

DEVELOPMENT OF PODOCYSTS OF THE SCYPHOZOAN,
CHRYSAORA QUINQUECIRRHA, AFTER REMOVAL OF
THE CYST COVERING

A Thesis

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Of the Requirements for the Degree of
Master of Arts

by

Lan-Ping Amy Tseng

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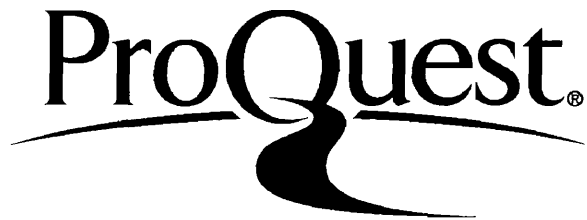
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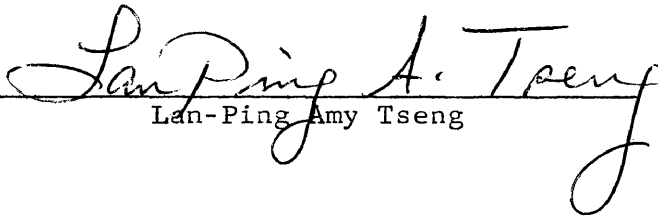
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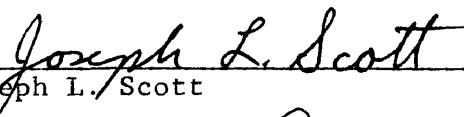
This thesis is submitted in partial fulfillment of
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Approved, August 1974


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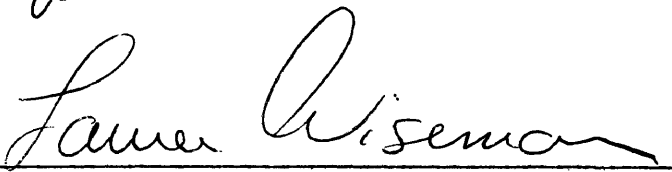

Lawrence L. Wiseman

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ABSTRACT

The podocyst of Chrysaora consists of an organized cell mass, surrounded by a chitin-protein outer coat. The cysts can remain dormant for long periods. When the outer covering is removed, most of the cysts are stimulated to develop into polyps. Some, however, reencyst by secreting a new covering and remain dormant. If the covering is removed from young incomplete cysts, only about 62% develop, while the remainder reencyst. When the covering is removed from cysts which are six months old, 84% developed, while 16% reencysted. If the second covering is removed from cysts which had reencysted, development is much more rapid than following removal of the first covering.

The new covering contains chitin, since it is insoluble in hot alkali and liberates glucosamine on hydrolysis. Both intact and denuded cysts incorporate exogenous glucose into acid-insoluble macromolecules, which probably include chitin.

Subdivision of cysts leads to the development of miniature, complete polyps. Fusion of two cysts usually produced polyps with two sets of oral structures, but in one case a giant, complete polyp was developed.

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INTRODUCTION

Five species of scyphozoan scyphistomae are known to produce podocysts at the point of their attachment to the substratum. Podocyst formation was first described by Hyde (1894). Herouard (1911) first recognized its importance as a means of asexual reproduction and survival of adverse environmental conditions. He reported that podocysts could withstand cold temperature better than could polyps. Cargo and Schultz (1966) found that in Chrysaora quinquecirrha of Chesapeake Bay, podocyst formation could be stimulated by extremes of salinity or temperature.

A podocyst is an organized cellular mass covered by a tough, outer coat. In the formation of a podocyst, in Chrysaora hysocella the basal epidermis invaginates and secretes a dome-like cuticle which overlies the epidermal cells. No gastrodermal cells are included. The cuticle is joined to the basal platform and the podocyst becomes completely closed. A similar process has been described in Aurelia aurita by Chapman (1970); however the latter author has also described the origin of Aurelia podocysts from amoebocytes which migrate to the basal region and secrete a cuticle. The cells of the podocyst are arranged in an outer layer, surrounding a central, clear zone, which is probably derived from the mesoglea (Chapman, 1970).

The podocyst covering is composed of a chitin-protein complex, which is tanned by phenolic substances. The material of the cuticle

is arranged in lamellae which are composed of microfibers. The internal covering next to the cells has a different structure from the outer covering, suggesting that the cells can continue to secrete covering during encystment (Blanquet, 1972).

The podocyst may be compared to an embryo, in the sense that on germination it develops into a polyp. Two modes of development have been described. Hadzi (1912) reported that in Chrysaora mediterranea a ciliated, planula-like embryo escaped and developed into a polyp, whereas Herouard (1908) found that a small, two-tentacled polyp developed directly.

The podocyst can remain dormant for a prolonged period. Herouard (1911) observed the germination of a podocyst of Chrysaora hysocella which had been in his laboratory for 3 years. Enright (unpublished) reported that at 25° C podocysts of Chrysaora quinquecirrha germinated after 1 to 24 months, with apparent modes at 9 and 18 months. Dormancy is broken in 80% of these podocysts by holding at 5° C for 23 weeks, followed by warming to 25°.

