matter. Three cell-types are present. Epidermis forms new epidermis; trophocytes supply or maintain an internal nutrient medium, but according to BRIEN play no part in morphogenesis; while cells of the vascular mesenchymatous septum proliferate and give rise to all the tissues of the new zooid with the exception of the epidermis.

Species—*Clavelina picta* VERRILL. Fig. 13

Source of material—Collected during winter months at Bermuda.

Remarks—The zooids of *Clavelina picta* are almost identical with those of *Clavelina lepadiformis*. The main difference between the two species lies not in the nature and structure of the zooids but in the manner in which they form a colony. It will be seen from fig. 13 that the ventral stolonic vessel is not only hypertrophied as in *Clavelina lepadiformis*, but is also branched, so that some branches pass anteriorly. These anterior vessels secrete test material and unite the zooids within a common mass, while other branches posterior to the zooids form a thick stalk. When buds are formed they lie therefore within the common mass of test and there is no tendency for them to become separate as in *Clavelina lepadiformis* and other forms.

Reproductive cycle—The sexual breeding season alternates with the period of asexual reproduction as in many other forms. The alternation differs only from such species as *Clavelina lepadiformis*, *Cl. oblonga*, and *Cl. huntsmani*, inasmuch as bud formation during the breeding season is of rare occurrence and the colonies are consequently much more uniform at any given period of the year.

The breeding season begins about midsummer and lasts for two months or so, while the process of bud formation is a relatively slow and prolonged process, lasting through most of the winter, bud development starting in early spring.

Formation of buds—During the early winter months the mature zooids gradually regress, accompanied by a migration of trophocytes into the stolonic vessels. The zooids are not sloughed off, except in rare cases, as in *Clavelina lepadiformis* following the migration of trophocytes. Usually the whole zooid undergoes autolysis, with the exception of the trophocytes themselves and of the stolonic vessels. Sometimes the autolysis or regression remains incomplete and the zooids may survive as relatively small compact masses—still in connection with the stolonic vessel, fig. 13 B. As regression proceeds the colony shrinks in size and the surface of the test becomes smooth and hard, and eventually is found to contain only stolonic vessels congested with trophocytes and occasional reduction masses of zooids themselves.

Development of buds—As in *Clavelina lepadiformis*, the development of bud masses begins only after connection with the parent zooid has taken place. In that species separation is sudden and development may commence even before the summer has passed. In *Clavelina picta* the prolonged process of regression delays the liberation

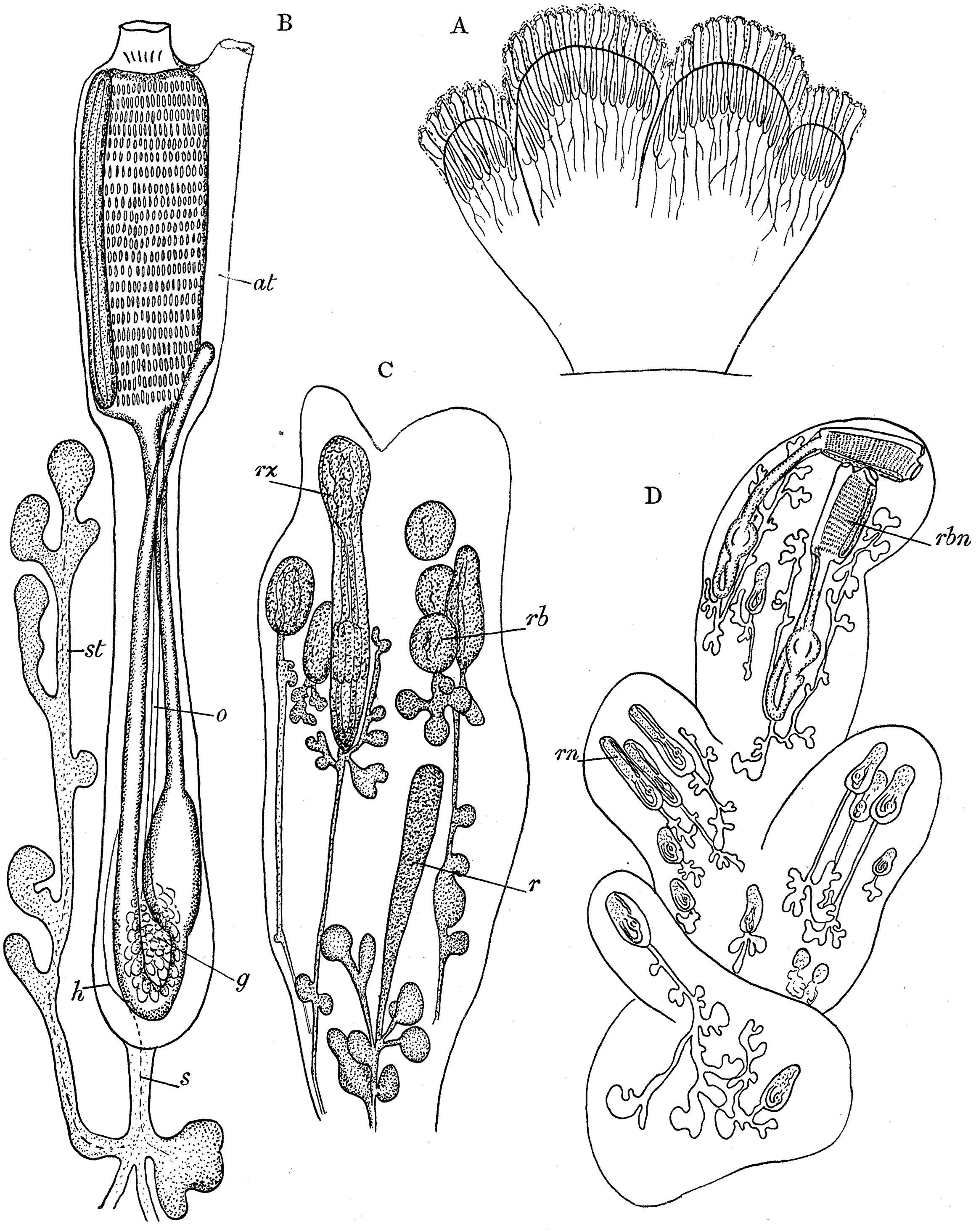

FIG. 13—*Clavelina picta* VERRILL. A, colony (continuous dorsal line represents level of thorax, which is often absorbed and reformed); B, mature summer zooid showing hypertrophied ventral vessel with anterior branches; C, part of colony collected in December, showing regression of zooids and opacity due to trophocytes; D, part of colony collected in March, showing bud development; *at*, atrial cavity; *g*, gonad; *h*, heart; *o*, oviduct; *r*, completely reduced zooid; *rb*, reduction body; *rbn*, zooid from reduction body; *rn*, zooid from stolon only; *rz*, partially reduced zooid; *s*, septum; *st*, stolon

of the buds and development hardly begins until the early spring. That the delay is due to the inhibiting presence of the parent zooids is shown by the fact that a piece of test sliced off from a sexually mature summer colony and containing only ampullae of the stolonic vessels is able to develop into new zooids at once. As in other species of *Clavelina*, epidermis forms new epidermis, the proliferating cells of the vascular septum give rise to all the other tissues of the new individual, and the trophocytes serve as nutrient matter. The zooids developing from the incompletely regressed zooids are at first much larger than those from the stolonic ampullae. In the latter type of development, where several ampullae are united together, only one develops, the rest apparently being inhibited.

Species—Chondrostachys cylindrica Q and G. Fig. 14

Source of material—British Museum.

Remarks—Chondrostachys is an Australasian genus and little is known of its reproductive cycle. Mature zooids resemble closely those of *Clavelina*, both in structure and size. They produce relatively fewer embryos at a time and there is a tendency for the posterior part of the atrial chamber to bulge outwards and form a distinct brood sac.

Formation of buds—The stolonic vessel is hypertrophied and fertile as in *Clavelina*, and trophocytes tend to accumulate in the terminal ampullae. The ampullae, however, are relatively very numerous and as many as fifty may form as a cluster in connection with a single vessel. Only when the vessel connecting the cluster with the parent zooid becomes detached does bud development begin, and again, as in *Clavelina*, only a single ampulla of a cluster develops, the rest being inhibited and their contents serving as nutriment to the one developing to form a new zooid.

The buds, while at first enclosed within the common mass of test, eventually grow and extend from the general surface and remain attached only by narrow stalks.

Species—Pycnoclavella aurilucens GARSTANG. Fig. 15

Source of material—Plymouth (Stoke Point and Mewstone Ledge) at various times of the year.

Remarks—Pycnoclavella is known only from the Plymouth district, and its structure, development, and budding are comparatively unknown. It is usually placed within the Clavelinidae, partly because of the free growth of the zooids as in *Clavelina lepadiformis*, a character that is now known to be of little significance. The structure of the zooid itself is shown in fig. 15 B, and while much smaller than *Clavelina* zooids it will be seen to differ in one more important respect. The ventral stolonic vessel is small and unmodified, resembling much more that of *Diazona*, synoicids, and especially the distomids *Eudistoma* and *Archidistoma*. It is, moreover, sterile, and altogether there is considerably more reason to include *Pycnoclavella* among the Distomidae than among the Clavelinidae.

