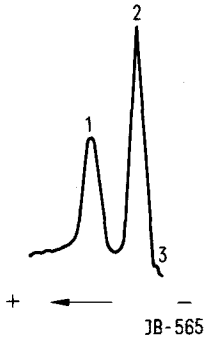

Figure 2. SDS-polyacrylamide gel electrophoresis of components not bound to DEAE-cellulose (peak 1, shadowed area of fig. 1).

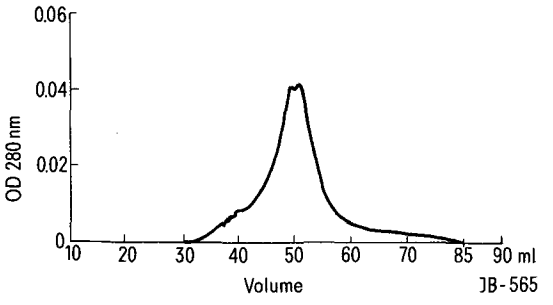

Figure 3. Gel filtration profile of cuticular collagen on Sephadex G-200 at high concentration (5 mg/ml). The protein was eluted as a single peak in the void volume.

tion of cuticle structure. Collagen does not bind to DEAE-cellulose under the experimental conditions used[8], and can be collected in the wash fraction (fig. 1, peak 1). The presence of hydroxyproline in the unbound fraction provides evidence that the protein part is collagen; it was entirely free of proteoglycans as judged by the absence of hexosamine. Bound fraction (peak 2) was non-collagenous as it failed to show the presence of hydroxyroline.

Electrophoresis of the collagen fraction revealed 2 major polypeptides with apparent mol. wts of 58,000 and 74,000 (fig. 2 and table). A 3rd, non-collagenous polypeptide was observed in many experiments. These results indicate the existence of at least 2 collagen polypeptides in the cuticle of *Gaigeria pachyscelis*. At a concentration of 5 mg/ml, the collagen was eluted in the void volume from the Sephadex column (fig. 3). The addition of a reducing agent (0.1% β-mercaptoethanol) had no effect on the elution pattern. At low concentration, (500 µg/ml), no protein could be observed in the void volume; it eluted much later from the column with a Ve/Vt value of 0.56. These results led us to believe that cuticular collagen in solution exists in two forms, a non-associated form at low concentration and an associated form at high concentration.

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A light and electron microscope study of spherical structures in the test cells of an ascidian *Ciona intestinalis* L.

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Summary. Spherical structures in the test cells that surround the embryos of the ascidian *Ciona intestinalis* L. (Tunicata, phylum Chordata) were studied by both light and electron microscope. Our data support the view that these structures are microorganism-like cells living in symbiosis with the test cells. Their possible role is discussed.

The test cells surround the ascidian egg throughout embryonic development until the larval stage. They are found between the egg membrane and the follicular cells from which they are separated by the chorion. Their name is due to Kupffer[2] who maintained that their role was secreting the larvae tunic, a transparent, extracellular structure which covers the epidermis of the larva.

Although they have been under study for a long time, their function is still not clear. Many authors[3–15] agree that they furnish nutritional material to the oocyte cytoplasm during oogenesis, but their role in embryogenesis is unknown. It should be remembered that the egg develops normally after their removal. Mansueto and Villa[16] have demonstrated that they incorporate radioactive precursors of proteins and RNA during embryonic development. Moreover, the incorporation of (^{3}H)-thymidine at the end of development, i.e., when their life seems to be concluded, is a peculiar feature. In fact, many of them are lost as a result of larval movements, even though some are firmly attached to the tunic.

We have reconsidered the test cells in the light of these results. The present research is aimed at providing further information on the structure and function of these cells in *Ciona intes-*

tinalis. This study has revealed the presence of spherical structures which could be interpreted as microorganims-like cells.

Material and methods. Adult specimens of *Ciona intestinalis* were collected in the Gulf of Palermo. Eggs and sperm were removed from the gonoducts, repeatedly washed in sterilized Syracuse dishes with Millipore-filters (0.45 µm porosity) in pasteurized seawater containing 100 µg/ml Parke Davis Chloromycetin. The gametes were mixed at 18 °C and rewashed. The stages considered were unfertilized and fertilized eggs, 16 cells, gastrulae, neurulae and larvae before and after hatching. Some eggs were dechorionated by hand with fine steel needles.

1% toluidine blue pH = 2.5 or Mayer's haemalum (C. Erba) in sterile seawater were employed. The eggs or embryos were placed on slides and examined with a Reichert microscope at a magnification of 200–1000 x, or with phase contrast. These observations were based on living material.