Conditions of dormancy are common in various life stages of microorganisms, plants and animals. Two types of dormancy are defined by Sussman and Halvorson (1966): constitutive, due to an innate property of the organism, and exogenous, imposed by environmental factors. Sussman (1966) points out that constitutive dormancy can be overcome only by a treatment that is not normally required by the organism for its vegetative existence. Such a treatment triggers the activation of the dormant stage and breaks the period of dormancy. Without such triggering, dormancy will persist, even under conditions

favorable to vegetative activity. By this definition, podocysts of C. quinquecirrha exhibit constitutive dormancy.

The mechanism by which dormancy is maintained has been investigated in a number of organisms, but not in the podocyst. Dormancy in plant seeds has been reviewed by Roberts (1969). In some cases true cellular dormancy is found in embryos, whereas in others the presence of the covering is required for maintenance of the dormant state. In the species Phalaris arundinacea Vose (1962) proposed that the husk is a barrier to gas exchange, and that internal anaerobiosis leads to the formation of a germination inhibitor. Germination inhibitors have been found in many plants, and most of these affect oxidative processes. Roberts attributes little importance to the direct restriction of oxygen availability by the seed coat; however, increased oxygen tension, as well as puncturing the seed coat, is effective in terminating dormancy in many species. In most seeds the seed coat is important in reinforcing dormancy, even when true cellular dormancy is present. Examples of true cellular dormancy in the absence of a heavy covering are found in buds. A review of bud dormancy is given by Wareing (1969).

Dormancy in fungal and bacterial spores is maintained in some instances by self-inhibitors of germination (Sussman and Halvorson, 1966). These authors have categorized three methods of triggering germination in spores: physiological, in which germination occurs in response to nutrients; chemical, in which germination is caused by an agent which is not metabolized; and mechanical treatments, such as the puncturing of spore walls. Similar plant seeds, the presence of a

covering on podocysts serves in some cases to restrict development.

In animals encystment and dormancy have been studied in only a few phyla. In soil amoebae encystment is caused by the absence of some growth factor from the medium, or by blockage of DNA synthesis. The encysted cell shows a continuous decline in respiration and in the activities of a number of enzymes, and it may remain dormant for years (Neff and Neff, 1969).

Fresh-water bryozoa produce dormant statoblasts, covered by chitinous material. In Pectinatella gelatinosa, a Japanese species, water in which statoblasts have been stored contains an inhibitor of germination (Mukai, 1974).

A state of cryptobiosis, or ametabolism exists in encysted, dehydrated embryos of the crustacean, Artemia salina (Clegg, 1967). Metabolism is rapidly resumed after placing the embryos in water (Muramatsu, 1960).

In diapausing pupae of the Cecropia silkworm, respiration is depressed (Schneiderman and Williams, 1953), and DNA synthesis is absent (Krishnakumaran and Schneiderman, 1963). Severe injury to the pupa temporarily increases respiration to the level of the post-diapausing embryo (Schneiderman and Williams, 1953) and increases RNA synthesis (Wyatt and Linzen, 1964), but does not result in the synthesis of DNA, which is a requirement for metamorphosis (Krishnakumaran et al., 1967).

It is apparent that in some encysted organisms, cellular dormancy arises by a decrease in biochemical activity following encystment. In such cases, it may therefore be presumed that cellular

activation must precede excystment and further development. In animals the cellular capabilities for growth following artificial removal of the cyst covering have not been investigated thoroughly in any organism, except the *Cecropia* moth, where true cellular dormancy is present, and injury to the covering does not lead to development.

The present investigation was primarily concerned with the effects of removing the covering on subsequent development of podocysts. If true cellular dormancy exists, one might expect that removal would either fail to stimulate development or that there would be a period of quiescence following artificial excystment before development could occur. On the other hand, if dormancy is solely an effect of the presence of the covering, then rapid development should take place upon its removal. Since most podocysts exist for long periods in the dormant condition, it seemed possible that a period of "maturation" might occur during encystment, before the cell mass could acquire the capability to develop into a polyp. In this case, removal of coverings from incomplete or very early cysts would fail to stimulate development. Cysts of differing ages have been examined to test this possibility. Most developing podocysts give rise to single polyps, indicating the possibility that the fate of the podocyst cell mass is determined prior to excystment. This possibility has been examined by subdivision and fusion of cell masses.

MATERIALS AND METHODS

Polyps of Chrysaora quinquecirrha were grown in the laboratory from gametes shed by medusae from the York River. The polyps were cultured at 25 °C in filtered York River water of 15-19 ‰ in large glass finger bowls. The cultures were kept in the dark to inhibit algal growth. The polyps were fed once weekly with nauplii of Artemia salina, and the water was changed one day after each feeding. To initiate a culture, the bases and the old podocysts were removed from polyps, which were then placed in a bowl and left undisturbed for about two weeks. During this time, most of the polyps become attached to the bottom of the bowl. Podocysts which were also attached to the bottom could be removed by gentle scraping with a scalpel. By removing them at regular intervals, podocysts of approximately known ages could be obtained.