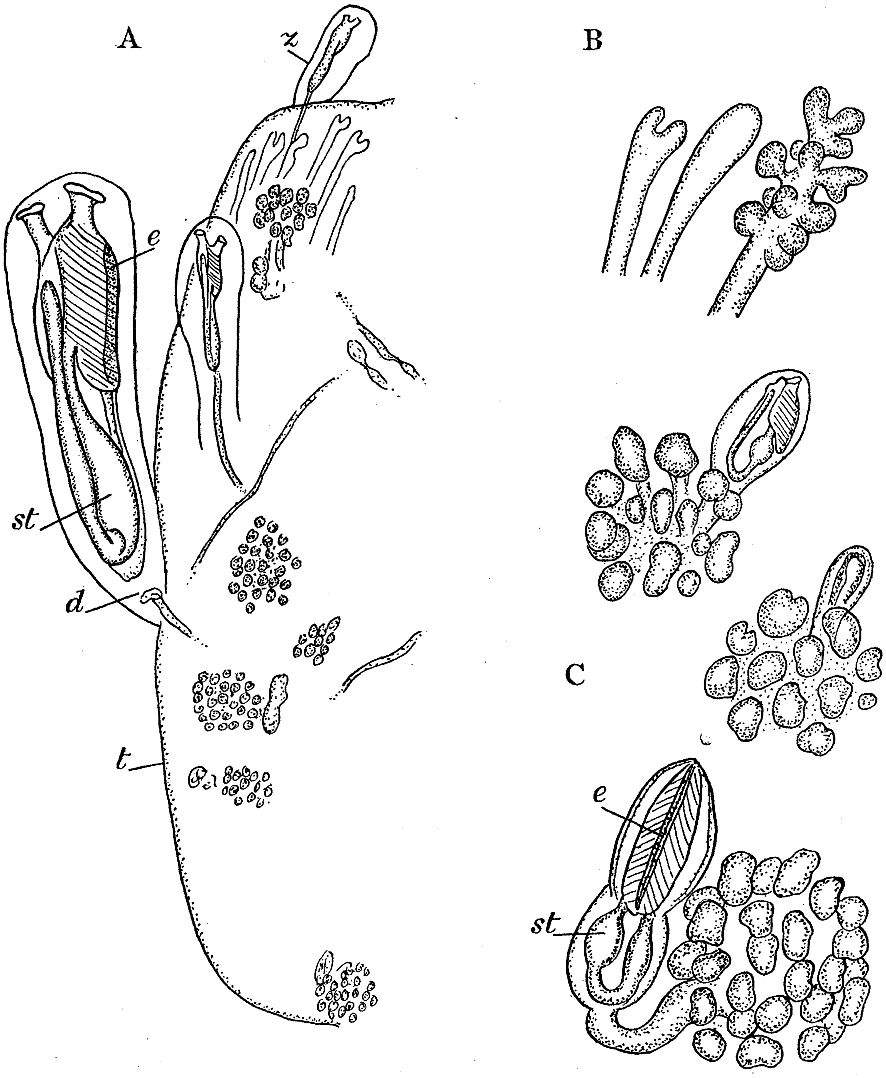

FIG. 14—*Chondrostachys cylindrica* Q and G. A, part of colony showing growth of zooids from general surface, and ampullary clusters of ventral vessels within ; B, terminal vessels from anterior part of colony showing lateral outgrowths ; C, development of bud from a single ampullary outgrowth in each cluster ; *d*, disjunction between zooid and stolon ; *e*, endostyle ; *st*, stomach ; *t*, general test surface of colony ; *z*, young zooid extending from general surface with stolonic attachment still intact

Reproductive cycle—The sexual breeding season begins in July or August and lasts for about six weeks. Asexual reproduction, as in most compound ascidians, is a winter phenomenon.

Formation of buds—In spite of an investigation of *Pycnoclavella* colonies taken at short intervals throughout the year, only a partial view of the process of budding

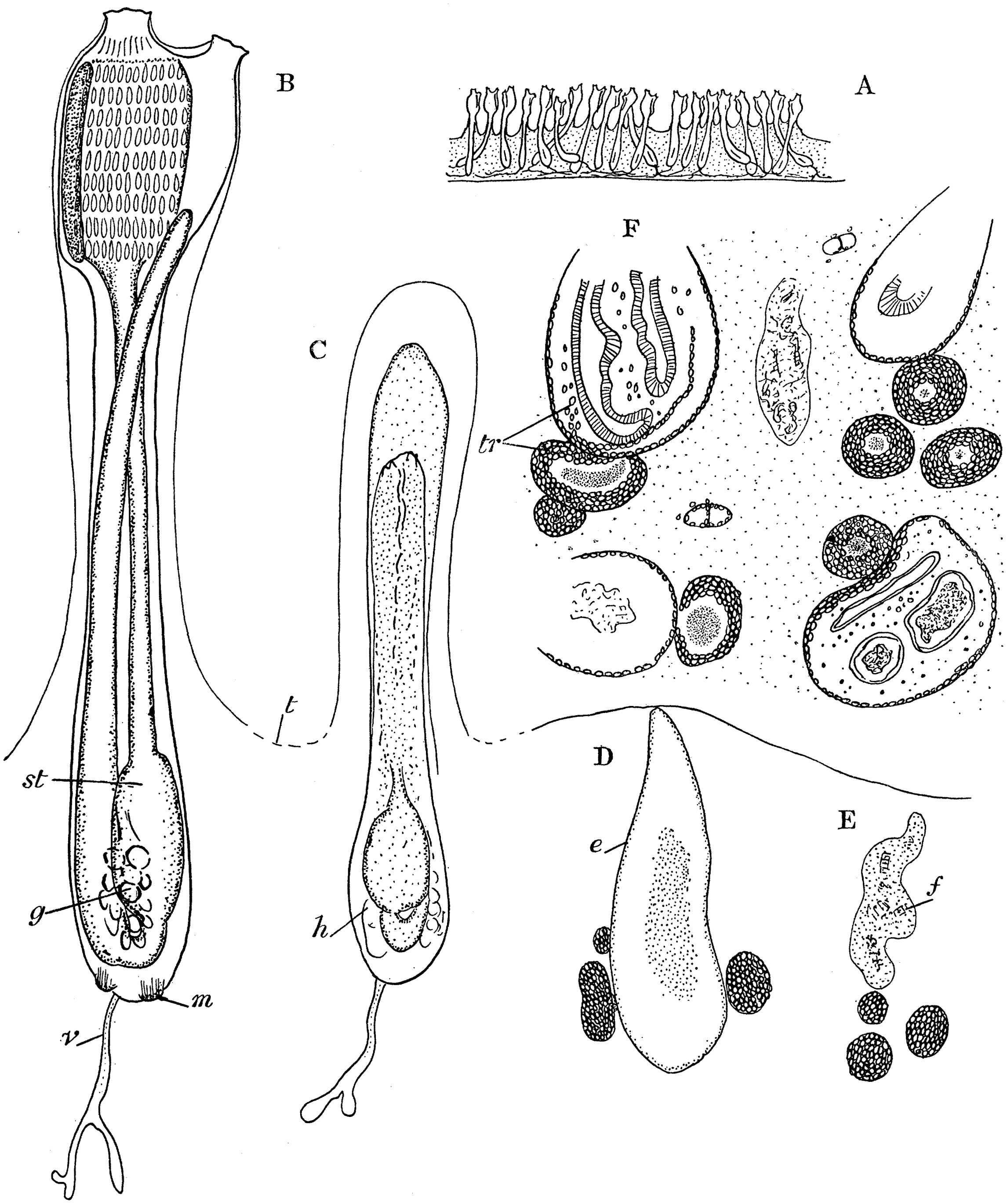

FIG. 15—*Pycnoclavella aurilucens* GARSTANG. A, colony, 1 cm high; B, mature zooid showing position relative to general surface of colony; C, zooid beginning to regress anteriorly; D, zooid regressed and autolysed with exception of epidermis, faeces, and lateral bud masses; E, final stage of zooid consisting of masses of trophocytes (buds) and faeces; F, section through base of winter colony showing formation of spheroidal masses of trophocytes outside zooid in colony test; *e*, epidermis; *f*, faecal matter; *g*, gonad; *h*, heart; *m*, insertions of longitudinal muscles; *st*, stomach; *t*, general test surface of colony; *v*, vascular stolon with septum

has been obtained, but this as far as can be determined is unique. The early stages of the process resemble those of many other forms. There is a degeneration of the mature zooid following the close of the breeding season and apparently brought about by the exhaustion of the tissues. Autolysis seems to occur and gradually the greater part of the zooid disappears, commencing anteriorly and progressing posteriorly. At the same time cells that can be looked upon only as trophocytes accumulate in large numbers at the posterior ends of the zooids. As the stolonic vessels are small and primitive there is no opportunity for the trophocytes to enter and congest them as in *Clavelina*. The process thus far is similar to that of the majority of forms so far described. There is a degeneration of the sexually exhausted zooid accompanied by a proliferation and posterior migration of trophocytes. The rest of the process is unique and unexpected. The autolysis of the zooid proceeds until not a trace remains of zooid or stolonic vessel except the undigested faecal remains, test substance closing in upon it as the soft tissues disappear. The only surviving substances seem to be the trophocytes, which, as the epidermis dissolves, come to lie outside the zooid as a whole. In particular, one or two spheroidal masses of trophocytes aggregate near the posterior end of the zooid, usually with sufficient connection to show their origin. As neither these masses nor any others have been seen to develop into new zooids, the evidence is not complete that they are the buds that will form the succeeding colony; but there seems to be no alternative. There is no epidermis surrounding them as in all bud masses of other forms, and at first there seems to be no other tissue connected with them that might take the place of the vascular septum or epicardial lining. Any cells present in the mass other than the yolk-packed trophocytes must either have migrated from the zooid at the same time or must be the test-secreting mesencyhme cells that are scattered through the test.

In any event, there appears in the centre of each ball of trophocytes a comparatively clear space which, in stained preparations, has the appearance of a mass of protoplasm containing numerous very minute nuclei. If nuclei they are so small that little or no structure can be observed, while cell boundaries between them have also not been seen. At the same time the central mass of clear protoplasm and nuclear material grows steadily in size at the expense of the surrounding layer of trophocytes, and while no morphogenesis of a new zooid was observed, these peculiar bodies seem to be the only possible source.

IX—BUDDING IN THE PEROPHORIDAE

The family Perophoridae contains the three genera *Perophora*, *Perophoropsis*, and *Ecteinascidia*. They differ from one another mainly in the size of the constituent zooids, length of the stolonic vessels, and in the number of rows of stigmata, this last character being an index of body-size throughout the Ascidiacea. *Perophora* species have four or five stigmata rows, *Perophoropsis* about ten, and species of

Ecteinascidia from fifteen to thirty. Otherwise the structure of the zooids and their mode of budding in the three genera may be discussed as one.

Species—*Perophora listeri* FORBES. Fig. 16 A
Perophera viridis VERRILL. Fig. 16 A
Perophora bermudensis BERRILL. Fig. 16 A
Ecteinascidia conklini BERRILL. Fig. 16 B, C
Ecteinascidia turbinata HERDMAN. Fig. 16 D

Source of material—*Perophora listeri* was examined at Plymouth, the other four species at Bermuda.

Remarks—Compared with the zooids of all the families previously discussed, those of the *Perophoridae* have undergone a profound change. There has been no dislocation of the heart and gonads to form a post-abdomen as in the *Synocidae* and *Euherdmania*, but the whole of the ventral stalk contents, in other words the intestinal loop, gonads and heart, have been moved anteriorly to lie at the sides of the thorax. The intestinal loop and gonads are now found lying along the left side of the branchial sac, the heart at the base and along the right side of the sac. The ventral stolonic vessel thus arises directly from the base of the thorax (fig. 16 E), and with its surrounding layer of test it is the only connection between adjacent zooids. The vessel itself is hypertrophied, as in *Clavelina*, *Colella*, and *Distaplia*, and like the two former genera is fertile.

Reproductive cycle—The sexual breeding season in all forms begins in the spring or early summer and lasts for about three months. Budding takes place to some extent throughout that period, but eventually the zooids of the colony slough off, leaving attached stolonic vessels that may survive through the winter. Most colonies, however, live for one or two years only, and the young oozoids born during the summer bud rapidly until the following spring, when the process dwindles and the zooids attain sexual maturity.

