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STUDIES ON THE COLONIAL ASCIDIAN *BOTRYLLUS SCHLOSSERI* A REVIEW OF SOME LINES OF RESEARCH

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ABSTRACT. — Different lines of research on inland cultures of *B. schlosseri* have been pursued in our laboratory. Some of them are reviewed here. An understanding of the colony as a physiological unit both in space and time was gained by studying: a) how a vascular network develops which connects the zooids of coexisting generations, those of each generation being at the same stage, so that protogyny suffices to prevent selfing; b) the relationships between succeeding blastogenic generations as expressed by the gradual attainment of sexual maturity, and the transfer of male and female germ cells from one generation to those following; c) the ability of the colony to regulate the number of buds per zooid within the generations, and the number of coexisting generations, according to naturally or experimentally altered environmental conditions; d) the transmission through generations of epigenetic characters like the experimentally induced reversal of *situs viscerum*.

Development and differentiation of the heart in the blastozooids, the steps in their regression and the cells involved in their resorption, at the change of generation, have been investigated. The intestinal epithelium differentiates into various cell types which have been characterized by their ultrastructure and their role in the extra- and intracellular food digestion and absorption. A comparative study of the intercellular junctions in the alimentary tract of different species has shown the constant presence of tight and gap junctions and the absence of septate junctions, while scalariform junctions and *zonulae adhaerentes* were also at times recognized. Special attention has been paid to specialization of the ciliated stigmatal cells, which probably influences the metachronal coordination of ciliary beating.

The blood cell types of *B. schlosseri* have been characterized and the polychromatism in which they are involved has been genetically analyzed. The ovoviviparity of this species has been compared with that of two other species as regards egg envelopes, ovulation, egg retention in the atrial wall in *Botryllus* and *Botrylloides* and egg segregation within the tunic in *Diplosoma*.

The genetic analysis of histocompatibility has shown that, unlike *B. primigenus*,

in *B. schlosseri* selfing and intercrossing of identical genotypes are feasible and segregation distortions in the progeny from compatible colonies are rather unusual.

Key words: *Sexual and asexual reproduction. Membrane specialization. Histocompatibility. Ascidians.* Botryllus schlosseri.

INTRODUCTION

Many years ago, one of the authors (A.S.), with previous experience of sexual differentiation and experimental sex inversion in vertebrates, thought that it would have been of some interest to investigate such problems as the relationships between somatic and germ lines and between asexual and sexual reproduction, as well as germ cell differentiation into male or female elements, in the hermaphroditic colonial protochordates.

Once inland cultures of the colonial ascidian *Botryllus schlosseri* were established (SABBADIN, 1955a; 1960) and maintained, the above-mentioned and other lines of research were pursued by the senior author and his coworkers. Some of them will be reviewed in this paper.

THE COLONIAL STRUCTURE OF *BOTRYLLUS SCHLOSSERI*

The colonies of the almost cosmopolitan ascidian *Botryllus schlosseri* are clones, engendered by metamorphosing larvae, whose development has been studied, among others, by BERRILL (1941a, b) and WATTERSON (1945). They consist of *three sequential generations* of zooids: the adults with primary, grown buds, which in turn bear secondary, junior budlets (Fig. 1). Buds arise as bilateral evaginations of the parental atrial epithelium coated by epidermis, and remain connected to the parents by an epidermic hollow

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Plate I

Figs. 1, 2, 4, 5. — B. schlosseri in vivo.

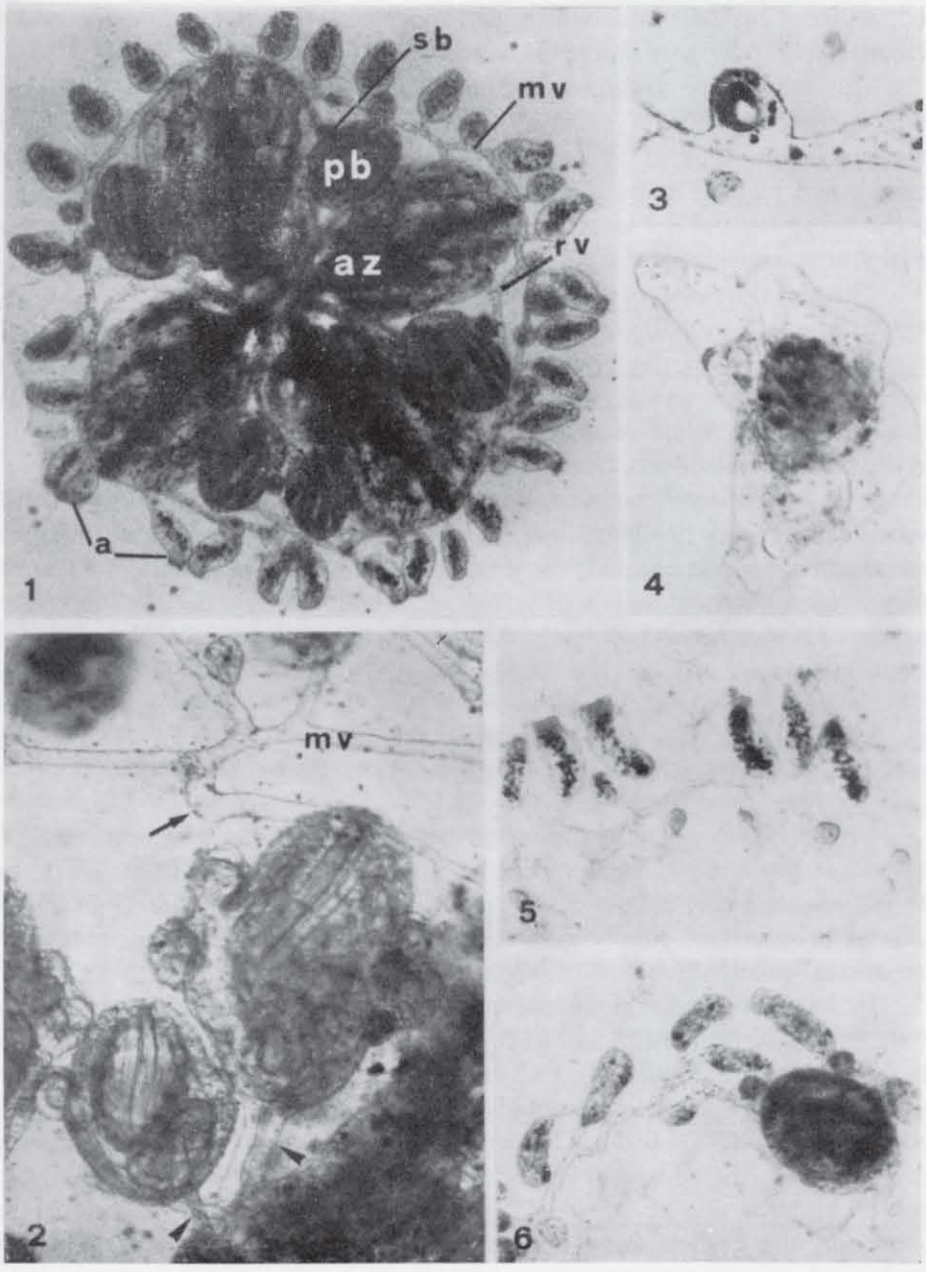
Fig. 1. — Young colony from the ventral side: adult zooids (**az**), primary (**pb**) and secondary (**sb**) buds within the tunic, the first two generations linked through radial vessels (**rv**) to the peripheral marginal vessel (**mv**), from which a crown of ampullae (**a**) branch out. (× 20)