In order to attempt to induce premature development of the cysts, the coverings were removed from the cysts. The covering was removed by slitting one side of the wall and gently forcing the cellular contents out by pressing on the top of the cyst. Cellular contents could be removed in this way with a minimum amount of disturbance to the cells. The cellular contents of 50 cysts were transferred by pipette to a small bowl and cultured at 25° C in 20 ml of York River water. Observations were made daily for up to 17 days. The "endpoint" of development was taken to be the formation

of a four-tentacled polyp.

In a low percentage of cases the cellular material reencysted, and further development did not occur. In such cases the reencysted cysts were held for one month, following which the second covering was removed, in order to compare the rate of development of these with the cysts described earlier.

A study was made of the effect of extreme cellular disorganization on the development or reencystment of the cyst material. In one batch of cysts a small hole was made in the top and the cells were squeezed out, so as to cause the maximum degree of disorganization. The results were compared with those obtained in the experiments stated earlier.

In one series of operations, a small portion of the cyst wall was removed from cysts, without removing the cellular mass from the covering. In this manner the cells were left totally undisturbed but exposed to the external medium because of the hole in the covering.

In all cases, after removal from their original covering, the cyst cells secreted a thin, new covering. Most of the cysts then "germinated" from this new covering or remained encysted, as indicated. It was of interest to determine whether this new covering contains chitin, as does the old one. Tests for chitin were done on both original and remade coverings by the method of Blanquet (1972). Original and remade covering structures were collected separately, placed in 10% disodiumethyldiaminetetraacetate (EDTA), pH 8.0, for 12 hours to remove any calcium which may have been present in the

cuticle. The podocysts were next washed in distilled water and then extracted at room temperature in 8.0 M urea (24 hours), 0.01 N NaOH (6 hours) and 1.0 N NaOH at 100°C for 6 hours. The insoluble transparent material was washed in distilled water until all traces of NaOH were removed. The cuticles were collected by centrifugation and hydrolyzed in 6 N HCl in sealed vessels at 100°C for 6 hours. The hydrolysate was evaporated and the residue was then taken up in 80% ethanol for chromatography. Hydrolyzed material was chromatographed along with glucosamine, on Eastman #6064 cellulose sheets, using n-butanol:ethanol:acetic acid:water (5:4:3:2 by volume). Glucosamine was visualized after spraying with ninhydrin, and heating at 105°C for 5 minutes.

In order to determine whether the cells incorporated glucose into the new covering, the cell masses were placed in 5.0 $\mu\text{C}/\text{ml}$ ^3H -glucose at 0, 24, 48, and 72 hours after removal from the original covering. After incubation at the labeled glucose for 24 hours, the cells were fixed in calcium acetate-formalin, embedded in paraffin and sectioned at 7-10 μ . Autoradiography was performed using Kodak NTB-2 emulsion and an incubation period of 15 to 20 days.

In one experiment, an attempt was made to determine, by a quantitative method, whether glucose is continuously incorporated into the covering by intact cysts. Cysts were incubated for 24 hours in the presence of 5 $\mu\text{C}/\text{ml}$ D-glucose-6- ^3H (7 C/mM, ICN Corporation), following which the coverings and cells were either counted immediately or after 10 days' incubation without label. Penicillin (1,000 units

per ml) and streptomycin (100 μ g per ml) were included in the incubation mixtures. The cysts were thoroughly washed with sea water before opening. The coverings were rinsed briefly in 1 N HCl before placing them in the scintillation vials. Cells and coverings were dried on filter paper discs at 60° C overnight before scintillation fluid was added.

Several experiments were done in which the cell masses of cysts were subdivided or fused. It was necessary to fuse cell masses almost simultaneously with their contact with the external medium, since after only a few seconds they became less sticky.

RESULTS

Following removal of the primary covering, the cell mass secreted a thin, new covering within 2 to 3 days. In most cases, the newly-encysted structure was greatly flattened and attached to the substratum. Excystment occurred in a high percentage of cysts within a few days; however, a small percentage remained dormant after reencystment. The process of excystment resembled that in normal cysts: a club-shaped protuberance developed, and formation of a mouth and tentacles occurred at the tip of this structure within a few days (Figure 1). In some cases a ciliated ball of cells was formed; this also developed into a polyp.

In comparing the development of cysts, the four-tentacled polyp was taken as the end-point, although development usually proceeds to at least eight tentacles when no food is provided. Developmental curves for cysts of different age are shown in Figure 2. Age-related differences are clearly visible, with six-month-old cysts showing a higher degree of development than the younger cysts, and incomplete cysts developing somewhat more slowly than cysts 0.5 to 2 months of age. The cumulative percentage of six-month-old cysts which developed was significantly greater ($p < .001$; Kolmogorov-Smirnov one-tailed test) than the 0.5 to 2-month-old ages, and the 0.5 to 2-month-old cysts significantly greater than the incomplete cysts ($p < .05$).