Formation of buds—Bud formation in *Perophora* has been the subject of considerable investigation, and in recent years especially by BRIEN (1927). Unlike *Clavelina*, the stolons of *Perophora* and of *Ecteinascidia* are capable of developing into new zooids while yet in organic continuity with the parent zooid. The epidermis hypertrophies locally at some distance from both the zooid and the tip of the stolon, while the cells of the vascular septum proliferate and form a hollow vesicle within the epidermal bulge. The process is thus similar to that of *Clavelina* except that there is no physical separation from the parent zooid, and there is no accumulation of trophocytes since the blood circulation in the stolon produced by the parent zooid supplies ample nutrition. Under adverse environmental conditions, according to J. S. HUXLEY (1921), the new zooid may grow at the expense of the old, in other

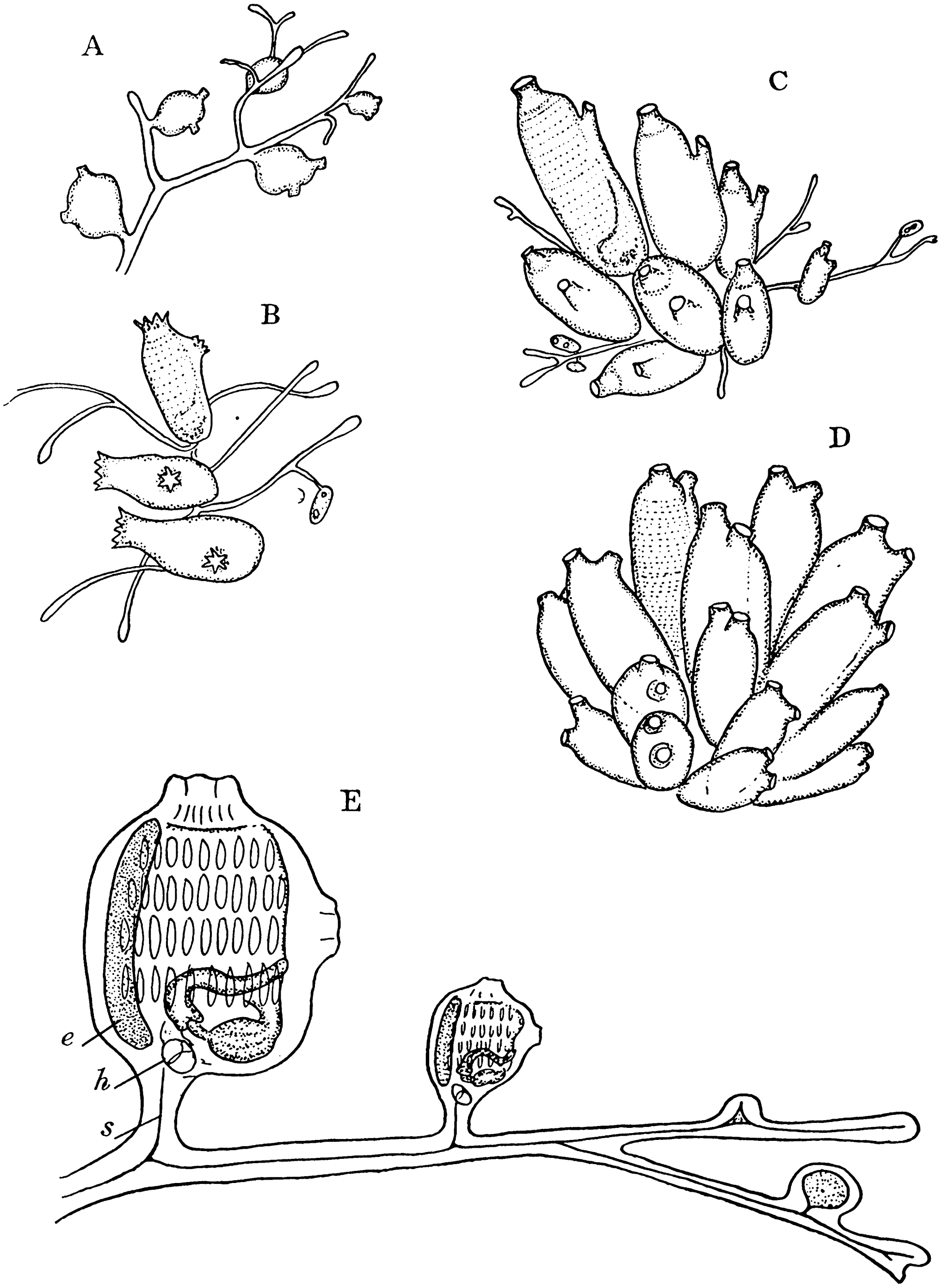

Fig. 16—*Budding in the Perophoridae.* A, part of colony of *Perophora bermudensis:* B, part of colony of *Ecteinascidia conklini minuta;* C, small colony of *Ecteinascidia conklini typica;* D, part of colony of *Ecteinascidia turbinata*, showing length of ventral stolonic vessels and influence on appearance of colony; E, *Perophora*, showing relation of buds to parent zooid, stolonic vessel, and vascular septum; *e*, endostyle; *h*, heart; *s*, septum of vascular stolon

circumstances the parent zooid may assert itself to such an extent that the bud is reabsorbed.

Two cell-types only are concerned in the development of the new individual. Epidermis forms new epidermis, the mesenchyme septal cells form everything else.

X—Budding in the Styelidae, including Botryllidae, Fig. 17

The Styelidae and also the non-budding orders Pyuridae and Molgulidae of the order Stolidobranchiata are widely separated on morphological grounds from the families so far discussed. As in the Perophoridae, the gut loop has been reflected anteriorly and lies along the left side of the branchial sac. But additional morphological differences separate these families even from the Perophoridae. The epicardium has become the renal vesicle and the mesenchyme septum of the test blood vessels has been lost. The large ventral stolonic vessel, in fact, no longer exists. So that any process of budding among these families that there may be is by the nature of the individual precluded from involving a post-abdomen, epicardial epithelium or vascular septum. Budding, in fact, in the only family with that faculty in the Stolidobranchiata is without doubt a re-acquirement and is markedly different from any of those methods so far described. At the same time among the Styelidae and the subfamily Botryllidae the mode of budding is uniform and yet so different from other types that the conclusion is unavoidable that the re-acquisition has occurred but once only.

The differences within the family are slight and concern for the most part rate of budding, length of stolons, etc., which while virtually without significance do have a very marked influence on the appearance of the colony as a whole and on the size of its constituent zooids.

Of the species examined *Polyandrocarpa tincta* forms colonies of four or five zooids so close that their outer walls are in close contact. Apart from the faculty for budding, the zooids are almost indistinguishable from the solitary individuals of the genus *Polycarpa*. In *Distomus variolosus* and *Stolonica socialis* budding is more rapid, with the result that the colonies are composed of smaller but much more numerous zooids. In *Symplegma viride* the zooids are smaller and more numerous again, and apart from the fact that each zooid has an atrial siphon opening independently to the exterior, they resemble closely colonies of *Botryllus* or *Botrylloides*. The zooids of the last two genera have atrial apertures opening into common cloacal cavities instead of independently to the exterior, and the zooids in consequence become arranged in systems.

Species—*Polyandrocarpa tincta* Van Name. Fig. 17 A
Stolonica socialis Hartmeyer. Fig. 17 B, J, K
Distomus variolosus Gaertner. Fig. 17 C, H, I
Symplegma viride Herdman. Fig. 17 D, E

Source of material—*Stolonica* and *Distomus* were obtained during summer months at Plymouth, *Symplegma* and *Polyandrocarpa* also in summer months at Bermuda.