Acid phosphatase activity was determined on unfertilized eggs and larvae following Gomori's method[17]. The eggs or the larvae, on a slide, were incubated at 37 °C for 30 or 60 min; the substrate was sodium glycerophosphate (Merck). Controls without substrate were also run.

For electron microscopic investigation unfertilized eggs of *C. intestinalis* were fixed in 2% glutaraldehyde in 0.1 M sodium phosphate buffer (pH = 6.9), for 2 h at 4 °C, followed by 1% osmium tetroxide in the same buffer, for 1 h at 4 °C. The specimens were dehydrated with an ethanol-propylene oxide series and embedded in Dow epoxy resins[18]. Sections, obtained with a MT-1 Porter-Blum ultramicrotome, were collected on copper grids, stained with uranyl acetate and lead citrate[19,20] and examined by a Siemens Elmiskop 1b and a Zeiss EM 9 transmission electron microscope.

Results. The initial observations were made on unfertilized eggs and swimming larvae of *Ciona intestinalis*. The unfertilized egg is surrounded by an extra-embryonic envelope that consists of test cells, follicular cells and chorion (fig. 1). The swimming larvae are enclosed in a transparent extracellular tunic, to which numerous test cells are attached. The test cells appear roundish or oval in shape with a diameter of about 7–8 µm; the nucleus is evident after staining with Mayer's haemalum. Large vacuoles and metachromatic granules can be observed in their cytoplasm when toluidine blue is used; some green granules of differing sizes can also be seen.

After gentle pressure on a coverslip covering a drop of sea water containing the larvae, spherical mobile particles with a diameter of about 1 µm can be observed in the test cells; the spheres are refractile and sometimes join together in pairs or in small chains (fig. 2, arrows). The mobile spheres are also present in unfertilized eggs, and in embryos of 16 cells, gastrulae, neurulae and tail-bud stages. Their number increases in relation to the vacuolization of the cells. In contrast, they are less numerous in larvae at the beginning of metamorphosis. The spheres are not seen in embryos from dechorionated eggs. If the eggs and the envelopes are squashed, the spheres remain near the test and follicular cell components. Rounded structures, about 4–5 µm in diameter, are seen mainly at the base of follicular cells after squashing; they appear surrounded by a wall and contain the refractile spheres.

The spherical particles of the eggs and the embryos are acid phosphatase-negative, indicating that they are not lysosomes. Electron microscopic observation of the test cell reveals a large nucleus with heterogeneously condensed chromatin, few typical mitochondria and several large cytoplasmic vacuoles generally containing highly electron-dense granules (figs 3–5), which may correspond to the so-called 'test-granules' of other authors[21,22]. Besides these, other structures, never previously reported, can be observed scattered throughout the cytoplasm of the test cells (figs 3–10, arrows). They appear to be roughly spherical particles with a diameter of about 1 µm and are surrounded by a plasma membrane alone which invaginates forming numerous interior tube-like projections (fig. 6), sometimes containing electron-dense material (about 300 Å in diameter) in their dilated end which resemble the mesosomes of bacteria (figs 7–9, arrowhead). In some sections it is possible to detect slender fibrils interpreted as DNA (about 3 nm in thickness) and granules of about 100 Å in diameter, probably corresponding to ribosomes (fig. 7). The spheres are sometimes located at the periphery of the test cells, where the test cells and the egg plasma membranes are in close contact (fig. 10). Figures 3 and 8 show the test granules close to the spherical particles.

Discussion. The observations reported above have shown that some hitherto undescribed particles are present in the test cells of *Ciona intestinalis*. They resemble miniature berries under the microscope and are in motion. Their diameter is about 1 µm and they exist singly, in pairs or in small chains. They are not detected in dechorionated eggs or embryos.

Electron micrographs of these spherical particles show that they are bounded by a plasma membrane which in many areas is invaginated. Grains 100 Å in diameter are seen; in some areas fibrils seem to be present. The grains may be ribosomes and the fibrils nucleoid material.

It is difficult to understand the identity of the spherical particles. They are acid phosphatase negative which indicates that they are probably not lysosomes. Their morphology and ultrastructure could allow them to be interpreted as procaryotes. The size and shape, the absence of surface envelopes external to the plasma membrane, the size of the ribosomes and the presence of nucleoid material are essentially the same characteristics as observed in mycoplasma[23,24]. The invaginations, resulting in an increase in the area of the plasma membrane, seem to be peculiar characteristics of the described particles, even if they could be interpreted as mesosomes. Electron dense material is detected in the wider end of the invaginations. The rounded forms, 4–5 µm in diameter, seen at the base of the follicular cells, could be interpreted as spores which are part of the biological cycle of a microorganism. The spheres of *Ciona intestinalis* test cells are present at all developmental stages.