Fig. 2. — Anterior and posterior buds, with their budlets, connected to the same parental zooid by a hollow peduncle (**arrowheads**) and to the marginal vessel (**mv**) by radial vessels (**arrows**). (× 70)

Fig. 3. — A vascular budlet is forming within a vessel in a hibernating colony of *Botrylloides leachi*. Haemalum-eosin. (× 240)

Fig. 4. — An isolated bud has formed its own vascular network. (× 40)

Figs. 5 and *6.* — One of the budlets isolated within the colonial matrix at the vesicle stage (Fig. 5), in the course of differentiation (Fig. 6). Fig. 5 (× 30). Fig. 6 (× 40)



stalk that conveys blood and nutrients. The three coexisting generations are strictly correlated throughout the stages of their life cycles. At 18°C a weekly change of generation takes place: the adults regress and are resor-

bed, replaced by their buds which gain functional maturity, while the budlets give rise to a new generation (SABBADIN, 1955a).

The zooids are arranged in star-shaped systems, the individual atrial siphons opening into a common cloaca. They are embedded within the gelatinous tunic crossed by a vascular network, that joins a marginal vessel from which stalked ampullae branch out (Fig. 1). These fasten the colony to the substrate and act as reservoirs of blood in whose circulation they share by rhythmic contractions.

The development of the vascular system running across the tunic has been studied in our laboratory by BRUNETTI and BURIGHEL (1969): eight ampullae, ventral to the branchial basket of the larva, during metamorphosis expand radially and give rise to a marginal vessel by fusing their stalks. Each new zooid, while maintaining its connection with the parent through the epidermic stalk, joins the marginal vessel through a radial vessel. At the change of generation (the « takeover ») the epidermic stalks outlive the resorbed adults and fuse into an interzooidic vascular network. When new systems of zooids are added, the fusing radial vessels of continguous zooids become intersystemic vessels. The colony, owing to this definite connection between its components, behaves like a physiological unit, as shown by the synchronous evolution of the zooids belonging to the same generation.

ASEXUAL REPRODUCTION: AN EXPERIMENTAL ANALYSIS

In the zooids there is an asymmetry of the blastogenic powers of the two sides: the bud primordium on the right side appears a little earlier than the left one and may subdivide into anterior and posterior buds (Fig. 2); the sinistral primordium subdivision occurs subordinately to the subdivision of the dextral primordium.

In laboratory conditions the posterior buds are often resorbed, those of the right side subordinately to those of the left; resorption can also affect all the anterior sinistral buds and exceptionally some of the anterior dextral buds, so that the number of zooids does not increase or even decreases in some generations (Table I). Competition between the coexisting generations may exceptionally add to this intrageneration competition: adults may regress before the due time, when their buds cannot yet gain functional maturity and the budlets have not yet reached the budding stage. Thus the number of coexisting generations is reduced to two, as though the adult generation could only supply the nutrient needs of its buds by being resorbed (SABBADIN, 1955a, b; 1958).

These observations suggested an experimental analysis of blastogenesis. Starting from young colonies consisting of the oozooid, its dextral bud and

Table I

The blastogenic powers of the two body sides expressed by the zooids of B. schlosseri in the first 8 blastogenic generations (SABBADIN, 1958)

Bud position	No. of buds produced	No. of buds resorbed
Dextral anterior	339	57 (16.8%)
Sinistral anterior	339	227 (67%)
Dextral posterior	81	74 (91.3%)
Sinistral posterior	14	14 (100%)

budlets, the latter were removed, except one, throughout a sequence of generations, so that a single zooid per generation was allowed to reach functional maturity. The number of new budlets, generated by the treated colonies was compared with that of the budlets generated in the control series by the anterior dextral buds, which are the most productive. The experiment showed that in treated colonies the percentage of posterior budlets doubled, and much fewer of them regressed before being removed, that is the blastogenic powers were enhanced and the differences between the two body sides greatly reduced (Table II). Besides, the treated zooids gained significantly greater size and their adult phase lengthened in time so that four, or even five, generations could come to coexist in the colony instead of the usual three generations. This confirmed the competition between generations.

Table II

The blastogenic powers of the dextral buds in the control set of colonies and of the residual buds in the experimental set (SABBADIN, 1958)

Set of colonies	buds No.	buds post.	posterior buds No.	posterior buds sinistral	posterior buds resorbed
controls	323	17.6%	57	15.8%	71.9%
experimental	514	37.3%	192	40.1%	14.1%

It had been shown (OKA and WATANABE, 1957a; 1959) that clusters of stem cells from the blood line can take on the function of buds in different botryllid species. This vascular budding, that in *Botryllus primigenus* associates spontaneously to the palleal budding and in *Botrylloides leachi*

occurs in hibernating colonies, as shown in our laboratory by BURIGHEL *et al.* (1976) (Fig. 3), necessitates the removal of all the zooids in *B. schlosseri* (Milkman, 1967): vascular buds then appear that are able to restore the colonial structure (Fig. 4) (SABBADIN *et al.*, 1975).