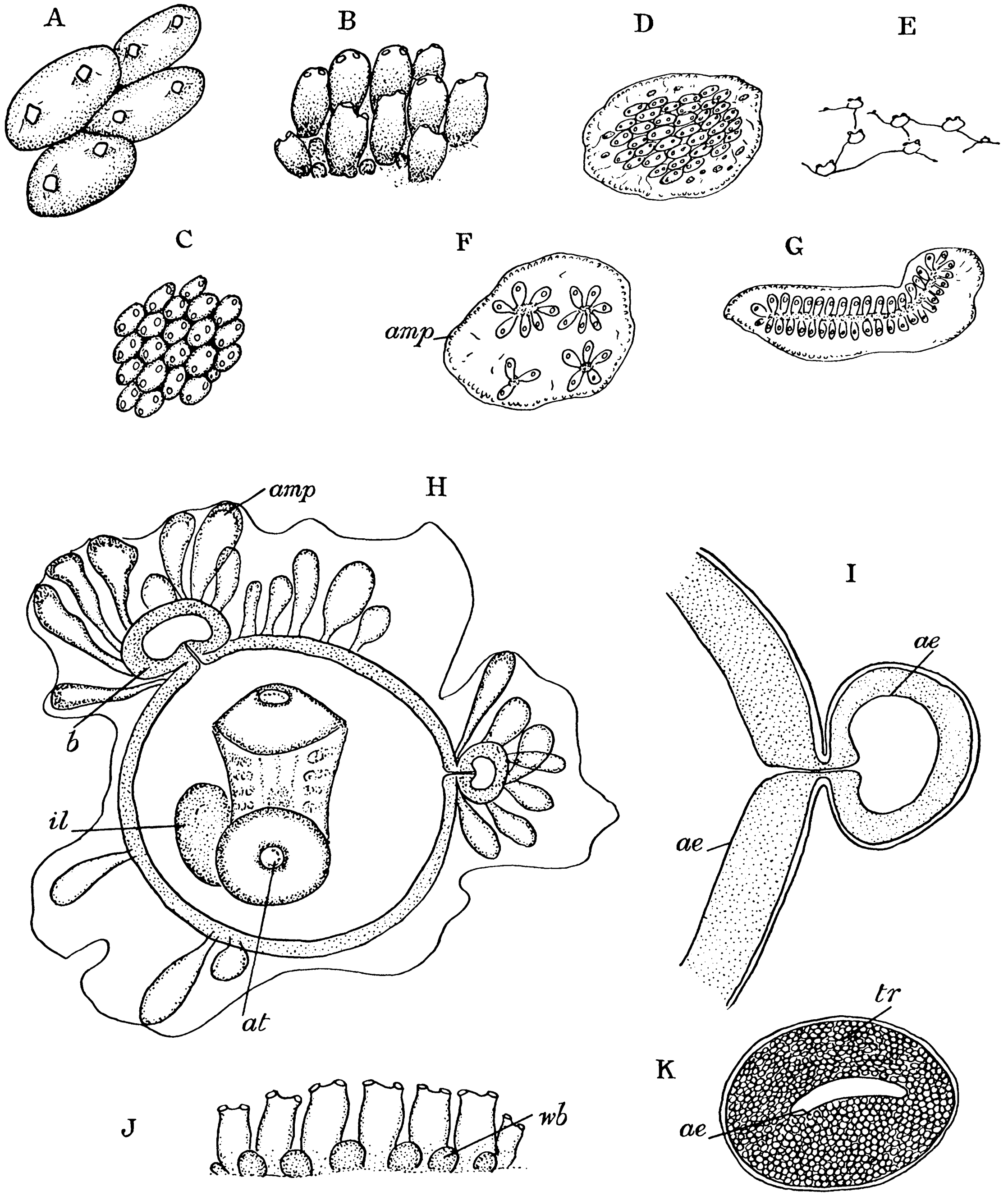

FIG. 17—*Budding in the Polystyelinae.* A–G, colonies slightly smaller than natural size ; A, *Polyandrocarpa tincta :* B, *Stolonica socialis* ; C, *Distomus variolosus :* D, *Symplegma viride :* E, *Symplegma viride stolonica :* F, *Botryllus schlosseri :* G, *Botrylloides leachii* ; H, young zooid (ten weeks) of *Distomus variolosus* showing two marginal buds amid clusters of vascular ampullae ; I, optical section through bud showing connection with epidermis, mesenchyme, and atrial lining of parent ; J, *Stolonica socialis* showing relative size of winter buds or statoblasts ; K, section through winter bud showing congestion with trophocytes, and cavity originally continuous with atrium of parent ; *ae*, atrial epithelium ; *amp*, epidermal ampulla ; *at*, atrial siphon ; *b*, bud ; *il*, intestinal loop ; *tr*, trophocytes ; *wb*, winter bud

Remarks—The mode of budding is so similar in all the above genera that a description of but one, *Distomus*, will suffice.

Formation of buds—There is no abdominal stalk as in *Diazona*, no post-abdomen as in the Synocidae, so that methods of budding involving such structures are not possible. There is no vascular stolon with a mesenchymatous septum, nor an epicardium with an undifferentiated epithelial lining, so that those two sources of totipotent cells no longer exist. The order as a whole, however, is composed of forms attached by the posterior surface, and since the gut-loop, gonads, and heart have all been shunted forwards, the posterior peripheral margin of the atrial cavities come into contact with the substratum, and their walls tend to grow radially outwards from the zooid and anchor it more firmly. Thus it is understandable that it is the lateral basal margin that becomes concerned in the new method of budding.

In *Distomus*, as in the other three genera, the outer wall bulges out in one or several places, each protrusion consisting of three layers, an outer epidermis, an inner layer continuous with the lining of the atrial cavity, and an intermediate mesenchymal layer. In *Distomus*, *Polyandrocarpa*, and *Symplegma viride*, the junction remains short and the buds develop in close contact with the parent. In *Stolonica* and in *Symplegma viride stolonica* the junction between parent and bud grows to a variable length and the zooids of a colony may be widely separated. In fig. 17 H is shown an oozoid of *Distomus* two months after settling, with two marginal buds being formed. The oozooid of *Stolonica* behaves in a similar manner. The mature zooids of *Stolonica* also produce marginal buds, but differ in that the mesenchymal layer becomes enlarged and congested with trophocytes. Such buds survive after the parent zooids have been sloughed, and form winter statoblasts. A cross-section of one of these winter buds is shown in fig. 17 K, but for further details and information the reader should consult the monograph by DE SELYS-LONGCHAMPS (1917).

Development of buds—The epidermis forms new epidermis, the mesenchymal layer such tissues as muscle, blood, heart, connective tissue, etc., while the nervous system, branchial and digestive tract, are formed from the inner or atrial layer. A new source of totipotent cells thus emerges, namely, the lining of the outer wall of the atrial cavity.

Species—*Botrylloides leachi* SAVIGNY. Figs. 17 G, 18 A–D
Botryllus schlosseri (PALLAS) var. *polycyclus*. Fig. 17 F

Source of material—Collected during summer months at Plymouth.

Remarks—The method of budding in these two genera is in all essentials similar to that of the Styelids proper. In the Styelids there is a fairly well-defined alternation between sexual and asexual phases more or less corresponding to summer and winter, although in *Symplegma* the asexual phase persists to some degree throughout the year. In *Botryllus* and *Botrylloides*, the alternation between phases is even more definite, but during the warmer half of the year at least there may be several, if not many, alternations in one and the same colony.

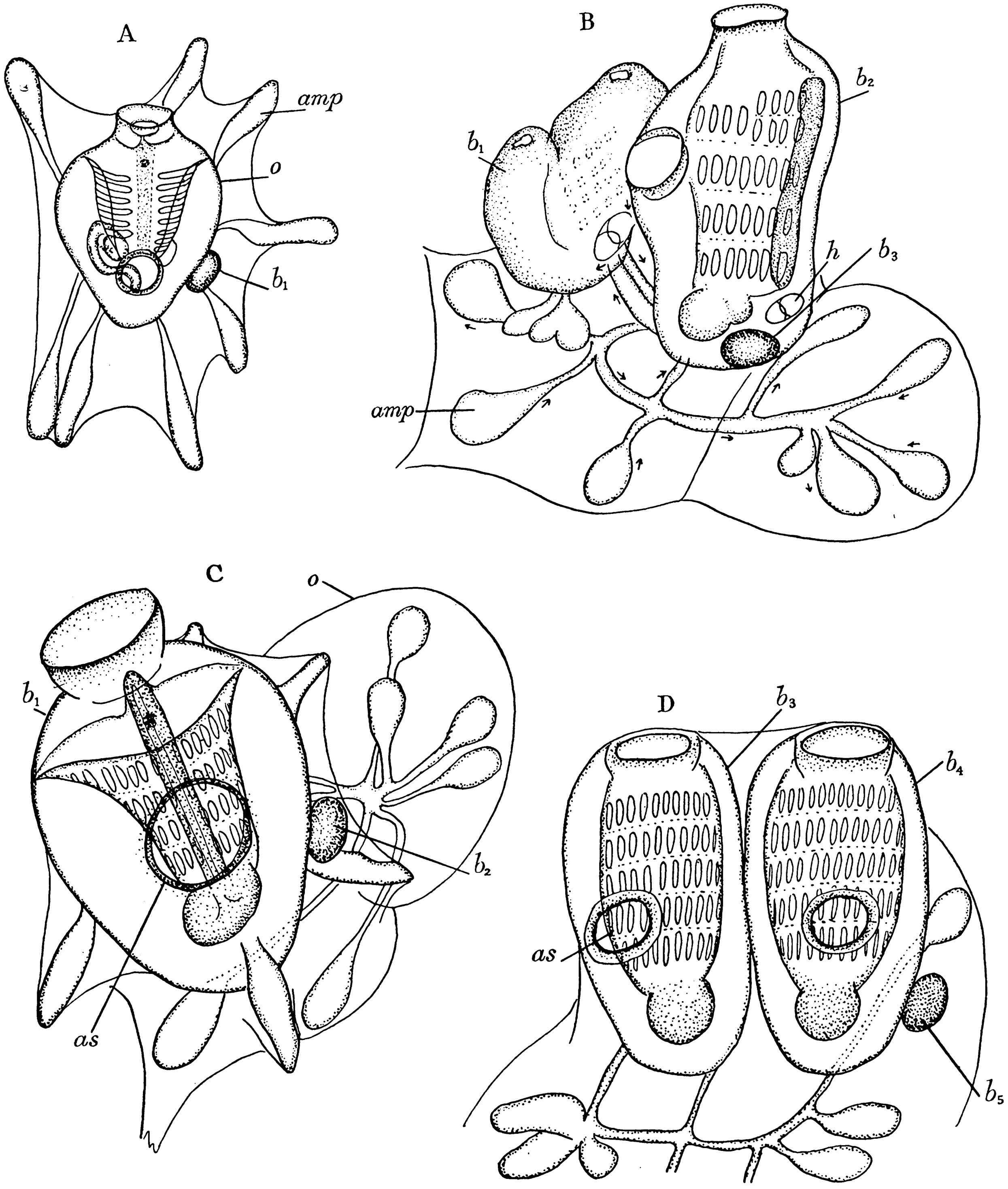

FIG. 18—*Betrylloides leachii*. First four generations of buds—A, oozooid with lateral bud on right side ; B, degenerated oozooid (only vascular ampullae surviving) with fully developed first bud, second bud showing on its right side ; C, second bud fully developed with third bud on its right side, and degenerating first bud on its left side ; D, third and fourth buds surviving side by side, with fifth bud on right side of fourth ; *as*, atrial siphon ; *amp*, epidermal ampulla ; *b*, 1st blastozooid ; b_{2-5}, 2nd, 3rd, 4th, and 5th blastozooids ; *h*, heart ; *o*, oozooid

Formation of buds—The method of budding in the adult colonies has been the subject of many studies, Pizon (1893), so that the illustrations shown in fig. 18 represent a somewhat different aspect, namely, the first few bud generations of the oozooid of *Botrylloides*. The sequence and time intervals in *Botryllus* and even the arrangement of the zooids at this stage, are so similar that it would not be possible to determine which genus was being observed if it were not definitely known. A single bud grows out from the right side of the oozooid and after growing very slowly for about a week becomes completely developed in the next three days, the oozooid being absorbed in the process. The first bud after several more days forms and gives place to the second bud in much the same way, and so in turn the second is replaced by the third. Only then is it found that the new bud can grow without necessarily absorbing the previous bud generation, and the third and fourth buds function together. There is very obviously a struggle for the available material.

As in the Perophoridae, the constituent zooids of a colony are united together by the blood vessels of the test and have a circulation in common. In fact, the colony in botryllids is more truly a super-organism than is that of any other sessile ascidian.

XI—Discussion

Budding in ascidians has been the subject of many papers and of much controversy. Outstanding are the early investigations of Hjort (1896) and of Pizon (1893), in later years of de Selys-Longchamps, and the recent series by Brien (1930). There should also be mentioned the theoretical discussion by Garstang (1928) in his paper on the morphology of the Tunicata. To a greater or lesser extent two problems have dominated or influenced all such investigations, namely, the significance of the germ layers and of the epicardium. Only in the accounts of Brien do these questions become relatively unimportant and histogenesis and morphogenesis loom large.

A discussion of the views expressed by the above-mentioned authors will be left until the various phenomena described in the preceding pages have been brought together and summarized. The addition of several new types of budding to those previously known radically alters the situation, especially as some of these may be considered as more primitive than any others.