The effect of removing the secondary coverings from cysts which

had reencysted and remained dormant for one month following removal of the primary covering is shown in Figure 3. The cumulative frequency distribution for reactivated cysts is significantly different ($p < .002$; Kolmogorov-Smirnov one-tailed test) from that of the control cysts. This difference is probably due to the delay in the onset of development of the control cysts and to the greater percentage which developed in the reactivated cysts.

The degree of development of cysts treated in different ways is shown in Table 1. Disruption of cells affected only slightly the percentage developing to polyps. When cysts were merely punctured or slit, without removal of cellular contents, however, only a very low percentage was induced to develop. In these cases, extensive healing of the puncture occurred, with the production of a thick covering, similar to that of the primary covering.

The experiment on disruption of the cell mass suggested that axes within the podocyst are not highly determined. Further experiments to test this hypothesis were performed. Subdivision of the cell mass into pieces resulted in the development of as many as 7 polyps from one cyst, while in one instance fusion of 2 cyst masses resulted in a large, normal polyp. In most of the attempts at fusion, polyps with dual mouths and sets of tentacles developed (Figure 4).

The ability of the cyst cells to secrete a similar covering was apparently greatly reduced when the cell mass was removed from the old cover. The primary covering is a chitin-protein complex (Blanquet, 1972), and a question existed as to the nature of the

secondary covering. Two experiments were performed to test the ability to synthesize a chitinous new covering. In the first, cell masses were incubated in ^3H -glucose for 24 hours, following which they were sectioned in paraffin and autoradiography was performed. Heavy glucose incorporation into acid- and ethanol-insoluble material was found in the outer cells, and in what may be covering material external to them (Figure 5). In the second experiment, coverings were isolated from 1,000 cysts and analyzed for chitin by a modification of the method used by Blanquet (1972). After washing in hot alkali, the cyst material was hydrolyzed in acid and chromatographed. The presence of glucosamine and two additional amino compounds were identified by spraying chromatograms with ninhydrin (Figure 6). The additional components of the secondary covering are apparently absent from hydrolyzates of the primary covering, and they have not been identified.

An attempt to determine whether intact, dormant cysts continually synthesize a covering was made by incubating cysts in ^3H -glucose, followed by either immediate or delayed analysis of cells and cyst coverings for radioactivity. It was expected that the initial radioactivity in the cells would be slowly incorporated into the coverings over a period of days. The results, shown in Table 2, are not interpretable, since even isolated coverings are heavily labeled (presumably because of external bacteria or fungi) on short-term incubation. Since the internal, cellular material was probably not contaminated with bacteria, however, it is possible

to conclude that glucose incorporation into macromolecules by the cells of the intact cyst does occur, even when the cyst is supposedly dormant.

DISCUSSION

The stimulation of podocyst development by removal of the covering is an indication that the covering is of direct importance in the maintenance of the dormant condition. A generalization of the probable role of the covering is that it permits (or causes) the cells to exist in an environment which differs from that outside the cyst. The cells may be bathed in an inhibitor, which diffuses away when the covering is removed. The covering may provide a barrier to the diffusion of oxygen or to ions, so that the internal milieu is changed by the metabolic activities of the cells. This is similar to theories of dormancy proposed by Vose (1962) and others (see Roberts, 1969).

Removal of the primary covering is followed by a delay of 3 to 6 days before visible development begins. By contrast, development of a four-tentacled polyp from a fertilized egg requires only 30 hours at 25°C (Black, unpublished). The extended period of apparent inactivity preceding development of a podocyst may in part be related to the necessity for activation of cellular processes following exposure of the cells to the external medium. This leads to the conclusion that the podocyst cells are intrinsically "dormant," at least insofar as developmental processes are concerned. That this intrinsic cellular dormancy does not come about gradually after prolonged residence inside the covering is indicated by the results

of removing cells from incomplete podocysts. These cell masses develop somewhat more slowly than those from month-old cysts (Figure 2). The cells must possess their dormant property even prior to their enclosure in the cyst, or acquire it during enclosure.

Additional support for the possibility of an intrinsic cellular dormancy is derived from experiments in which the second covering was removed from reencysted podocysts which had remained dormant for an additional month following removal of the first covering. These cysts developed much more rapidly than did those after removal of the first covering (Figure 2). Evidently removal of the first covering had partially activated these cysts, and the activated state must have persisted for a month.

Blanquet (1972) proposed that podocyst cells are capable of producing additional chitinous covering material after encystment. Analysis of the new covering produced by cysts of Chrysaora in this study indicates that it is probably chitinous, since it is insoluble in hot alkali and has glucosamine as a major component. The primary covering also contains protein; the secondary one has not been analyzed for this component, but it is resistant to the action of chitinase, as is the primary one.

The dormancy of the cyst may be related to its capability to secrete or maintain a covering of sufficient quality. When only a small hole is made in the primary cover, it is nearly always repaired, and dormancy persists (Table 1). When the entire cover is removed, the new cover is always extremely thin. In those cysts which reencyst for a prolonged period, the covering is frequently amber in color,

although it also is thin. In these cysts a certain degree of tanning has apparently occurred. Blanquet (1972) has shown that enzymes for tanning the cuticle are present in the cyst. The thinness of the covering must result from depletion of precursors from which the covering may be made. The availability of a limited supply of precursors may be one factor which determines the length of the dormancy period of intact cysts.