It may be concluded that *B. schlosseri* colonies are highly homeostatic systems that can withstand a range of diverse environmental conditions by adjusting the number of zooids per generation and the number of coexisting generations, and by raiving the vascular budding.

Oozooids bring to maturity only the dextral bud, the development of the sinistral bud being stopped at a stage preceding organogenesis. We observed that the removal of the dextral bud close to the time of colonial takeover causes the atrophic sinistral bud to resume development; however its congestion with the resorption products of the adult sometimes disturbes the course of organogenesis, with the possible consequence being the inversion of *situs viscerum*: the heart becomes positioned on the left side and the gut on the right. Once installed in the colony this anomaly is transmitted to the buds from generation to generation and involves also the inversion of the blastogenic powers, the left side becoming more productive than the right side (SABBADIN, 1958; 1960).

The following experiments were devised in order to study this phenomenon more closely (Figs. 4-6): a) young buds were isolated in the tunic, before organogenesis began, by removing the other zooids, and were used as controls (Figs. 5 and 6); b) other buds at the same stage were grafted from colonies with normal *situs* into colonies with inverted *situs viscerum* and vice versa; c) the development of vascular buds was induced in colonies of either type of *situs viscerum*.

It resulted that the buds always developed the *situs viscerum* of the parental colony. We suggested that the bilateral asymmetry is predetermined and impressed on the palleal buds by the enveloping parental epidermis and on the vascular buds by the vessel wall, an epidermic derivative, from which their primordia are enveloped. As for the antero-posterior polarity, it seemed to be conditioned by vascularization, the entrance point of the tunic vessel that reached the isolated or grafted buds, or the point of the tunic vessel from which the vascular buds projected, becoming the posterior end of the new zooid (SABBADIN *et al.*, 1975).

Buds connected to the vascular network of the tunic through their radial vessel can outlive the removal of the parents. If also the radial vessels are removed, most buds regress; but a few can survive and develop a new extracorporeal vascular system which eventually allows reconstruction of the colonies (Fig. 4). That is, blastozooids can undertake a task that in normal development of the colony is carried out by oozooids, whose genetic complement they share (ZANIOLO *et al.*, 1976).

BLASTOZOOID DIFFERENTIATION AND RESORPTION

Blastozooid development commences from the palleal bud primordium, a thickening of the parental atrial epithelium covered by the epidermis, which then arches into a prominent emisphere and eventually into a double-walled vesicle. Folds of the inner wall cause the cavity to subdivide into a central pharyngeal and two peribranchial chambers. A posterior evagination of the pharyngeal chamber gives rise to the intestinal tract (BERRILL, 1941a; BURIGHEL, 1970).

We focused attention on: a) differentiation of the heart and vascular system; b) differentiation and functions of the alimentary tract epithelium; c) blastozooid resorption at the generation change.

Heart and vascular system (BURIGHEL, 1979; NUNZI *et al.*, 1979). The heart primordium, which in the larva results from a congregation of mesenchymal cells discending from the sides of the brancial chamber, in the blastozooid first appears as a compact mass of cells on the right side of the atrial chamber between the rudiments of the endostyle and gut (Fig. 12). This mass then hollows and the resulting vesicle elogantes to give a tubular monolayered structure; its wall invaginates along a dorsolateral line forming an inner vesicle, the myocardium, that remains attached to the enveloping pericardium. In the cells of the myocardium, myofibrils develop which recall the larval tail musculature in the striation type and filament orientation characteristic of the chordates (BURIGHEL *et al.*, 1977; SCHIAFFINO *et al.*, 1976).

The bud is already vascularized from its early stages through the parental epidermic stalk. Though blood circulation occurs in lacunae and sinuses without a sharply delimited endothelial wall, it follows a well defined pattern including the mantle sinuses — intersiphonal, lateral and periatrial — the dorsal and interstigmatic branchial sinuses, and the perivisceral lacunae, the three sections being interconnected by the main subendostylar sinus in prosecution of the heart (BURIGHEL and BRUNETTI, 1971).

Alimentary tract (Fig. 7). Histological, electron microscopical and cytochemical investigations have provided a comprehensive view of the alimentary tract development, cell type differentiation and functions (BURIGHEL, 1970; BURIGHEL and MILANESI, 1973; 1975; 1977). The ciliary beating of mucous cells replaces the missing peristalsis in making the food cord progress; extracellular digestion in the stomach, carried out by zymogenic cells, is backed by intracellular digestion in the main type of gastric and intestinal cells, the vacuolated cells, which, besides absorbing the digested material, are also able to ingest, in endocytotic vesicles, and then digest macromolecules, as shown by using horseradish peroxidase (HRP) as a tracer (Fig. 8) (BURIGHEL, 1979b). A possible regulatory function, which in vertebrates is

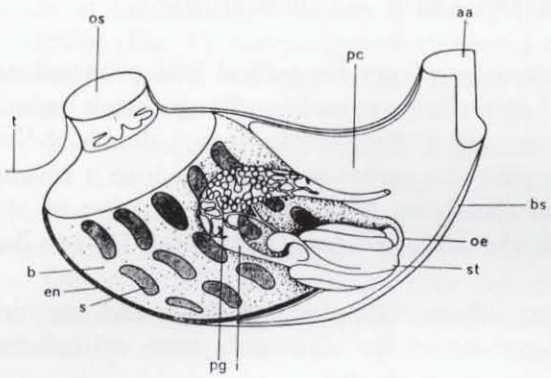


Fig. 7. — Schematic drawing of a *B. schlosseri* zooid seen from the left side. **aa**: atrial aperture; **b**: branchial sac; **bs**: blood space; **en**: endostyle; **i**: intestine; **oe**: oesophagus; **os**: oral siphon; **pc**: peribranchial cavity; **pg**: pyloric gland; **s**: stigma; **st**: stomach; **t**: tunic.

devolved to different organs, seems to pertain to endocrine cells scattered in the gut epithelium, and to plicated cells, so-called because of the enormously extended plasmalemma infoldings; for these last cells the demonstration of scalariform junctions, typical of cells involved in active transport of ions and/or liquid, has confirmed the previously suggested osmoregulative function (BURIGHEL *et al.*, 1985a).