Reproductive Cycle

In all the forms examined the phases of maximum sexual and asexual reproduction alternate, although in some, such as *Clavelina*, *Botryllus*, *Perophora*, etc., asexual reproduction is not completely suppressed during the sexual breeding season. The main factor in this separation in time of the two modes of reproduction is that usually the asexual type involves structural changes in the zooid that interfere with its normal functioning and especially with the production and passage of eggs.

As a rule, asexual reproduction occurs during winter months, sexual reproduction during summer months, a correlation typical of most groups in possession of both reproductive faculties. But it can be demonstrated that, while the approximate confinement of reproduction by budding to the winter may best serve the survival needs of a colony, the factor responsible for its commencement is not the falling temperature or diminishing food supply of autumn but the internal condition of sexual exhaustion. The sexual breeding season, as in *Morchellium*, *Sidnyum*, *Clavelina*, etc., may end while the external temperature is still rising and the food supply ample, yet asexual reproduction begins as soon as the other wanes.

In those forms in which budding interferes in no structural way with the integrity of the zooids, as in *Perophora*, *Ecteinascidia*, *Symplegma*, and *Botryllus*, reproduction by budding is always in process, although it may be relatively subdued, probably as a result of the drain upon nutritive resources by the developing germ cells. J. S. Huxley (1921) has shown that there is a genuine struggle for domination by the parent zooid and the young buds connected with it by the vascular stolon. In Botryllus, though not apparently in other polystyelids, the later development of the buds involves a degeneration of the preceding generation of blastozooids, the old material being needed for the growth of the new. This struggle between successive bud generations in *Botryllus* and *Botrylloides* is especially evident in the first few generations arising from the oozoid.

Colonies of *Botryllus*, *Distaplia*, and *Diplosoma* taken during summer months may be in any state of either asexual or sexual reproduction and it is evident that several alternations may occur within the year.

The above observations apply for the most part to fully formed colonies, but it seems probable that in all ascidians able to bud, as also in the polychaete *Filograna* (Faulkner, 1930), the oozooid forms a colony by budding before there is any tendency to become sexually mature. This may be because low winter temperatures inhibit sexual development but not asexual reproduction. On the other hand, the culmination of the developing egg may be the colony and not the single zooid, and sexual maturity a character of the colony as a whole and not of the oozooid.

Regression

There is a marked tendency among most ascidians able to bud for the anterior end of the zooid to undergo regression following the end of the sexual breeding season. This may occur in many forms whenever the environmental conditions become in any way adverse, but in general it seems to result from the state of exhaustion connected with breeding. The regression sometimes involves not only the thorax but even the whole of the zooid, as in *Distaplia*, *Pycnoclavella*, and *Clavelina*.

Thoracic regression alone occurs in *Diazona*, *Archidistoma*, and sometimes in *Euherdmania* and in *Diplosoma*. In the synoicids, in *Tylobranchion* and usually in *Euherdmania*, all forms with a post-abdomen, regression of the oesophagus and abdomen also occurs. The degeneration of the zooids of Botryllids is not so much a regression as an absorption of the tissues by the developing buds, as J. S. Huxley has shown to occur under certain conditions in the *Perophora*.

Where it occurs at all, regression of a zooid commences at the anterior end of the thorax and gradually proceeds posteriorly. There is a very definite antero-posterior gradient.

The regression of the zooid may be a direct result of a state of sexual exhaustion, but it also seems to be the preliminary to the phase of asexual reproduction. This, however, is understandable if the regression is considered to be a destruction of organization and the asexual reproductive processes a modified regeneration. At the same time, one result of regression is to produce a rich nutrient medium for any such reproductive activity that may be latent. There may be a complete breakdown of the tissues by autolysis, as in *Distaplia* where it precedes bud development and as in *Botryllus*, where it coincides with bud development. Or, as regression continues, certain cells become distended with yolky materials and migrate from the degenerating tissues to become stored throughout the posterior region of the zooid. These are the trophocytes, and in *Diazona* and in *Archidistoma*, where there is neither a post-abdomen nor an enlarged stolonic vessel, the abdominal and oesophageal regions become congested with them. Where there is a post-abdomen, as in *Tylobranchion*, *Euherdmania*, and the synoicids, it is that region that receives them, thus allowing the abdomen to undergo regression as well as the thorax. In the clavelinids there is no post-abdomen, but the stolonic vessel is hypertrophied and the trophocytes are able to enter it, again allowing the abdomen to regress. The trophocytes always migrate posteriorly until a physical barrier at the posterior end of the zooid intervenes. In *Pycnoclavella*, a form without post-abdomen or hypertrophied stolonic vessel, the trophocytes accumulate in the abdominal region, but as this part undergoes regression they migrate into the surrounding test and form rounded masses.

The various nutritive mechanisms associated with bud development are accordingly as follows :—

(*a*) autolysis without trophocyte formation	*Distaplia.* *Colella.*
(*b*) autolysis with trophocyte formation and migration preceding bud development.	*Diazona.* *Tylobranchion.* *Archidistoma.* *Euherdmania.* *Synoicidae.* *Clavelina.* *Chondrostachys.* *Pycnoclavella.*
(*c*) direct connection with parent zooid—	
(1) by ventral blood vessels	*Perophora.* *Ecteinascidia.*
(2) by atrial wall with included blood vessels	*Botryllus.* *Symplegma.* *Polystyelids.*
(3) within parent	*Diplosoma.*

In *Eudistoma* there is no visible evidence of either autolysis or trophocyte formation, merely a temporary condensation of the tissues followed by an expansion and apparent rejuvenation.

Isolation of Buds

In all animals in which budding occurs, the bud is a fragment which has in some way been isolated from the whole organism and which is capable of regenerating the missing parts. The isolation may be physical or physiological, and among ascidians both methods are to be found.

From the foregoing descriptions, it seems evident that the principal agent in the isolation of parts of a zooid to form buds is the epidermis. In *Diazona* and *Archidistoma* epidermal constrictions appear in the abdominal and oesophageal regions, and it was shown that the epidermal cells actively change in shape and size during the constricting process. The constrictions deepen until the zooid becomes completely divided. In *Morchellium*, *Sidnyum*, *Aplidium*, and other synoicids, and also apparently in *Tylobranchion* and *Euherdmania*, epidermal constrictions also develop, but in the post-abdominal region rather than in the abdominal. In *Eudistoma*, constrictions are also to be found, but only one divides the zooid completely, between the thorax and oesophagus, though not even that invariably happens. In *Colella*, epidermal constrictions divide neither abdomen nor post-abdomen but the ventral stolonic vessel. Also in *Clavelina* it is the activity of the epidermis that results in the separation of the stolonic vessel, and its ampullae from the remainder of the zooid. Thus in *Diazona*, *Archidistoma*, *Eudistoma*, *Morchellium*, *Aplidium*, *Sidnyum*, *Colella*, and *Clavelina*, isolation of buds is brought about by transverse epidermal constrictions akin to strobilization. In *Distaplia* and *Diplosoma* a proliferation of cell masses from the epicardia coincides with an associated extension of the epidermis; but it is difficult to say whether one activity induces the other, or whether some more obscure factor is responsible for both. In *Diplosoma* the bud masses are able to develop without physical isolation from the parent, though with the result that the presence of the original zooid limits the extent of reorganization. In *Distaplia* the buds are removed from any parental influence by the complete degeneration of the original zooid. It is a striking fact that the autolysis of the zooid as a whole does not involve the epicardial cell masses. Similarly in *Pycnoclavella* the masses of trophocytes survive the general autolysis following degeneration. In *Perophora* and *Ecteinascidia* the ventral stolonic vessel is long and branched, and buds are formed by it while yet physically united to the parent zooid. Isolation must accordingly be physiological rather than physical. A proliferation of epidermal and mesenchyme cells to form a bud rudiment occurs always at some distance from the zooid, later buds developing from the growing stolon at similar distances from one another. In the perophorids the buds never form from the stolon tip, but in the botryllids and polystyelids they develop always from the terminal bulge of the atrial outgrowth, whether the outgrowth is so short that the bud is in contact with the parent, as in *Botryllus* or *Polyandrocarpa tincta*, or at the end of a long stolonic process, as in *Symplegma viride stolonica* or *Stolonica socialis*. In the last-mentioned form the connecting stolon tends to separate and disappear.

Thus in the Perophorids, Styelids, and Botryllids, isolation of the potential buds is physiological, whereas in *Diazona* and in most Synoicidae, Clavelinidae, and Distomidae, it is physical and of the nature of an epidermal strobilization, the inner tissues being passively cut through.

Constitution of Buds

It is evident from the above that the tissues composing a bud will vary with the region of the zooid from which the bud is formed. Where buds are formed by transverse epidermal constrictions the bud will consist of epidermis plus various inner tissues according to whether the constrictions are oesophageal, abdominal, post-abdominal, or stolonial. The isolated segment may thus contain a part of the digestive tube and epicardium, or epicardium alone, or merely the mesenchyme septum of the vascular stolon. Or in the Styelids and Botryllids it contains none of these tissues but instead a prolongation from the atrial epithelium.

Where, however, the buds become physically isolated from the parent zooid, they are composed of at least three kinds of tissues, epidermis, trophocytes, and some tissue of a non-differentiated nature. This last comes always from one of three sources. In *Stolonica*, *Distomus*, *Polyandrocarpa*, *Symplegma*, *Botryllus*, and *Botrylloides* it is the cells derived from the epithelium of the atrial chamber. In *Clavelina*, *Chondrostachys*, *Colella*, and in the forms *Perophora* and *Ecteinascidia* where the buds do not become isolated physically, the tissue is the mesenchymatous septum. While in *Morchellium*, *Sidnyum*, *Aplidium*, *Euherdmania*, *Eudistoma*, *Distaplia*, *Diplosoma*, *Tylobranchion*, and *Diazona* the epicardium gives rise to it. In each of the three types it should be noted that the tissue is an indifferent epithelium merely serving to separate one cavity from another and is analogous to the coelomic epithelium of other animals. Trophocytes are characteristic of buds that have become physically isolated and so cut off from any continuous nutrient supply by the parent zooid, *i.e.*, in Synoicids, Clavelinids, *Diazona*, and the winter buds of *Stolonica*. The subsequent development of a bud depends upon whether part of the digestive tube is present or not. When present the process is more one of regeneration, when absent it is more truly a reorganization of available material.

If a part of the tube is included, as in *Diazona*, it survives as part of the tube of the new zooid; but the remainder of the digestive tube and branchial sac are formed not from the old but from the proliferating epicardial cells. When no part of the original digestive tube is present, the whole is formed anew by the epicardial cells, if the epicardial tissue is included in the bud. Buds from any source are composed of two layers, an outer vesicle of epidermis, an inner mass of indifferent cells that develop a central cavity, and an intermediate nutrient medium either in direct communication with the haemocoel of the parent, or else congested with trophocytes. The outer epidermal layer, as HJORT long ago demonstrated (1896), forms only new epidermis. All other tissues, whether of ectodermal, mesodermal, or endodermal origin in egg development, arise from the inner mass or vesicle, showing that the germ layers are only of significance in the embryo.