The fate of a cyst from which the covering has been removed appears to be determined by the outcome of competition between two opposing processes: the secretion and tanning of a new covering and cellular activation leading to development. In those cysts in which a sufficient degree of activation occurs early, development will follow. On the other hand, if the cells secrete a sufficient covering before development has progressed too far, then dormancy will persist. Preliminary observations suggest that glucose metabolism may be central to these two opposing processes. Exogenous, labeled glucose, is rapidly incorporated into macromolecules by cells of intact cysts (Table 2) and by cells minus their coverings. In the latter, autoradiography shows that all cells, except those which are probably cnidoblasts, are active (Figure 4). Glucose derivatives are precursors of chitin, while the catabolism of glucose provides ATP, pentose phosphate and other compounds essential for development. The relationship between the opposing pathways of chitin synthesis and glucose catabolism may be of primary importance in determining the fate of the podocyst.

The apparent absence of a high degree of axial determination

in the podocyst is indicated by cellular disruption, subdivision and fusion of podocysts. It should be noted that no conclusion can be drawn about the extent of cellular determination, since the cells are probably capable of movement within the developing cell mass. Since eclosion always occurs by the formation of a small hole in the top of the podocyst, it would be of interest to know whether certain cells are destined to produce an eclosion enzyme. If all the cells have this capability, then differences in the thickness of the covering or in the degree of contact between cells and covering may determine the site of eclosion and consequently the establishment of the oral-aboral axis of the polyp. The cells at the tip of the bud emerging from this hole develop into the oral disc and tentacles of the polyp. These cells are probably the first to be activated by contact with the external medium.

If only some of the cyst cells are capable of producing the eclosion enzyme, these cells may migrate to the tip of the bud, even when the cell mass is disrupted. Some evidence for this possibility exists: in most of the attempts to fuse cells from different podocysts, polyps with dual or multiple oral regions are produced (Figure 3). This would be expected if the tip cells which are the ancestors of oral structures were already determined prior to the opening of the podocyst. Further experiments, using marked cells, are necessary to distinguish between these possibilities.

TABLE 1

FATES OF CYST CELL MASSES AFTER VARIOUS TREATMENTS

After each treatment, the cysts were observed for at least two weeks.

Treatment	N	% Developed	% Reencysted	% Died
Remove 1 ^o covering, from 1.5 month cyst, minimum disruption	600	70	25	5
Remove 1 ^o covering, 1.5 month cysts, extreme disruption	200	56	44 ^a	
Remove 1 ^o covering from 6 month cysts	200	84	16 ^a	
Remove covering from very young, incomplete cysts	200	62	38 ^a	
Remove 2 ^o covering from cysts which had reencysted for 1 month	150	81	19 ^a	
Punctured or slit 1 ^o covering, cells not removed from covering, 1.5 month cysts	200	6	94	

^aNo dead cysts identified.

TABLE 2
 INCORPORATION OF ^3H GLUCOSE INTO CELLS AND
 COVERINGS OF INTACT, DORMANT CYSTS

Intact cysts or isolated coverings were incubated 24 hours in 5 $\mu\text{C/ml}$, following which the material was washed and counted. Cysts were held for either 10 hours or 10 days in the absence of glucose before processing for counting.

Treatment	Counts per minute	
	Cells	Coverings
Isolated coverings incubated in glucose, counted after 10 hours		26,269 32,735
Intact cysts incubated in glucose, counted after 10 hours	98,200	25,717
Intact cysts incubated in glucose, counted after 10 days	28,594	10,533

FIGURE 1

Development of a podocyst at 25° C following removal of the covering. X 240.

- A. After 5 to 10 minutes the contour of the cell mass is irregular because of injury during the operation.
 - B. At 30 minutes the cell mass has already begun to round up.
 - C. After 24 hours.
 - D. After 50 hours an elongated growth has appeared at the top of the cell mass.
 - E. At 60 hours the presumptive hypostome at the tip of the growth is seen as a ridge surrounding an invagination at the site of the future mouth.
 - F. By 72 hours a feeding polyp has been formed.
- Development to this stage may require as long as 8 days in some cysts.