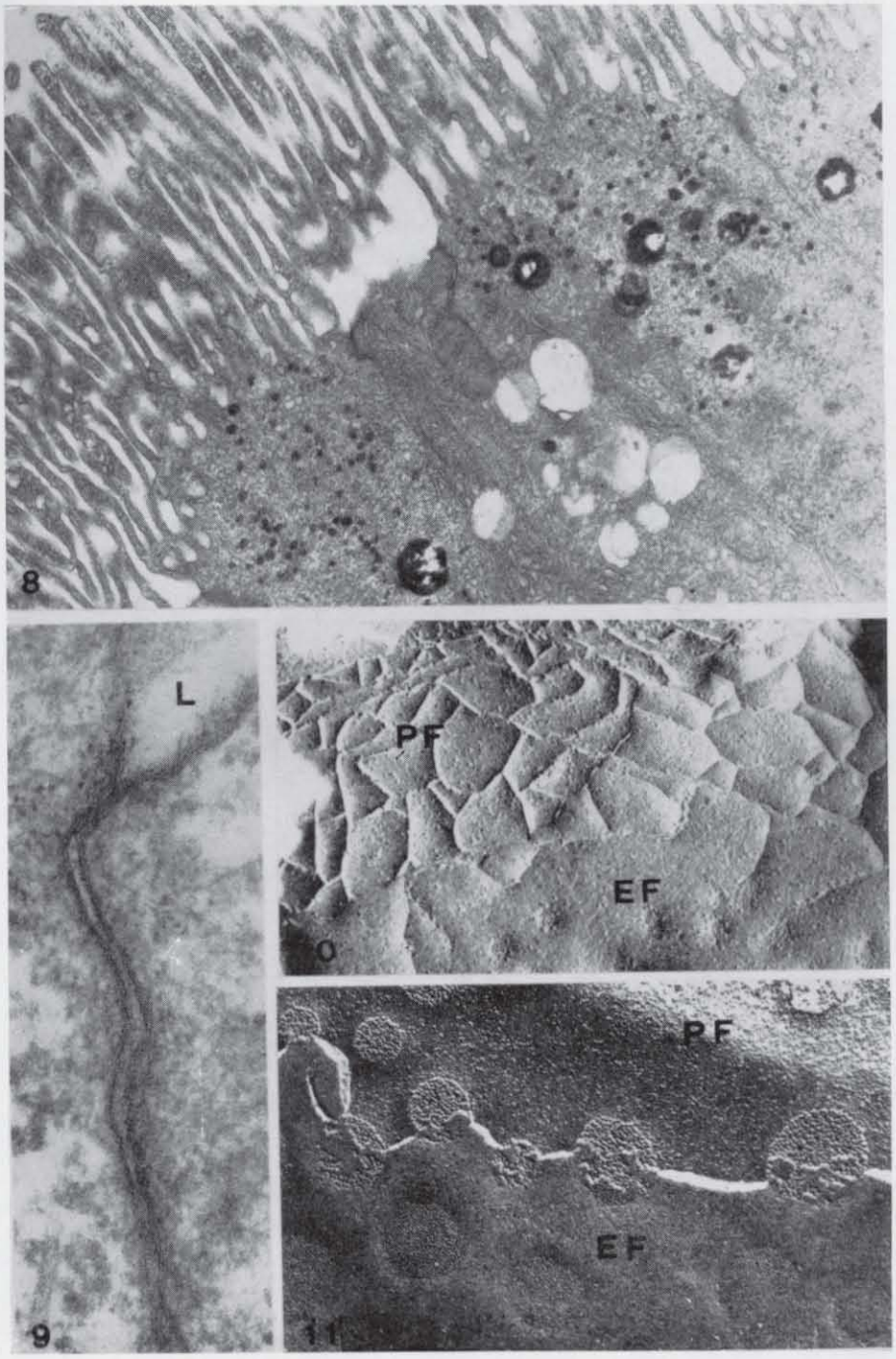
In order to get a better insight into the cell interactions in the alimentary tract, the intercellular junctions were studied comparatively in different genera and species (Figs. 9-11). Gap junctions, allowing controlled intercellular communication, tight junctions, acting as permeability barriers, but no septate junctions were found in all the species examined (LANE *et al.*, 1986). Extended tight junctions were also observed in the branchial basket between the ciliated stigmatal cells involved in strong mechanical stress, thus giving evidence of an adhesive function of these junctions. In the Aplousobranchiata and Phlebobranchiata, stigmatal cells are also connected by *zonulae adhaerentes* which seem to be lacking in the Stolidobranchiata (MARTINUCCI *et al.*, 1988b).

Plate II

Fig. 8. — Electronmicrograph of gastric cells of *B. schlosseri* ten minutes after feeding the zooids on HRP. The dark reaction product of HRP is seen in pynocytotic invaginations and vesicles at the apical region of vacuolated cells. It is absent in the contiguous zymogenic cells where several secretory granules are recognizable. For the experimental procedure see BURIGHEL, 1979. (× 10,500)

Fig. 9. — Section through a tight junction of the gastric epithelium of *B. schlosseri*. **L**: lumen. (× 92,000)

Figs. 10 and 11. — Replicas of the alimentary tract of *Clavelina* (Fig. 10) and *Lissoclinum* (Fig. 11). In Figure 10 the tight junction features a network of IMPs fracturing on P-face (**PF**) of the membrane and occasionally on the E-face (**EF**) grooves. In Figure 11 the numerous round gap junctions are seen to fracture IMPs on the P-face (**PF**) and correspondent pits on the E-face (**EF**). Fig. 10 (× 46,000). Fig. 11 (× 40,000)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Each stigma in all the ascidian species is a modular structure consisting of seven rows of flattened cells, each one with a single row of cilia beating in metachronal coordination (DALLAI *et al.*, 1985; MARTINUCCI *et al.*, 1987; BURIGHEL *et al.*, 1988). In the stigmatal cells the basal ciliary shaft presents a dense material lying between the ciliary membrane and the axonemal doublets, fuzzy coat, and clusters of intramembrane particles (IMPs) on the protoplasmic P-face of the membrane. These specializations suggest a regulatory role in ciliary beating (BURIGHEL *et al.*, 1986; MARTINUCCI *et al.*, 1991).

Blastozooid resorption. In the colonies at the takeover phase the adult zooids close their siphons with cessation of the filtering activity, and contract following the activation of the cytoplasmic microfilaments. Their tissues then begin to degenerate while circulating and fixed phagocytes are activated. Tissue debris and entire cells are attacked and disposed of by fixed and circulating phagocytes (BURIGHEL and SCHIAVINATO, 1984), resembling what happens to the larval tail during metamorphosis (SCHIAFFINO *et al.*, 1974).