Epidermis thus gives rise to epidermis but to no other tissues. HJORT suggested that it is too highly differentiated to do otherwise All other tissues may arise from either epicardial cells, mesenchyme cells, or atrial epithelial cells, although there is survival of any part of the digestive tube that may be present, and, according to BRIEN (1927), the cerebral ganglion and neural gland develop from the dorsal cord if any part of that tissue is present. Accordingly, it appears that non-differentiated or slightly differentiated tissues are capable of rapid proliferation and of differentiation into any kind of tissue, that moderately differentiated tissue like epidermis can proliferate, but only to form more tissue of the same kind (or with dorsal cord tissue* to form more highly differentiated tissue but of a related type such as ganglion and neural gland), and that highly differentiated tissue, such as that of the digestive tube, is unable even to proliferate, at least with the rapidity necessary in regeneration. These facts tend to show that the capacity for cell-division is inversely proportional to the degree of differentiation, while plasticity, or totipotency is to be correlated with the absence of previous differentiation. Such conclusions are supported by the facts of regeneration in other phyla, though it is not proposed to review here the extensive literature of this subject.

Before discussing the significance of certain aspects of budding and regeneration in ascidians, it is considered advisable to examine the method of budding in the Thaliacea and its possible connection with any of the types found among the ascidians proper. It is generally recognized that the Thaliacea are by no means primitive and have evolved from a sessile ascidian stock. It is possible, therefore, that the type of budding should also have arisen from a mode found within that stock, unless it has arisen *de novo*. GARSTANG (1928), however, considers the Thaliacean budding to be more primitive than any ascidian type, even though he recognizes the probability of a sessile ascidian ancestory of the class itself. The Thaliacea are free-swimming colonial forms in which the individual zooids have an hypertrophied branchial apparatus, a relatively reduced digestive tube, but no ventral abdominal stalk containing the viscera. In general organization the zooid resembles more those of Didemnids and Distomids, for the heart is not reflected as in *Diazona* and *Ciona*. There is no post-abdomen as in Synoicids and no shifting of the intestine and gonads along the side of the branchial chamber as in the Perophoridae, Ascidiidae, Styelidae, and Botryllidae. At the same time there may have been a shifting of the gut-loop and adjacent heart towards the ventro-posterior end of the pharynx, so that upon a basis of adult anatomy one might expect the Thaliacea, and especially the less specialized zooid of *Pyrosoma*, to have evolved

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* According to BRIEN (1927), the dorsal cord is a structure absolutely independent of the cerebral ganglion and is a posterior continuation from the hypophysial tube. It is composed of various histological elements, but is neither ganglionated nor fibrillar. The cells of the dorsal cord that regenerate the cerebral ganglion and neural gland are relatively undifferentiated and show no histological evidence of nervous function.

from the Didemnid-Distomid-Clavelinid stock, and the method of budding to have some resemblance to a type found within that group.

In the Thaliacea budding has assumed an importance and complexity far beyond that of any of the ascidians, much as in the Siphonophora as compared with the rest of the Hydrozoa. Above all it has been developed as in *Perophora* and *Ecteinascidia*, so that the process does not interfere with the normal life of the parent zooid.

When the process is examined in *Pyrosoma*, *Doliolum*, and *Salpa*, it becomes eviden that there is a fundamental similarity among the three forms. The process in the adult is more specialized than in the embryo or larva, and the process in ascidiozooids of *Doliolum* is more like that of the larva than of ascidiozooids of *Salpa* and *Pyrosoma*. In the oozooid of each form the epidermal stolonic outgrowth contains a pharyngeal tube. In *Pyrosoma* and *Salpa* the tube is median and forms as a diverticulum from the wall of the pharynx just below the endostyle. In *Doliolum* there are two diverticula, one on either side of the middle line, again just posterior to the endostyle. There is accordingly little doubt that the paired or single diverticulum, as the case may be, represents the epicardia, for it appears in the correct position, and there is no other sign of that structure in the organism. The pair of pharyngeal tubes persists in the ascidiozooids of *Doliolum*, but in *Pyrosoma* and *Salpa*, the lumen of the diverticulum disappears and a solid strand of cells is proliferated from the lower end of the endostyle. The change is merely a shift in the relative position of the central stolonic tissue, for in oozooid and ascidiozooid (blastozooid) the cells composing it are of an indifferent type derived from the pharyngeal wall. *The essential nature of the stolon in each genus is thus an epidermal outgrowth with a central mass of indifferent cells derived from the same part of the pharynx that in other forms gives rise to the epicardia. The buds are formed from the outgrowth by transverse epidermal constrictions that cut through the passive inner tissue.* There is thus a very marked resemblance to the mode of budding in the larvae of *Distaplia* where the epicardia gives rise to one or two buds that become separated from the parent by epidermal constrictions. The epidermal activity in connection with the isolation of buds is therefore general among tunicates, while in both Thaliacea and *Distaplia* there is a precocious activity of the epicardia to form the central strand or vesicle of the buds.

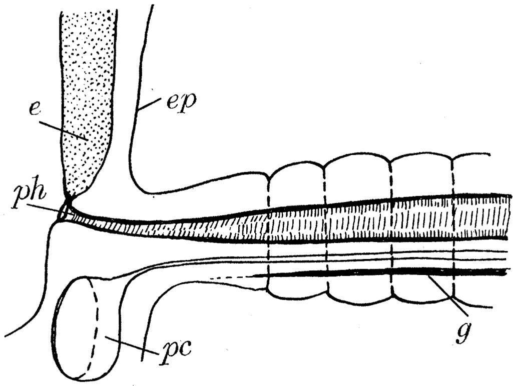

FIG. 19—Diagram to illustrate the relations of the building stolon of the Thaliacea, *e.g.*, *Salpa*. *e*, endostyle; *ep*, epidermis; *g*, gonadial strand; *pc*, pericardium; *ph*, pharyngeal diverticulum

The outstanding difference between budding in the Thaliacea and a form such as *Distaplia* is that in the latter the pharyngeal or epicardial tissue gives rise to the whole of the new organism with the exception of the epidermis. In the Thaliacea additional tissues are present in the stolon and play an active part. As in many forms, mesenchyme is present in the bud of *Pyrosoma* and gives rise to the gonads, although in *Salpa* and *Doliolum* they arise indirectly from the pharyngeal tissue. The more exceptional stolonic structures are found in the stolons of the larval forms of all three A strand or tube passes into the stolon from the pericardium and gives rise in the developing bud to the heart. In *Doliolum* the strand originates in a mass of mesenchyme cells just below the heart. In *Pyrosoma* and *Salpa* it arises from the wall of the pericardium itself. The strand lies ventrally to the pharyngeal or epicardial tissue in the stolon. Other stolonic tissue arises as outgrowths from the posterior end of the atrial or peribranchial chambers, which extend into the stolon as a pair of lateral tubes. In *Doliolum* each peribranchial tube is reflected, so that there is formed a pair of lateral tubes and a dorsal median fused tube. The lateral tubes give rise to muscle, the median tube to the nervous system. The pharyngeal tubes also divide to form two distinct and one median fused tube, the distinct tubes giving rise to the gonads, the median tube to the pharynx and digestive tube.

In the blastozooids of all three genera conditions are somewhat simplified. In *Pyrosoma*, the epidermal stolon contains, in addition to gonad-forming mesencyhme, only a solid strand derived from the base of the endostyle, the peribranchial and pericardial connections having disappeared. In *Salpa* the pericardial connection persists, but the peribranchial, gonadial, and mesenchymal tissues arise from the strand of cells proliferated from the base of the endostyle. In *Doliolum* the peribranchial and pericardial connections persist, as do also the two pharyngeal diverticula; but there is no doubling of the peribranchial and pharyngeal tubes.

Summing up one can say that isolation of buds in the Thaliacea is effected by the general method of transverse epidermal constriction, that the central vesicle of the bud arises from a pharyngeal outgrowth equivalent to the epicardia and thus resembles the larval budding of *Distaplia*, and that various other tissues have been incorporated into the stolon. These additional tissues are those in closest proximity to the stolon base, namely, the posterior ends of the peribranchial chambers and the pericardium, in each case a proliferation of relatively indifferent cell types.

The facts so far presented throw light upon two major problems, the morphological homology and the histology of budding within the Tunicata. In the first one is dealing with a question peculiar to the Tunicates, in the second a part of the general problem of the relation between differentiation, plasticity, and cell division.

Morphology of Budding

Many classifications of Tunicate budding have at various times been evolved, from the simple grouping under stolonial and pallial budding to schemes that reflect every morphological variant of the process. Much confusion has arisen, and in some text-books very jumbled and incorrect accounts are to be found. Altogether too great an emphasis has been placed upon the morphology of the tissues or organs giving rise to the totipotent cells in the bud, and it is no more logical to make a classification including every type than to evolve a similar classification of polychaete regeneration based upon the various sources of the neoblasts and other regenerative cells.

There has also been controversy as to whether budding in Tunicates has a multiple or only a single origin. On theoretical grounds there seems to be no valid reason why a faculty for budding should not have arisen on many occasions within a sessile phylum. The older classifications of the Tunicata, however, were based upon the assumption that budding is monophyletic, an opinion more recently stated by Van Name (1921). It is generally recognized at present that the pallial budding characteristic of the Botryllidae and Styelidae is a reacquisition of the process by a stock that had lost the faculty, although Garstang (1928) attempts to derive both the botryllid pallial budding and that found among other compound ascidians from the complex budding of the Thaliacea. Brien believes the perophorids to be forms that have reacquired a lost faculty for budding.

As a result of the important part played by epicardial tissue in the formation of buds, and the existence of a ventral budding stolon in the Thaliacea, *Clavelina*, and in *Perophora*, there has arisen a conception that has won wide acceptance, namely, that the budding stolon or *stolo prolifer* is a primitive structure of Tunicates, and also that the epicardium is primarily an organ of budding. These are believed both to be misconceptions, although Garstang as a protagonist argues the case well (1928, pp. 156–178). Considerable misunderstanding has arisen concerning the nature of the septum in the stolon of *Clavelina* and in that of *Perophora*. In development the septum arises near the base of the heart and epicardium, and it is considered (MacBride, 1914, p. 638, Garstang, 1928, p. 157) to be a prolongation into the stolon, for budding purposes, of the epicardium, the cavity of which is compressed out of existence. The function of the septum as a membrane separating an afferent and efferent blood flow is thought to be secondary (Garstang, 1928, p. 172).