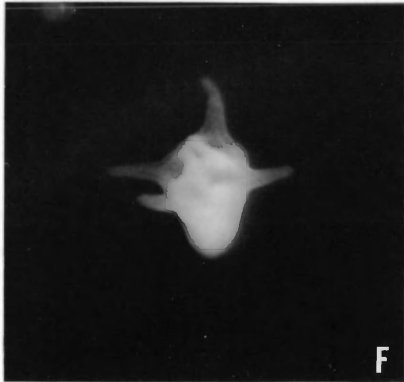
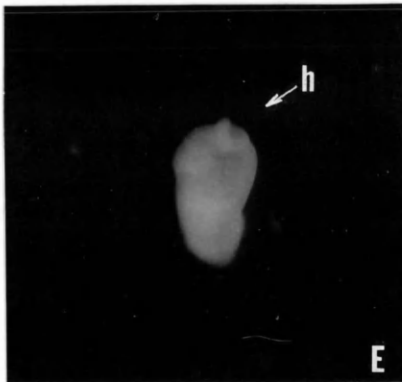
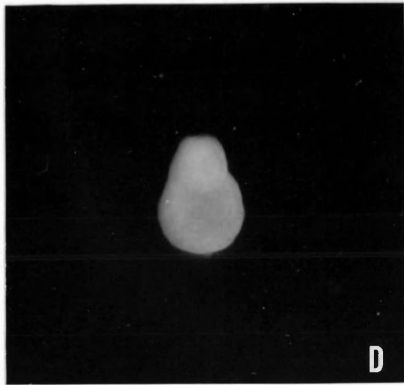
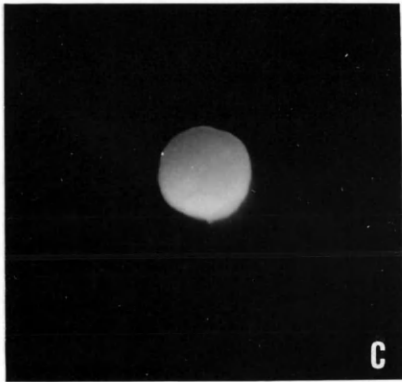
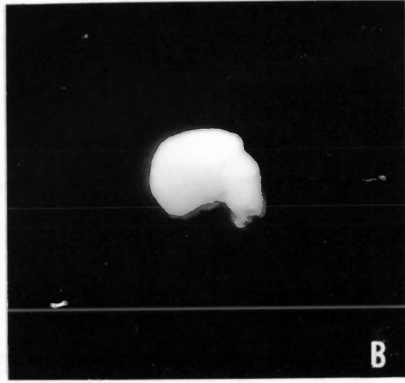
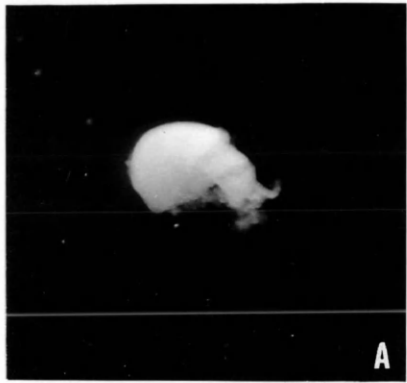


FIGURE 2

Development of the four-tentacled polyp of cysts of different ages after removal of the primary covering. (●) Cysts 0.5 to 2 months after deposition (n = 600); (□) Cysts 6 months after deposition (n = 200); (▲) Incomplete cysts (n = 200). Bars represent standard error of mean.