A STUDY OF SEXUAL REPRODUCTION

In grown colonies bilateral gonad primordia appear in the youngest generation as clumps of undifferentiated cells adhering to the atrial epithelium (Fig. 12). Their lateral portion becomes an ovary, whose differentiation is completed in the primary buds, in the form of a loose structure with oocytes of different size and stage. The mature oocytes are ovulated and after pas-

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Plate III

Figs. 12-16. — B. schlosseri.

Fig. 12. — Transverse section of a young bud at the beginning of inner wall infolding. On both sides, gonad primordia adhere to the presumptive atrial epithelium. Heart anlage (**arrowheads**) is located between epidermis (**ep**) and the endostyle (**en**). Haemalum-eosin. (× 350)

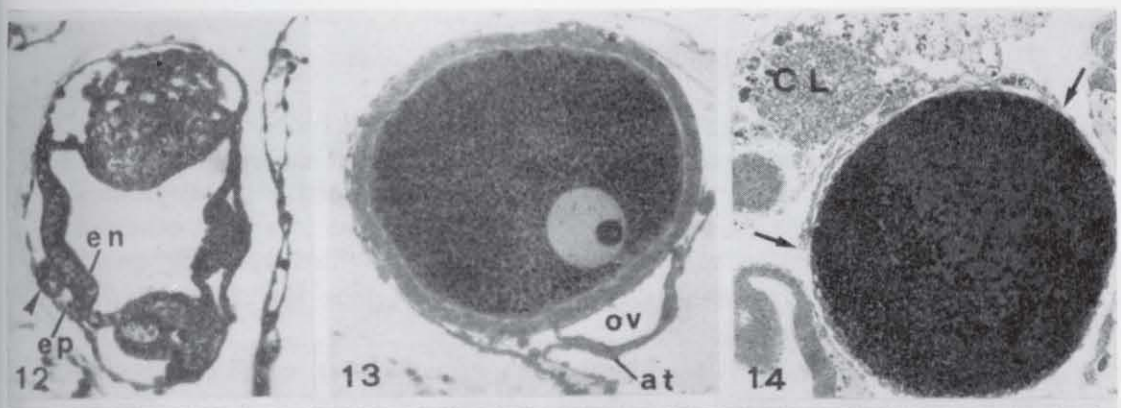
Fig. 13. — The empty vesicular oviduct (**ov**) interposed between, and closely contacting, the large oocyte and the atrial epithelium (**at**). Toluidine blue. (× 230)

Fig. 14. — Immediately after ovulation, the egg is held in the atrial chamber by means of a cup-like 'placenta' whose limits are indicated by **arrows.** The remnant of the outer follicle cells forms the corpus luteum (**CL**) in the mantle. Toluidine Blue. (× 230)

Fig. 15. — Electronmicrograph showing details of an oocyte during vitellogenesis. Numerous microvillar extensions are seen to penetrate the vitelline coat (**VC**). **TC**: test cell; **IFC**: inner follicle cells; **OFC**: outer follicle cells. (× 6,200)

Fig. 16. — Sagittal section of a spermatid of *B. schlosseri* passing along the fusiform dense nucleus. The mitochondrion is not shown. (× 35,000)

Fig. 17. — *Diplosoma listerianum*. Transverse sections of spermatozoa at various levels. Sections of the main components of the head are recognizable: the dense groove (**arrows**), the long mitochondrion (**mt**) associated with the dense nucleus, and the endoplasmic derivatives (**arrowheads**). (× 13,000)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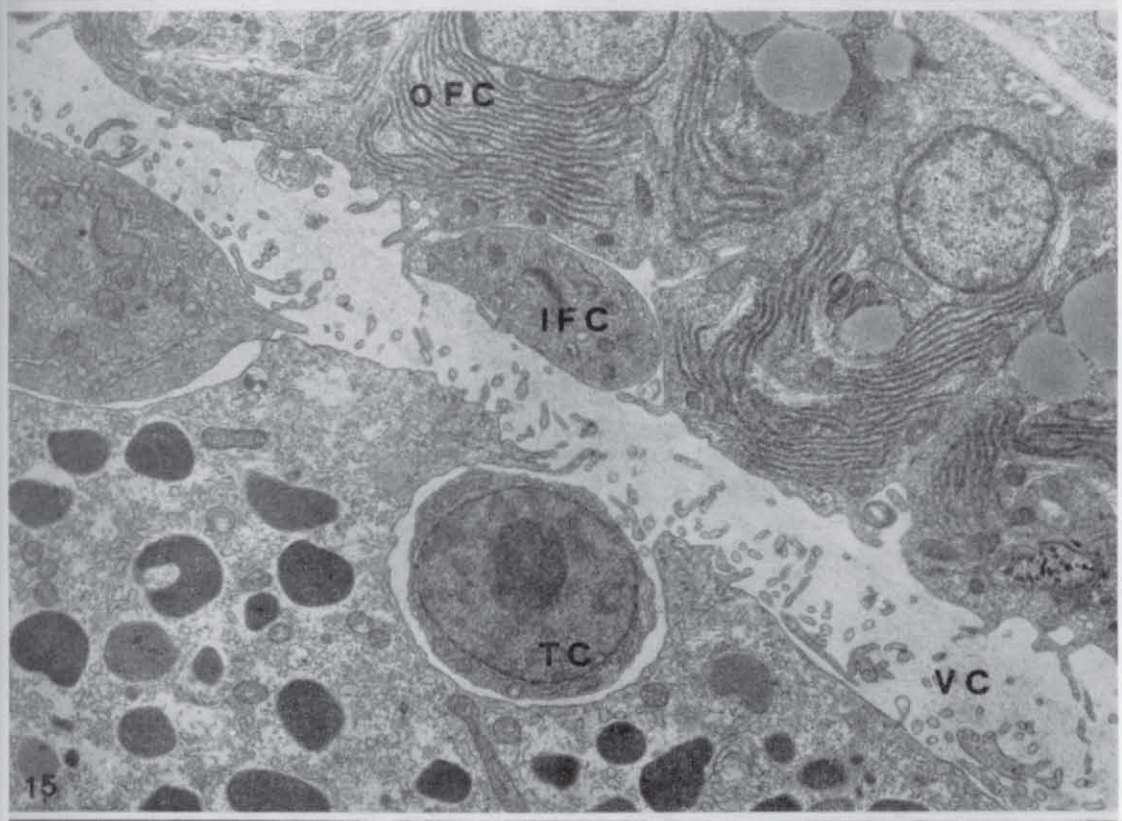
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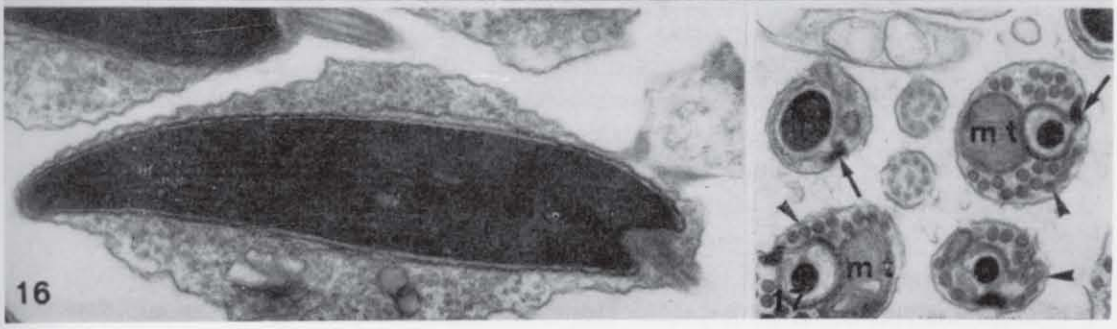
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sing through the oviduct, are kept in a newly formed brood pouch on the wall of the atrial cavity, where they are fertilized when the opening of the siphons allows the entry of sperm. The medial portion of the gonad primordia differentiates into a testis, in the grown buds, in the form of a coherent structure which reaches maturity in the adults and discharges the sperm one to two days after ovulation, thus avoiding selfing (MILKMAN, 1967; SABBADIN and ZANIOLO, 1979).