Brien (1927, 1930) has recently shown that the septum in blastozooids of both *Perophora* and *Clavelina* is mesenchymatous in nature and origin and without connection with the epicardium, and as there is apparently no epicardium even in the oozoid of *Perophora* there remains only the possibility that the septum arises from the epicardium in the oozooid of *Clavelina*. If it does not, then there is no evidence whatever that the septum is epicardial. There is certainly no evidence that the stolonial septum at one time possessed a lumen continuous with that of the epicardium, for the appearance of a lumen in the part of the septum concerned with bud development is only a necessary stage in that development.

The statement frequently made that the septum arises from the posterior end of the epicardium in the oozooid of *Clavelina*, although in bud development it is recognized that it arises from the base of the pericardium, is due to the description of the development by Van Beneden and Julin. These authors (1886, p. 301), state that the two epicardial tubes form a single posterior *cul-de-sac*, the cavity of which is reduced until the walls form a horizontal cellular plate. The plate extends into the stolon as the vascular septum. In the developing bud, according to these authors (1886, p. 313), the septum arises as a membranous continuation of the pericardium. At the same time, the figures (plate X, I*a*-I*g*) quoted in evidence of the first of the above two statements merely show that the base of the epicardium does not extend as far as the base of the heart, and therefore in the stage illustrated the septum either does not arise from the epicardium or else has not yet appeared.

On other grounds an epicardial origin is to be doubted, for it would radically alter the course of the blood leaving the heart and returning from the stolon to the body. Consequently it has seemed advisable to reinvestigate this point. With this object a young oozooid (four days after fixation of the tadpole larva) of *Clavelina picta* was sectioned. The results are shown in fig. 20, where it may be seen that the posterior horns of the epicardial cavity disappear well anterior to the base of the heart and that the vascular septum of the stolon is continuous with the "raphe" of the heart at the posterior end of the pericardium. The septum arises actually from the condensation of mesenchyme cells in contact with its base. But most decidedly in this species the septum does not develop from the lining of the epicardium. Neither does it in *Ciona*, according to ÅRNBÄCK and BRIEN (1932).

There is thus no justification for the assumption that the septum is a continuation of the epicardium into the stolon, and therefore less reason to suppose the epicardium itself to be an organ evolved for the purpose of budding. GARSTANG (1928, p. 169) suggests that the epicardia themselves evolved as a pair of regenerative pockets at the base of the thorax.*

Reviewing the constitution of the bud throughout the Tunicata, one finds that the outer layer of the bud is always epidermal, and that the new epidermis develops from the old. The inner layers of the bud, on the other hand, are very variable, although free mesenchyme cells are almost always included. In *Perophora*, *Clavelina*, and *Colella*, the inner tissues develop from the mesenchyme septum of the stolon. In the Synoicidae, *Diazona*, *Distaplia*, and Didemnidae, they develop from the epicardial lining, while in the Polystyelids, and Botryllids they develop from a continuation of the atrial epithelium. So that a classification of budding based upon the nature of the inner layer would be septal, epicardial, and pallial, although in the Thaliacea the bud may contain pallial as well as epicardial tissue. It is not the purpose of the present paper to discuss in detail the adult morphology of the tunicate zooids or the nature and function of the epicardium apart from the part it plays in budding. At the same time, it should be noted that only in *Diplosoma* and in the Thaliacea are its normal morphological relationships disturbed and its importance in budding paramount, and these two forms represent the most complex types of budding within the phylum. In other words, in those forms in which budding is most specialized and highly developed, there has been a specialization of the epicardium in connection with that process. It is probable that the stolon in the Thaliacea primitively contained only mesenchyme and the pharyngeal (epicardial) outgrowths, and that the peribranchial and pericardial elements

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* A full discussion of the nature and structure of the epicardium of Tunicates will be presented in a later paper.

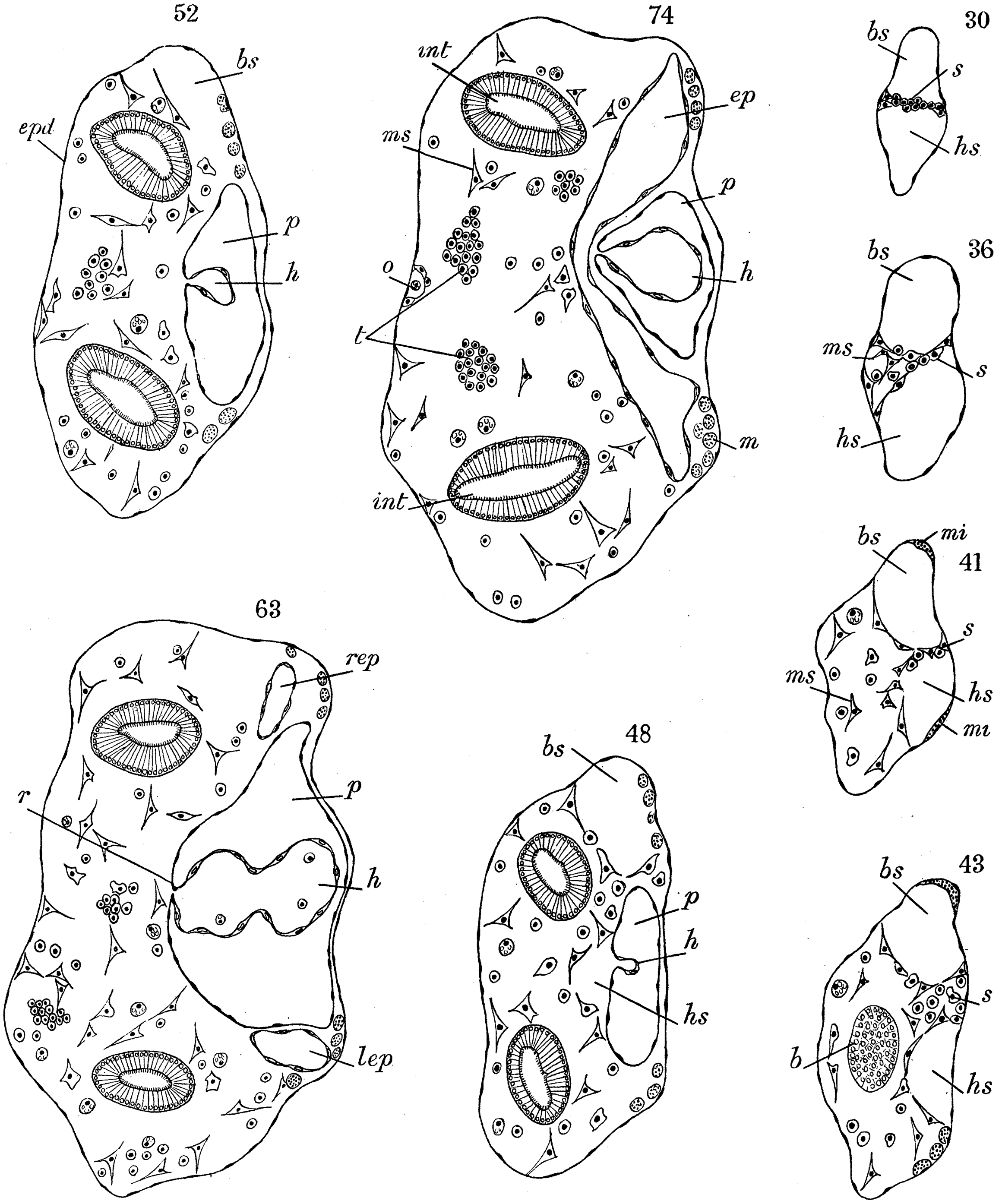

FIG. 20—Transverse sections of a newly functional oozooid of *Clavelina picta*, showing the origin of the vascular septum of the stolon from the mesenchyme near the base of the pericardium, and the lack of any connection between the septum and the epicardium. The sections were cut at a thickness of 7·5 μ, and the numbers refer to the serial number commencing at the tip of the stolon; *b*, base of intestinal loop; *bs*, blood sinus from stolon to abdominal haemacoel, *ep*, epicardium; *epd*, epidermis; *h*, heart; *hs*, blood sinus from heart to stolon; *int*, intestine; *lep*, left posterior horn of epicardium; *m*, muscle; *mi*, muscle insertion; *ms*, mesenchyme; *o*, rudiment of ovary; *p*, pericardium; *r*, "raphe" of heart; *rep*, right posterior horn of epicardium; *s*, mesenchymatous septum of stolon; *t*, rudiment of testis

entered the stolon because they might be drawn there in the normal process of growth, because they are relatively unspecialized or indifferent tissues, and because budding is of such great importance in these forms that it becomes one of the main functions of the zooid, especially in embryonic and larval stages when the peribranchial extensions are most obvious.

The isolation of buds seems to be primitively a process of transverse fission brought about by constricting activity of the epidermis. The constrictions appear in the stalk of fixation rather than the more anterior thorax, and accordingly the nature of the inner tissues of a bud depends on the relation of the different organs of the zooid to the stalk. In *Diazona*, *Archidistoma*, and *Eudistoma*, the posterior end of the gut loop reaches the base of the stalk, the ventral blood vessel being very small and insignificant. Therefore the constrictions cut through both oesophageal and intestinal limbs of the loop, and bud development is primarily a process of regeneration of the missing parts by the epicardial tissue and the epidermis.

In the Synoicidae the heart and epicardium reaches the base of the long stalk, but the gut loop only descends a relatively short distance, so that the buds usually contain only a mesenchyme and epicardial tissue. Since there is no rigid persisting section of the gut, the epicardial tissue reconstitutes the whole new organism apart from the epidermis. In *Distaplia* and *Diplosoma* budding is precocious and two masses of cells are proliferated from the upper ends of the epicardia. They are contained in epidermal outgrowths, to form buds in *Distaplia* when the zooid degenerates, to regenerate to form a double zooid in *Diplosoma* where the original zooid persists.

In *Colella*, *Clavelina*, and *Perophora*, the ventral blood vessel is hypertrophied to form the stolon and contains a mesenchymatous septum. Epidermal constrictions isolate buds in the first two, in *Perophora* epidermal activity results in bud formation while in organic continuity with the parent zooid. In each the contained tissue within the bud is free and septal mesenchyme cells.

In the free-swimming Thaliacea the stolon apparently represents the stalk of fixation, or at least its ventral continuation such as that of *Perophora*. It is entered by epicardial and other tissues already discussed, but again isolation of buds is effected by transverse epidermal constrictions.

A continuity in the budding process of the above forms can accordingly be visualized, the primary activity being transverse epidermal constriction, the inner composition of the buds so isolated varying with the nature of the zooid.