Some earlier studies have provided evidence for an association involving Bacteria-Tunicata, Algal-Tunicata and Nephromyces-Tunicata[25–31] common in marine environments, but similar investigations of ascidian embryonic development are limited. Julin[32] described some granules in the oocyte test cells of pyrosomes, where the test cells develop into the luminous organs of the adult. Pierantoni[33] suggested that the structures described by Julin were microorganisms living in symbiosis with the test cells and are responsible for the luminosity of the animal. According to the author the microorganisms, combined in spores, enter the blood system, reach the ovary, and penetrate the test and follicular cells of the egg. Reverberi[34,35] described some structures in the test cells of *Molgula impura*,

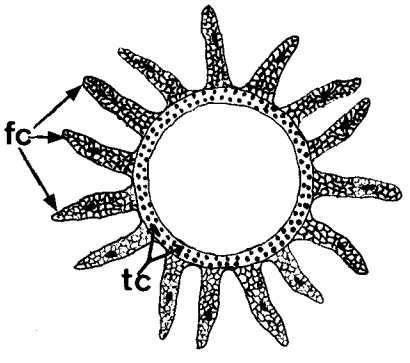

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Figure 1. Diagram of unfertilized egg of *Ciona intestinalis* showing the test (tc) and follicular cells (fc).