The gonadogenic powers, like the blastogenic powers, vary in the zooids according to their dextral or sinistral, anterior or posterior origin, but, at variance with them, they are significantly higher on the left side of the body as regards the presence, size and maturability of testes, and the number of eggs and of developing embryos. The opposite condition occurs in the colonies with the bilateral asymmetry of viscera reversed (SABBADIN, 1960; SABBADIN and ZANIOLO, 1979).

Gonads are not present in the oozooids, nor in the first blastogenic generations. Some generations follow in which the gonad primordia do not differentiate or the oocytes do not complete vitellogenesis and testes disappear before ripening (SABBADIN and ZANIOLO, 1979).

It has long been known from *in vivo* observations that the loose structure of the ovaries allows the individual oocytes to be captured by the blood stream and conveyed to other zooids. Their recycling through successive generations has been considered by some authors to be a necessary condition for their maturation. Nothing was known about a possible recycling of male elements.

An experimental analysis of germ cell fate in the colonial cycle was devised (SABBADIN and ZANIOLO, 1979): we prepared a series of parabiotic pair fusions between colonies of the opposite genotypes *AAbb* and *aaBB*, with reference to the dominant and recessive alleles of two pigmentation characters (SABBADIN and GRAZIANI, 1967a). After being fused for some days the two partners of each pair were separated and individually crossed with the double recessive genotype *aabb* for many successive generations. Offspring phenotypically *aB*, from *AAbb* parents, and phenotypically *Ab*, from *aaBB* parents, were considered to have arisen from germ cells received from the partner during the fusion period. In 16 out of 25 crosses, in which ex-parabionts were used as males, and in 15 out of 28 crosses, in which they were used as females, part of the offspring were heterochtonous offspring resulting from germ cells exchanged during parabiosis (Table III). Heterochtonous offspring were still collected at the 14th and 15th generation starting from the youngest of the generations coexisting in the colonies when fusion was interrupted. In some cases the entire offspring from certain generations were heterochtonous. These results suggest, in addition

Table III

Ex-parabionts, genotypically AAbb and aaBB, individually crossed in the double way with colonies aabb, give high percentages of heterochthonous offspring phenotypically aB and Ab, respectively (SABBADIN and ZANIOLO, 1979)

Parental genotypes	No. of crosses	Offspring total	Offspring heterochth.
♀ AAbb × ♂ aabb	16	244	(aB) 36
♀ aaBB × ♂ aabb	12	266	(Ab) 105
		510	141 (27.6%)
♂ AAbb × ♀ aabb	15	478	(aB) 188
♂ aaBB × ♀ aabb	10	492	(Ab) 185
		970	373 (38.4%)

to the circulation of oocytes, a circulation of already determined primordial germ cells that will evolve into gametes of either sex.

Some conclusions can be drawn about the reproduction of *Botryllus* colonies. These are genetic individuals whose successive blastogenic generations, while each performing a total ontogenesis, must be considered as physiological stages, some of them able to bring to an end what previous generations did not succed in doing, as, for instance, germ cell production and/or maturation. Colonies tend to perpetuate their own genotype through sequential sexual cycles. Besides, the colonial matrix is also able to memorize and transmit epigenetic experiences, such as the reversed *situs viscerum*.

COMPARATIVE ASPECTS OF THE SEXUAL REPRODUCTION

B. schlosseri is ovoviviparous, like most colonial ascidians, with eggs of large size enveloped by an outer and inner layer of follicle cells, which a loosely fibrillar vitelline coat isolates from the test cells encrusted on the egg surface (Figs. 13, 15). Unlike solitary ascidians of this species, as well as other colonial ascidians we studied (*Botrylloides leachi* and *Diplosoma listerianum*), the outer follicle cells form a thick layer and their ultrastructural features are consistent with intense protein synthesis. They are discharged at ovulation to form a sort of transient corpus luteum (Fig. 14). In *B. schlosseri* the egg, enveloped by the inner follicle, enters a vesicular oviduct, forces the atrial epithelium reaching the atrial chamber, in which it hangs by a cuplike structure, formed by the atrial and oviductal epithelia and the inner

follicle (Fig. 14) ZANIOLO *et al.*, 1987); in *Botryllodies leachi*, on the other hand, the atrial epithelium segregates the egg within a brood pouch (ZANIOLO *et al.*, 1990). In both species fertilization occurs in the water filling the atrial chamber and therefore it may be referred to as external fertilization.