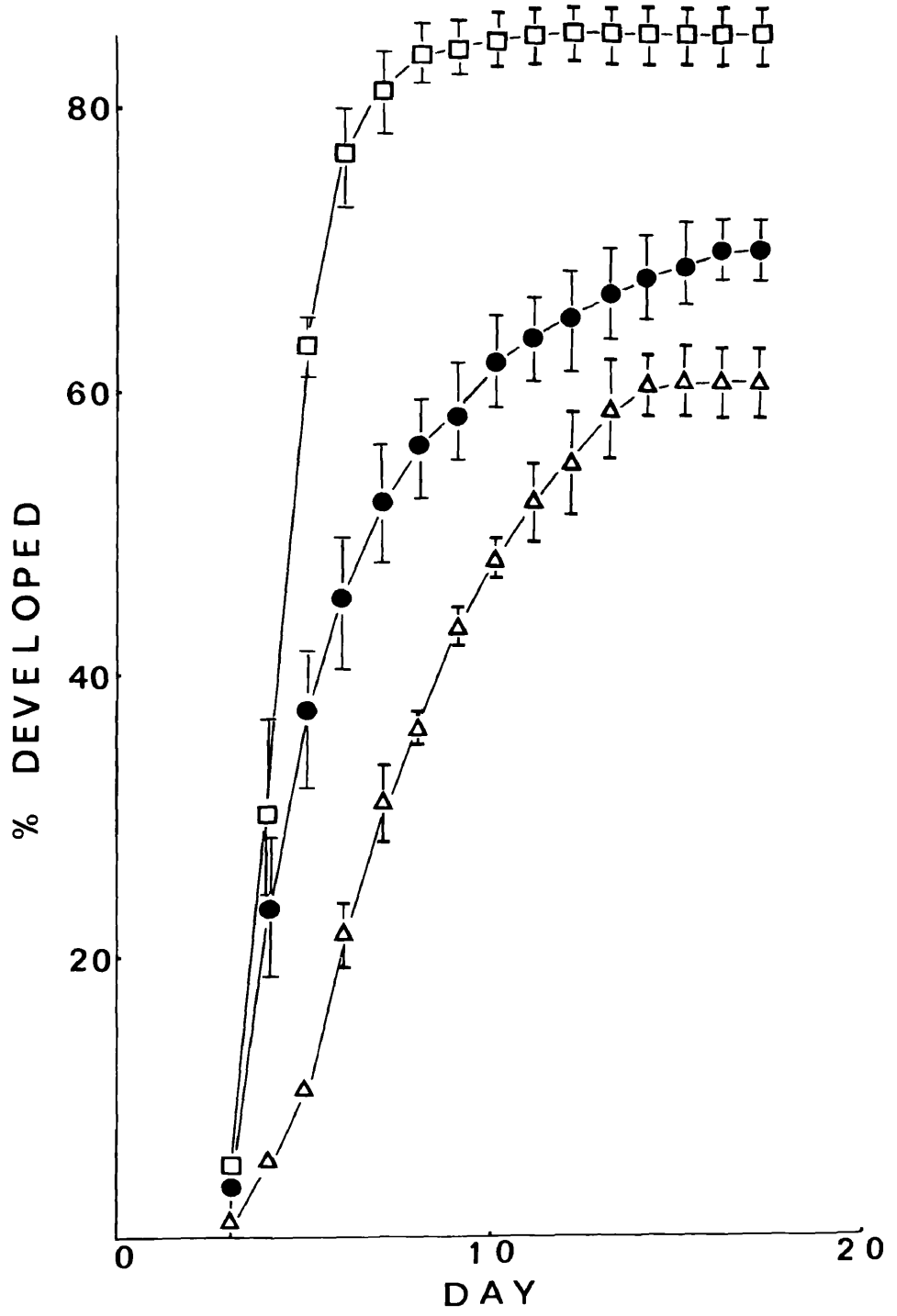


FIGURE 3

Development of cysts after removal of first and second covering. (●) Cysts from which first cover was removed (n = 600). These are the same as in Figure 2. (○) Cysts from which the second cover was removed. These had reencysted and remained dormant for a month following removal of the first covering (n = 150). Bars represent standard error of mean.

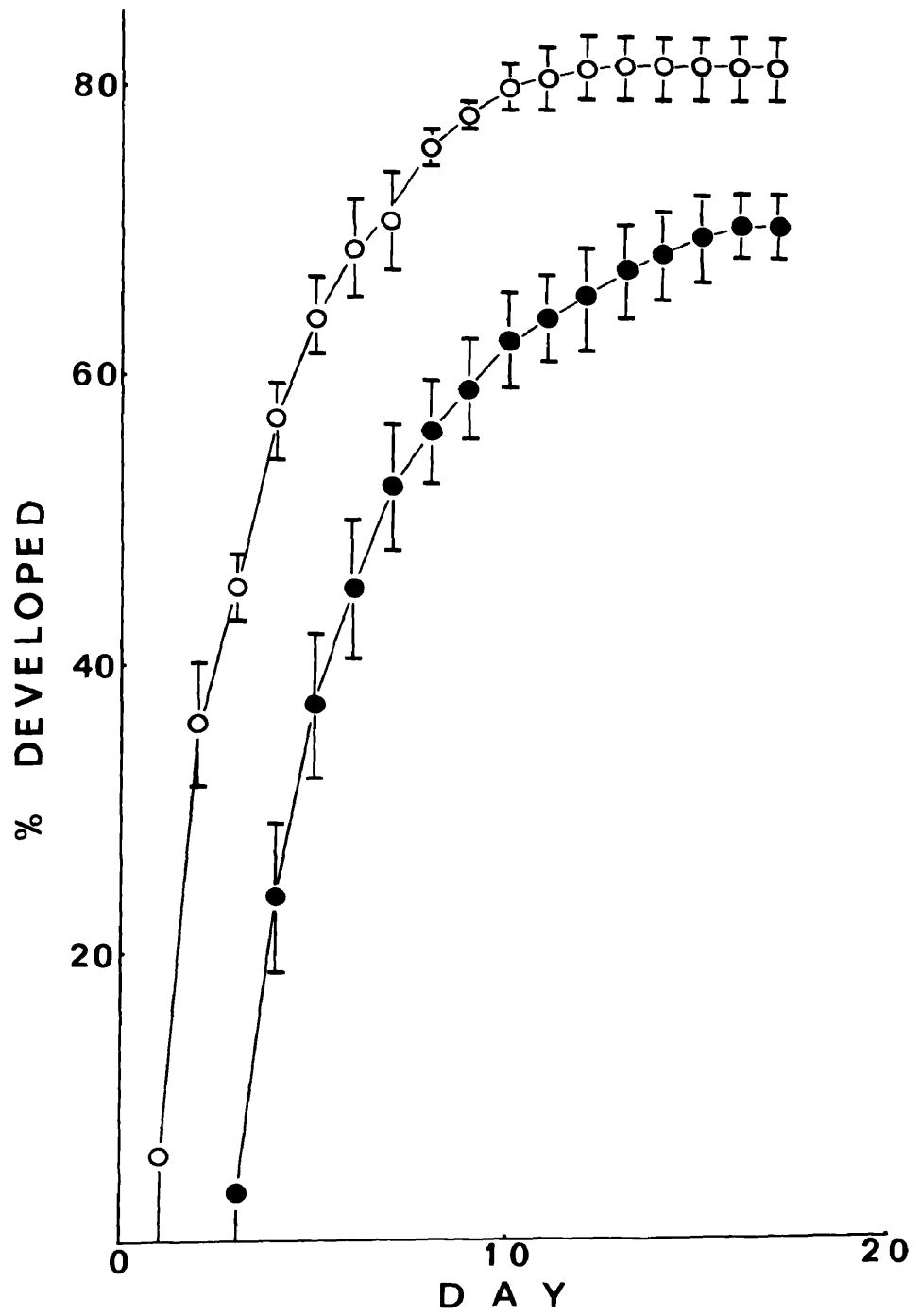


FIGURE 4

Formation of a two-headed polyp after fusion of the cell masses from two cysts. X 380.