Only in the Polystyelids and Botryllids does budding seem to be fundamentally different, for in these the bud is a lateral protrusion of the body involving epidermis, mesenchyme, and the atrial epithelium. It would seem to be a reacquisition of a lost faculty for budding, to be associated with the suppression of the stalk of fixation, so that the lower margin of the atrial wall serves as an organ of attachment, and so tends to extend laterally and form buds.

The morphological relationship between the various types of budding as here conceived are shown diagrammatically in fig. 21.

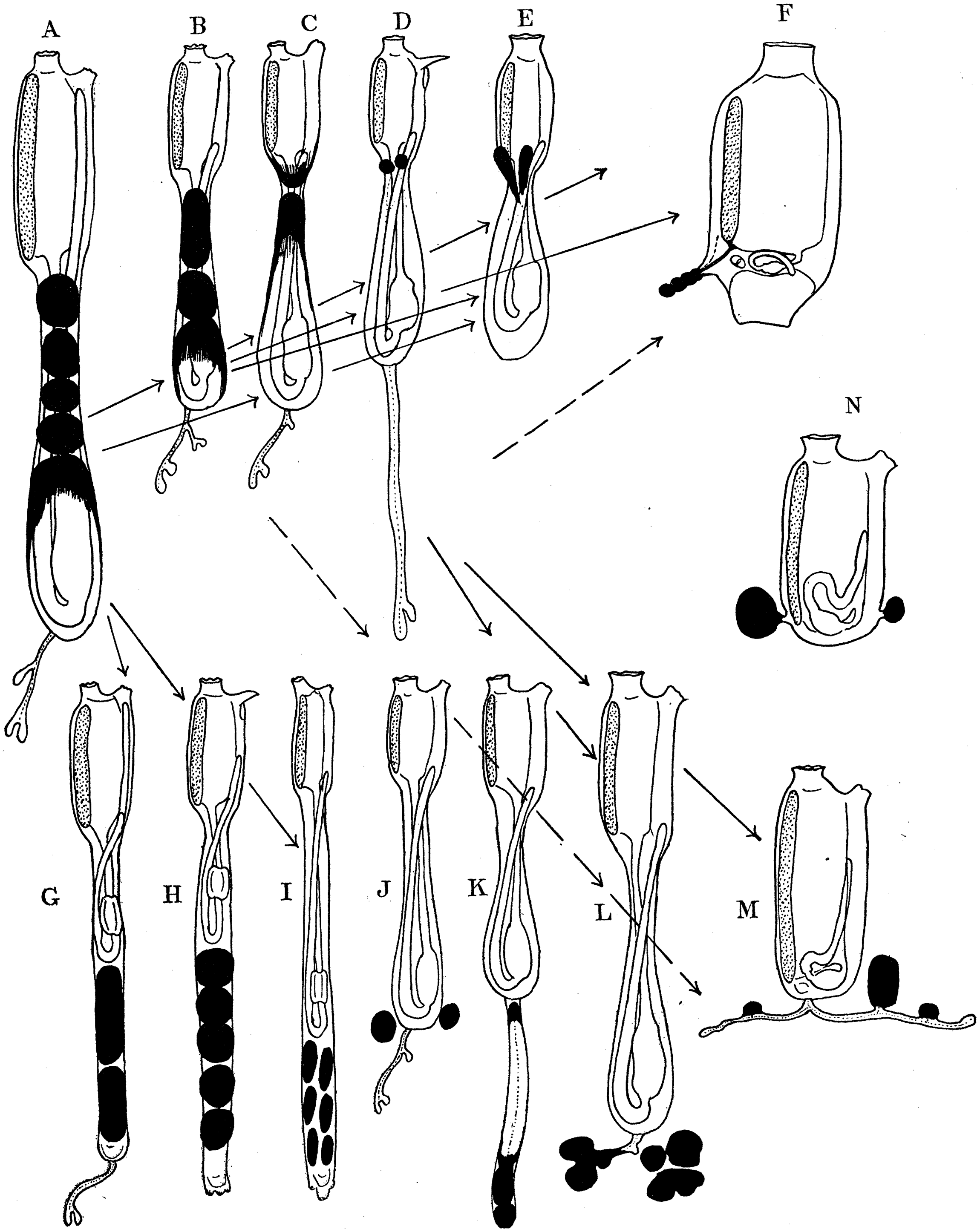

FIG. 21—Diagram to show mutual relations of the various methods of budding among the Tunicata. The types represent—A, *Diazona*; B, *Archidistoma*; C, *Eudistoma*; D, *Distaplia*; E, *Diplosoma*; F, a thaliacean; G, *Tylobranchion*; H, *Morchellium* or *Aplidium*; I, *Euherdmania*; J, *Pycnoclavella*; K, *Colella*; L, *Clavelina*; M, *Ecteinascidia* or *Perophora*; N, a Polystyelid or Botryllid. The arrows show the probable directions of specialization in the course of evolution, the discontinuous lines possible relationships

Histology of Budding

The histology of the buds or regenerating fragments is readily reviewed. In every species, with the possible exception of *Pycnoclavella*, the whole of the epidermis of the new organism develops from the outer epidermal layer of the bud, but in no recorded species does the epidermis from a fully formed zooid ever take part in the development of any other tissue. Mesenchyme cells are usually included in a bud, and these play a variable part, often forming muscle, heart, and gonads. The inner vesicle or tissue, whether it be stolonial septum, epicardium, or atrial epithelium, can give rise to all kinds of tissues, except that it does not form epidermis since it is not necessary. Digestive epithelial tissue, if included in the bud, survives as a part of the new digestive system, but does not itself regenerate new tissue.

The potency of the tissues may accordingly be graded as follows :—

Highly differentiated tissue, *e.g.*, gut epithelium, can survive but not proliferate (except the rectal buds in *Diplosoma*, which form more rectal tissue).

Moderately differentiated tissue, *e.g.*, epidermis, pericardium, can proliferate rapidly, but can give rise only to more epidermal and pericardial tissue.

Lowly differentiated tissue, *e.g.*, mesenchyme septum, epicardium, atrial lining, can proliferate rapidly and give rise to all kinds of tissue, nervous, digestive, muscle, gonadial, heart, etc., if there be need.

These statements suggest that the maintenance of a high degree of differentiation is incompatible with cell division, at least rapid enough to be of use in regeneration ; that moderate differentiation and rapid cell division are compatible although the direction of differentiation seems to be irreversible ; while totipotency is to be associated with tissues, such as limiting inner membranes that have never become moderately differentiated.

It is obvious from the foregoing pages that the primary germ layers have no significance in bud development and that only the degree of differentiation of a tissue is of importance. The germ layer theory was, in fact, shown to be invalid with regard to adult organisms and to be merely part of embryonic mechanics by Hiort (1896), in his famous monograph.

XII—Conclusions and Summary

The nature of budding is described for twenty genera of ascidians, in eight for the first time.

The newly described forms are *Diazona*, *Tylobranchion*, *Morchellium*, *Euherdmania*, *Eudistoma*, *Archidistoma*, *Pycnoclavella*, and *Chondrostachys ;* those reinvestigated are *Distaplia*, *Colella*, *Clavelina*, *Diplosoma*, *Perophora*, *Ecteinascidia*, *Stolonica*, *Distomus*, *Polyandrocarpa*, *Symplegma*, *Botryllus*, and *Botrylloides*.

In the majority there is a regular alternation between phases of sexual and asexual reproduction coinciding approximately with summer and winter, but it is held that the onset of budding is dependent more upon a certain physiological state following the cessation of sexual breeding rather than upon direct environmental stimulation.

Budding usually follows or coincides with a tendency on the part of the zooid to degenerate, either by a general autolysis or by an antero-posterior regression. The regression is to be associated with the formation of yolk-laden trophocytes and their migration to the posterior end of the body.

The extent of the migration posteriorly of the trophocytes depends upon the nature of the zooid. In primitive types with no post-abdomen nor enlarged ventral vessel, the abdominal and oesophageal regions become congested. Where there is a post-abdomen the trophocytes accumulate in that region and the abdomen is allowed to undergo regression. Where the ventral vessel is hypertrophied the trophocytes are able to enter it and the whole of the zooid may regress.

The nutritive medium for the developing bud may be fluid, where there is physical continuity with the parent or general autolysis, or may be supplied by trophocytes, as is usual where there is physical isolation of buds.

During regression or autolysis the least specialized types of tissue survive the most readily, and the presence of at least one unspecialized or undifferentiated type is necessary for development to form a new individual.

Isolation of buds is effected by transverse epidermal constrictions. In this process the epidermal cells become enlarged and columnar. The inner tissues are passively cut through by the constricting epidermis.

The tissues included within the epidermal layer of a bud depend upon the region of the zooid constricted by the epidermis. In *Diazoma*, *Archidistoma*, and *Eudistoma* there is no post-abdomen nor enlarged ventral vessel, and the buds contain part of the digestive tube as well as epicardial, mesenchyme and other tissues. In *Tylobranchion*, *Euherdmania*, and the Synoicidae, only the post-abdomen is constricted, the rest regresses, and the buds contain epicardial and mesenchymal tissue, but no part of the digestive tube. In *Clavelina* there is no post-abdomen, but an enlarged ventral vessel is constricted off containing the mesenchymatous septum.

Where part of the old digestive tube persists in a bud, development is primarily a process of regeneration of the missing parts. When there is no trace of the digestive tube, development is primarily a reorganization of all the contained tissues to form a new organism.

Highly specialized tissue, such as digestive epithelium, can survive but is unable to proliferate even to form more of its own sort. Moderately specialized tissue, such as epidermis, can proliferate readily but only to form more epidermis. Unspecialized tissues, such as epicardial, septal, or atrial, can proliferate and differentiate into any kind of tissue.

In buds isolated from the oesophageal, abdominal, or post-abdominal regions, epicardial cells serve as the totipotent tissue. When isolated from the ventral vessel, the mesenchyme cells of the septum are the active tissue; while when formed from the thorax the atrial epithelial cells are observed to be totipotent.

It is concluded that, contrary to the accepted view, the vascular septum of the ventral stolon has no connection even in embryonic development with the epicardium and cannot be considered as extension of the epicardium into the stolon for purposes of budding.

Assuming the mode of budding as expressed in *Diazona*, *Archidistoma*, and *Eudistoma* to be primitive, it is argued that there have been two major trends in specialization away from that mode, the one culminating in *Distaplia*, *Diplosoma*, and possibly the Thaliacea, in which budding has become localized, epicardial, and oesophageal, the other through the posterior extension of zooids and buds to form the Synoicids, Clavelinids, *Colella*, *Pycnoclavella*, and possibly the Perophoridae. It is believed that the mode of budding in the Botryllidae and Polystyelidae represents a reacquisition of that faculty and is not phyletically continuous with any other type.

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