On the contrary, in *Diplosoma listerianum* the growing oocytes strech the ovarian wall into a hollow peduncle thus projecting into the tunic within an epidermic sac (BURIGHEL *et al.*, 1987). Here the eggs are reached by sperm entering the ovary through a short oviduct communicating with the atrial chamber; in this species, at variance with botryllids, sperm show a complex structure, possibly related to this internal fertilization (Figs. 16 and 17) (BURIGHEL *et al.*, 1982; 1985b). The fertilized eggs develop within the tunic, surrounded only by the epidermis (MARTINUCCI *et. al.*, 1988a).

It is known that test cells play a debatable role. We have studied their differentiation during oogenesis and embryogenesis in *Botryllus* (ZANIOLO *et al.*, 1989). Test cells appear in the previtellogenic stage as derivatives from the primary follicle cells; during vitellogenesis, they show a well developed Golgi apparatus composed of a large number of elongated cisternae, and an increasing number of electrondense granules form in the cytoplasm. After fertilization, they loose progressively their homogeneity and density; in the embryo at the tail bud stage many of them are released from the cells and are observed to adhere to the outer border of the tunic primordium, apparently contributing the electron-dense material which forms its cuticular layer. In the late tadpole stage, shortly before hatching, test cells with little or no granules are observable outside the tunic.

Table IV

Normal or reversed situs viscerum (S.V.) expressed by treated budlets according to the paternal situs viscerum (SABBADIN *et al.*, 1975)

Budlet type	Parental S.V.	S.V. of buds	
		normal	reversed
isolated	normal	106	—
	reversed	—	82
transplanted	donor & recipient reversed	—	22
	donor normal, recipient reversed	5	—
vascular	normal	33	1
	reversed	—	15

Table V

Genetic *analysis of histocompatibility between colonies of* B. schlosseri (SABBADIN *et al.*, 1992)

Parental genotypes	No. of crosses	Offspring genotypes			
AB × CD	11	AC	AD	BC	BD
		64	57	68	63
AC × BC	9	AB	AD	CB	CD
		62	60	68	50
AB × BD	9	AB	AD	BB	BD
		82	78	73	84
AB × AB	4	AA	AB	BB	
		30	79	32	
AA × BD	6	AB	AD		
		88	81		
BB × BD	16	BB	BD		
		190	193		
AA × AA	10	AA			
		186			

BLOOD CELL LINE AND CHROMATIC POLYMORPHISM

In the blood plasma of ascidians there are different cell types that we have characterized in *B. schlosseri* by light and electron microscopical studies Figs. 18-21) (SABBADIN, 1955b; MILANESI and BURIGHEL, 1978; BURIGHEL *et al.*, 1983). They can be derived from a lymphocyte-like stem cell in the embryonic mesenchyme, which in this species gives rise to two series of hemocytes: (a) micro-and macrogranular granulocytes, signet ring cells as phagocytes, compartment cells, morula cells and nephrocytes represent a series of constant elements, while (b) there are vacuolated pigmented cells — orange, blue and reddish cells — whose presence/absence is genetically determined by three Mendelian independent gene loci. Two other loci have been found to control the distribution of nephrocytes around and between the siphons according to defined patterns. The various combinations of these characters give rise to an arrow of colour morphs (SABBADIN, 1959; SABBADIN and GRAZIANI, 1967a), differing in both geographical and ecological distribution (SABBADIN and GRAZIANI, 1967b; SABBADIN, 1978), which have proved

useful as markers in certain experiments (SABBADIN, 1971; SABBADIN and ZANIOLO, 1979).

HISTOCOMPATIBILITY

Many species of colonial ascidians are known for their « colony specificity »: brought into contact they either show a compatibility expressed by fusion of the tunics and anastomosis of the vascular systems, or an incompatibility expressed by a « nonfusion reaction », often in the form of rejection entailing tissue necrosis (Figs. 22-24) (see TANEDA *et al.*, 1985 for a review). In *Botryllus*, fusion or rejection occurs spontaneously whenever the peripheral vascular ampullae of two facing colonies come into contact. OKA and WATANABE (1957b; 1960) showed that in *Botryllus primigenus* fusion and rejection are controlled by a polymorphic gene locus with many codominantly expressed alleles: fusion occurs when even a single allele is shared: four classes of offspring with 75% fusibility — AC, AD, BC, and BD — are obtained by crossing two incompatible colonies, AB and CD. We have shown that the same holds true for *B. schlosseri* (SABBADIN, 1962; 1982). No selfing occurs in *B. primigenus* and moreover sperm sharing their allele with the egg envelopes are sterile: the double-way cross between F_1 BC and BD colonies yields F_2 offspring only segregating into two classes — BC, CD and BD, CD respectively. Therefore in this species all the colonies are heterozygous at the fusibility locus.

Things are different with *B. schlosseri*, in which even selfing, normally prevented by protogyny, can be made possible by crossing pieces of the same colony at different sexual stages (SABBADIN, 1971). Therefore, in this

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Plate IV

Figs. 18-24. — *B. schlosseri.*

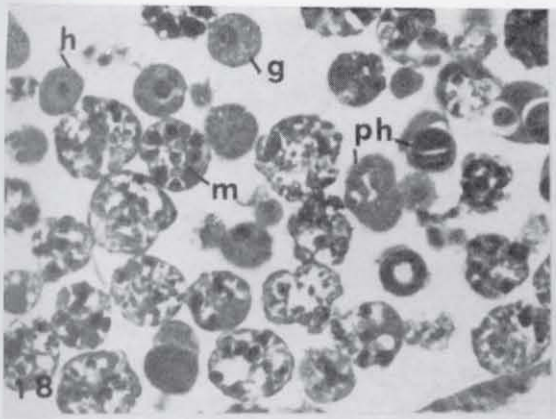
Fig. 18. — Among the different blood cell types, hemoblast (**h**), granulocytes (**gr**, morula cells (**m**) and phagocytes (**ph**) are recognizable. Toluidine Blue. (× 1,000)