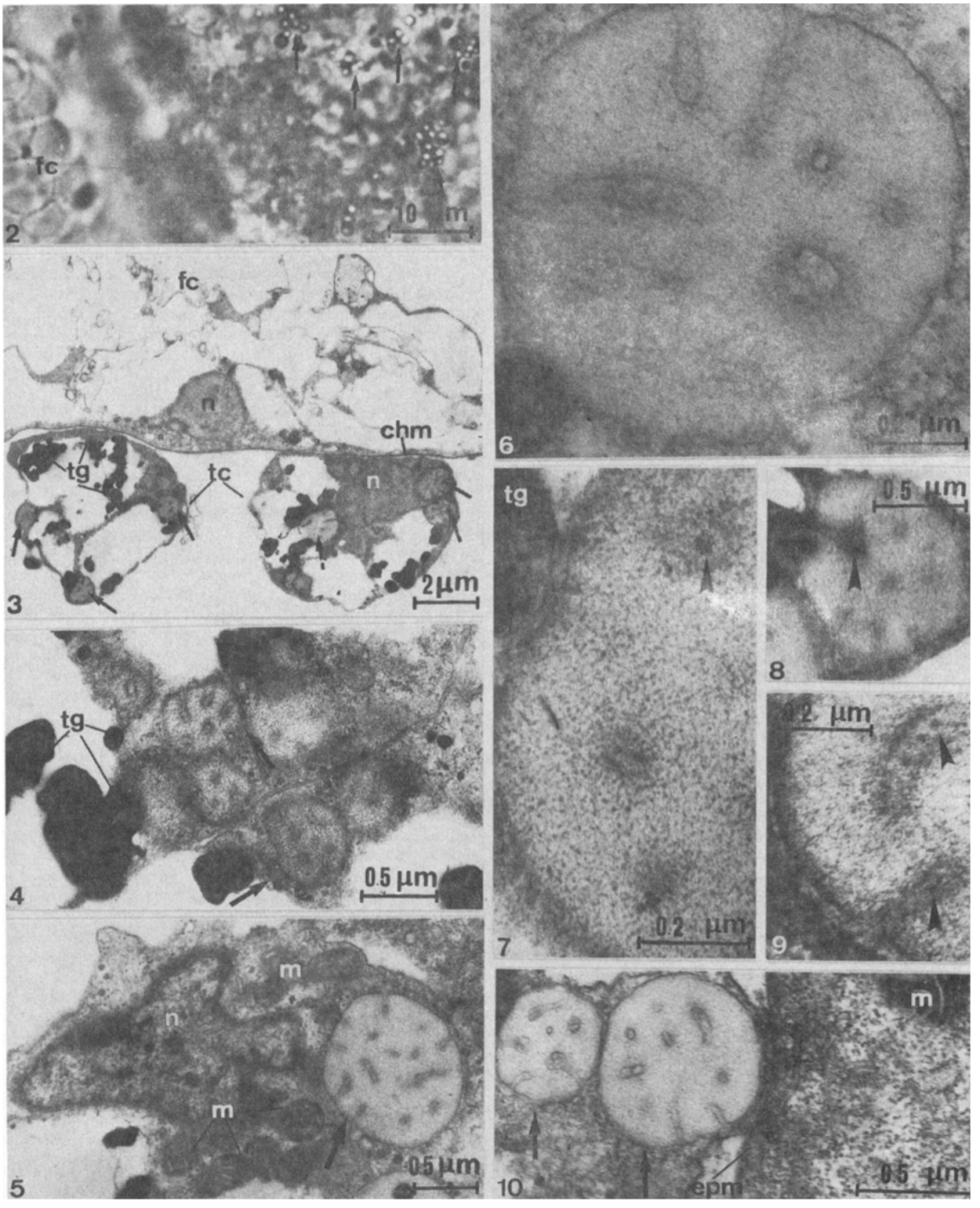

Figure 2. Light micrograph of the envelopes of a living unfertilized *Ciona intestinalis* egg; fc, follicular cells; arrows indicate the spherical particles.

Figure 3. Electron micrograph of the envelopes of unfertilized egg of *Ciona intestinalis;* chm, chorial membrane; fc, follicular cell; n, nucleus; tc, test cell; tg, test granule; arrows indicate spherical particles.

Figures 4 and 5. Electron micrographs showing the presence of spherical particles within the test cells of unfertilized eggs of *Ciona intestinalis;* m, mitochondrion; n, nucleus; tg, test granule; arrows indicate spherical particles.

Figures 6–9. Magnifications of the spherical particles; arrowheads indicate electron dense material contained in the invaginations of their plasma membrane.

Figure 10. Electron micrograph of unfertilized egg of *Ciona intestinalis* showing the close contact between the test cell and the egg plasma membranes; epm, egg plasma membrane, m, egg mitochondrion; arrows indicate spherical particles.

which he interpreted as microorganisms transmitted through the generations by the test cells.

If the spherical particles are considered to be microorganism-like cells, what relation-ship could they display with the test cells of *Ciona intestinalis?* We suggest that the test cells could function as filters against bacteria or other microorganisms to protect the egg and the embryo. On the other hand, the particles of the test cells are present in all stages of development from the unfertilized egg onwards, and in spite of their presence the test cells keeps its integrity until the larval stage. We propose that the particles could be transported by the test cells with which they live in symbiosis.

Goldberg et al.[36] found high concentrations and incorporation of vanadium in the ovarian follicles of *Ciona intestinalis* and *Ascidia ceratodes,* suggesting that this element may become part of a metabolic system that is present from the beginning in the developing embryo. Moreover, it has been demonstrated that the test cells of the oocyte and the unfertilized egg of *Ascidia pygmaea* and *Ciona robusta* accumulate vanadium and iron in some test granules and are thence absorbed by the oocyte[22-37]. By which mechanism are metals absorbed in the inclusions of the test cells? Since the electron micrographs show that the test granules are close to the microorganism-like cells, perhaps the latter could be the site for selective absorption and concentration of particular metals.

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Demonstration of a sexual dimorphism in the olfactory pathways of the drones of *Apis mellifica* L. (Hymenoptera, Apidae)

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Summary. An important sexual dimorphism is demonstrated in the drone at the level of the first central relay of the antennal olfactory pathway (antennal lobe of the deutocerebrum), represented by large and easily identifiable glomerular complexes. This preparation seems to be an excellent model for a functional study of the olfactory system.

In both useful and noxious insects, many behavior patterns which have a great biological and economic importance have an olfactory determinism. In order to study the mechanisms of olfaction in insects it seems that drones are potentially good experimental models. This is because their single known function is the mating of the females (queens), in the course of which olfactory clues play a fundamental role[1-3]. Their highly developed olfactory abilities justify a detailed functional study of their olfactory system.

Amongst insects the olfactory information is collected by the primary neurons of specialized sensilla along the antennae and reaches the first central relay, the antennal lobe of the deutocerebrum, along some tens of thousands of axons of the antennal nerve which converge there by means of synaptic connections onto the dendritic processes of some hundreds of deutoneurons at the level of specialized neuropilar areas, the glomeruli[4-6]. Amongst some insects, for example in the cockroach, *Blaberus craniifer,* and in the moth, *Mamestra brassicae,* it appears that these glomeruli are morphologically identifiable and moreover invariant[7,8]. Thus, it is possible to study the general mapping of these structures. Moreover, a sexual dimorphism in the deutocerebrum of the males of some species has been shown: in cockroaches and in moths, the antennal lobe contains specific structure(s), the so-called 'macroglomerulus'[7,9,10] or 'macroglomerular complex'[8,11] which is (are) involved in the specific processing of the pheromonal information[10-12].

In fact, in insects there are 2 distinct sub-systems, on the one hand during the detection of the odorants (at the level of the antenna) and on the other hand, during the processing of the olfactory information (at the level of the central nervous sys-