FIGURE 5

Autoradiograph of cyst incubated in 5.0 μ C/ml 3 H-glucose for 24 hours after removal of the covering. Heavy labeling is seen at the periphery of most of the cells and in the cytoplasm of some. X 1200.

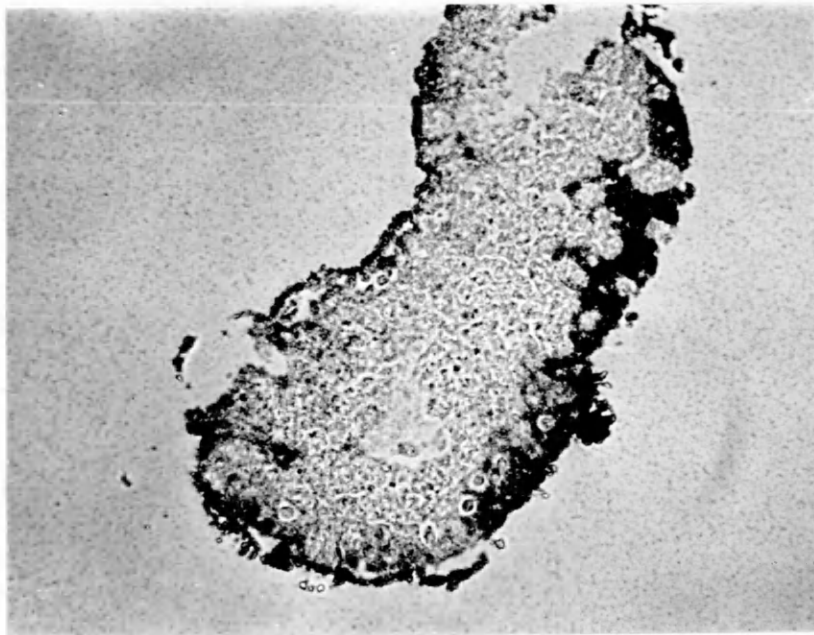
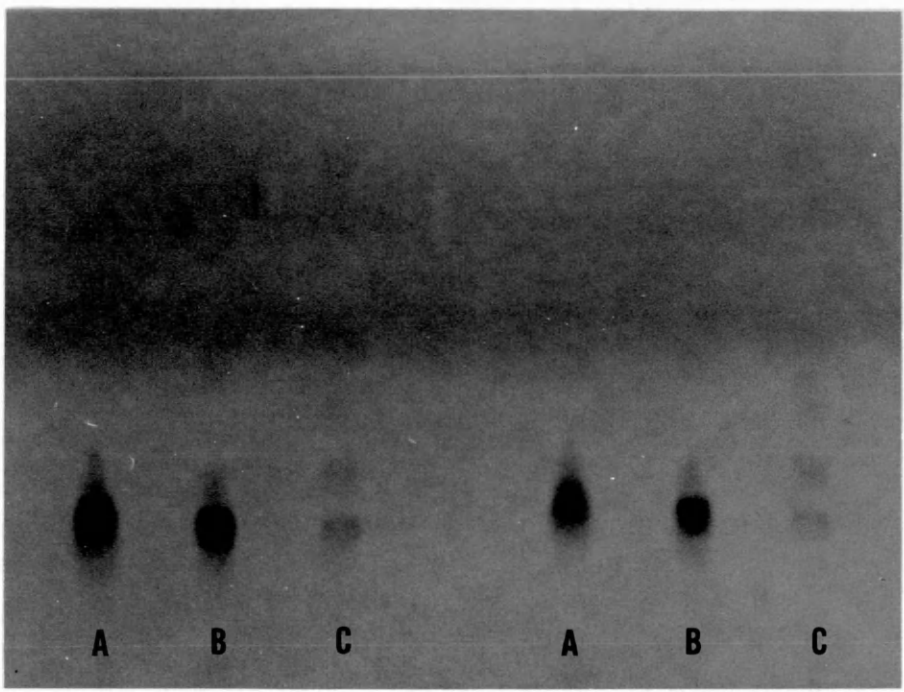


FIGURE 6

Thin-layer chromatograms of hydrolyzates of cyst covering material which was insoluble in hot alkali. Spots were visualized by spraying with ninhydrin and heating to 105° C for 5 minutes.

- A. Authentic glucosamine.
- B. Primary covering.
- C. Second covering recovered 6 days after removal of the first. Note ninhydrin-positive spots not present in first covering.



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