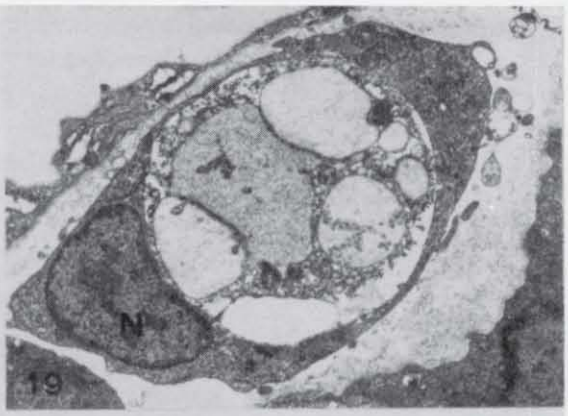
Figs. 19-21. — Electronmicrographs. The phagocyte in Figure 19 is characterized by its giant vacuole filled with heterogeneous material, whereas the microgranular granulocyte of Figure 20 shows numerous small, dense and round membrane-bound granules. In Figure 21 detail of a pigmented cell. The granules contained in the large vacuoles correspond to the *in vivo* « reddish-blue » granules whose genetic determinism has been defined. **N**: nucleus. Fig. 19 (× 10,500). Fig. 20 (× 9,000). Fig. 21 (× 16,000)

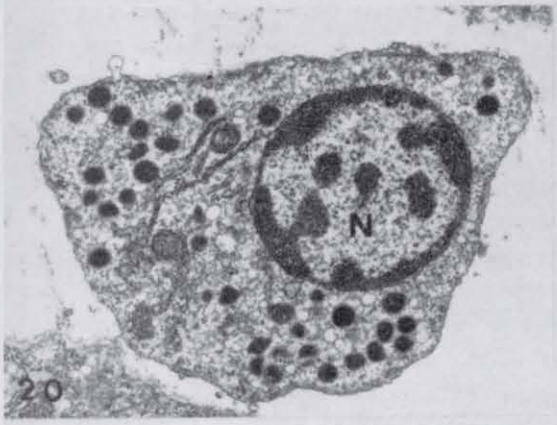
Fig. 22. — Chimera derived from the fusion of two differently pigmented colonies. (× 10)

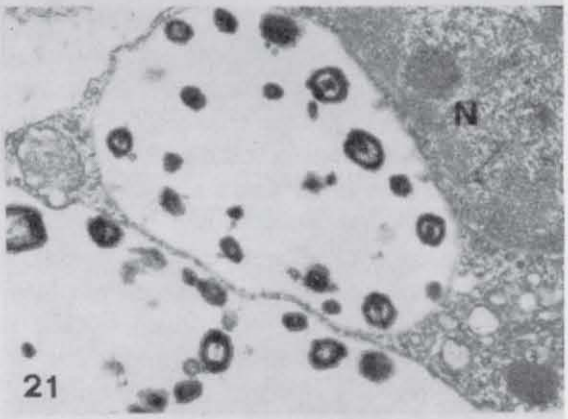
Fig. 23. — Necrotic spots at the contact area between two incompatible colonies. (× 12)

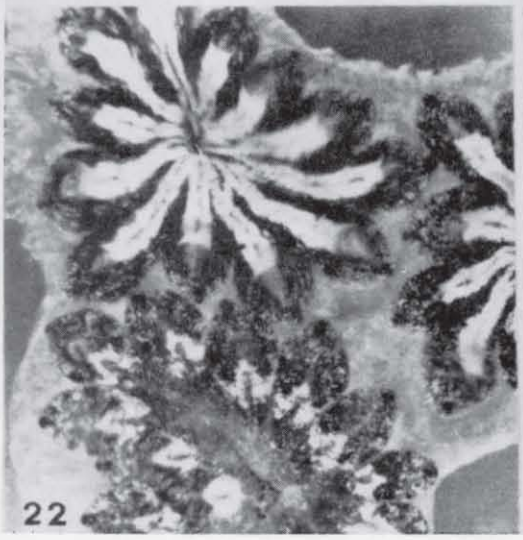
Fig. 24. — A blood cell mass has crossed the ampullar epithelium at the contact area (**arrowheads**) between two incompatible colonies. (× 420)


h
g
ph
m
18

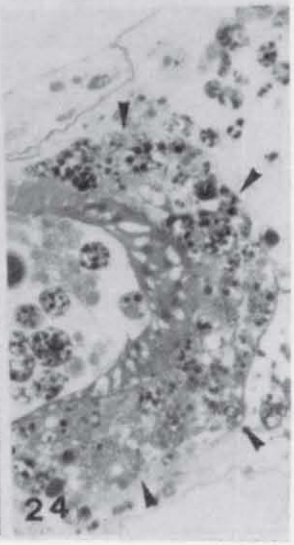

N
19


N
20


N
21


22


23


24

species the genetic analysis of fusibility does not suffer from the restrictions met in *B. primigenus*: homozygous colonies have been obtained from our crosses (SABBADIN, 1982) and homozygous lines are maintained in our cultures, although a segregation distortion due to sperm sharing their allele with egg envelopes does occasionally occur (SABBADIN, 1989; SABBADIN *et al.*, 1992).

Colonies differing in one allele and separated after having been fused for some time show an alteration of their original fusibility (SABBADIN and ASTORRI, 1988), thus confirming the role of the blood in fusibility control, previously demonstrated in *B. primigenus* by various approaches (TANEDA *et al.*, 1985).

A comparison between fusion/nonfusion and graft retention/rejection must be made. In *Botryllus* we have shown that early buds transplanted from a colony in the tunic of another colony can be vascularized and can complete their development only if the two colonies are fusible (SABBADIN, 1982). Allograft rejection of pieces of tunic has been studied in solitary ascidians (REDDY *et al.*, 1975; RAFTOS *et al.*, 1987 a, b). It proved to be a chronic reaction taking several weeks, whereas the nonfusion reaction in *Botryllus* is an acute reaction usually accomplished within a couple of days.

It has been shown that the responsibility for the recognition and distruction of allogeneic tissue in the graft experiments pertains to lymphocyte-like cells. Indirect evidence of the intervention of these same cells in the nonfusion reaction is given by the delay of the reaction in X-irradiated *B. primigenus* colonies (TANEDA and WATANABE, 1982), although the bulk of the necrotic material is represented principally by morula cells that have infiltrated from the opposite ampullae. Further experimental work is needed in order to elucidate the genetical and cytological relationships between these two types of histoincompatibility.

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