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Electron Microscopic Analysis of Tunicate (*Halocynthia roretzi*) Hemocytes

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ABSTRACT—Hemocytes from hemolymph of the tunicate, *Halocynthia roretzi* were investigated by transmission electron microscopy (TEM). Nine types were identified according to their ultrastructural characteristics: phagocytes or macrophages (PH cells), granulocytes with small granules (GS cells), granulocytes with large granules (GL cells), vesicle-containing cells (VC cells), fibrous material-containing cells (FM cells), vacuolated cells type 1 (VA1 cells), vacuolated cells type 2 (VA2 cells), basophilic cells (BA cells) and lymphoid cells (LY cells). Among these hemocyte types VC cells and FM cells were unique and novel. One functional assay, i.e. phagocytic activity against sheep red blood cells (SRBC) and rat red blood cells (RRBC) was developed. This investigation resulting from improved fixation has served as the basis for standardizing hemocyte types and for defining future analyses that can be used in functional assays.

INTRODUCTION

Tunicate hemolymph contains many hemocyte types often referred to as coelomocytes or coelomic cells. They have long been investigated by light and phase contrast microscopy in diverse species [1–9]. In addition, more recent studies have correlated fine structure with certain functional assays [10–13]. Despite attempts to define certain hemocyte functions such as coagulation, excretion, nutrition, immune responses and tunic formation [14–19], as summarized by Wright (1981), most of these functional analyses require more extended experimentation [20].

The ultrastructural characterization of hemocytes has been established, in some species [21–23], leaving us with confusion in terminology with respect to common features of hemocytes from different species. Recently we classified hemocytes of the tunicate, *Halocynthia roretzi,* into ten groups according to morphology by light, phase and fluorescence microscopy [24]. The present study was undertaken for several reasons. First, confusion in reaching a consensus concerning hemocyte types may result from inadequate fixation. Second, to rectify this, improved fixation revealing fine structural features of hemocytes was then investigated. Third, structure was viewed together with phagocytic activity as one assay for hemocyte function.

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MATERIALS AND METHODS

Tunicates

Tunicate, *Halocynthia roretzi,* a Urochordate was collected in the Mutsu Bay, Aomori prefecture, Japan.

Harvesting hemocytes

Hemocytes were harvested in tubes from live

tunicates by cutting the tunic near the point of attachment. Hemolymph was centrifuged at 250 g for 5 min and the pellets were fixed for electron microscopy.

Preparation for transmission electron microscopy (TEM)

Since any single method of fixation was not always suitable for all cell types (generally one fixative was reasonably good for only a few), we tried several approaches changing composition and concentration of fixatives and buffers. After several trials, we found that a mixture of 3% glutaraldehyde and 3% paraformaldehyde preserved many cell types reasonably well. In most cases hemocytes were fixed in a mixture of 3% glutaraldehyde and 3% paraformaldehyde in 0.2 M sodium cacodylate, pH 7.4. To examine the effect of pH on preservation of hemocyte structure, fixatives at four different pH, 6.4, 7.4, 8.0 and 8.5 were often used. Hemocytes were post-fixed in 2% osmium tetroxide in cacodylate buffer for 1 hr, and dehydrated in alcohol or acetone. The specimens were embedded in Epon 812, ultrathin sections cut with an LKB Ultrotome Nova, stained with uranyl acetate and lead citrate and then examined with a JEM-200CX electron microscope (Japan Electron Optics Ltd.).

Phagocytosis

Fresh tunicate hemolymph was mixed with SRBC or RRBC immediately after harvesting and incubated at room temperature for 20 min. Hemocytes were then collected, fixed in 3% glutaraldehyde/paraformaldehyde for 2 hr, post-fixed in 2% osmium tetroxide and embedded in Epon. In other experiments 0.35–3.5 ml of 10% RRBC, which were first fixed with 2% glutaraldehyde and paraformaldehyde, then injected into the coelomic cavity via papillae and hemocytes prepared for TEM by the usual method.

Young tunicate (stage of organogenesis)

Specimens of young tunicates 8–10 days after hatching were supplied by Drs. Yasuo Sugino and Yu Ishikawa. These were also examined as described above to ascertain the ontogeny of hemocytes.

RESULTS

CHARACTERIZATION OF EACH HEMOCYTE TYPE

General features

Many different hemocyte types were observed in tunicate hemolymph. By TEM we classfied them into nine groups: phagocytes (or macrophages; PH cells), granulocytes with small granules (GS cells), granulocytes with large granules (GL cells), vesicle-containing cells (VC cells), fibrous material-containing cells (FM cells), vacuolated cells type 1 (VA1 cells), vacuolated cells type 2 (VA2 cells), basophilic cells (BA cells) and lymphoid cells (LY cells). This classification was based on observations of hemocytes fixed in a mixute of 3% glutaraldehyde/paraformaldehyde in 0.2 M sodium cacodylate, pH 7.4. Correlation of TEM with LM classification together with TEM studies in different species is summarized in Table 1.

Phagocytes or macrophages

Phagocytes had numerous cell shapes and often pseudopodia. We found lysosomal granules and many vesicles with contents of variable electron-density in their cytoplasm (Figs. 1–3). Some phagocytes had a tube-like anastomosing structure which contained high electron-dense substances (Fig. 1). Phagosome-like large vesicles containing amorphous substances or myelinated figures were often observed. Active phagocytic activity was observed when hemolymph was incubated with SRBC or RRBC. Their phagocytic activity was also demonstrated by experiments using an *in vivo* system when fixed RRBC were injected into the coelomic cavity (Fig. 3). Phagocytized RRBC were found in phagocytic vacuoles and electron dense tubular or globular structures were often encountered around the engulfed RRBC (Fig. 3).

Granulocytes

Granulocytes with small granules (GS cells) were generally spherical and contained many granules which varied in electron density and size (Fig. 4). The granules were usually smaller than 0.5 μm. GS cells had several mitochondria, rough-endoplasmic reticulum (RER) with long slender cisternae and numerous small vesicles. Golgi complex was also observed. Granulocytes with

TABLE 1. Characteristics of nine types of hemocytes in the tunicates, *Halocynthia roretzi* and correlation with other reports

Cell types in this report *H. roretzi* by TEM	Major characteristics	Sawada et. al. [24] *H. roretzi* LM	Fuke [32] *H. roretzi* TEM	Milanesi and Burishel [23] *Botryllus schlossori* TEM	Overton [21] *Perophora viridis* TEM	Wright [20] Review
Phagocytes (Macrophages) (PH)	lysosome pseudopodia tubular structure phagocytic	Phagocytic cells type 1 (p1) and type 2 (p2)	Fine granular amoeboid cells	Macrophages	Phagocytes	Hyaline leucocytes?
Granulocytes with small granules (GS)	small granules (0.5 μm)	Granular cells type 1 (g1)	Minute granular amoeboid cells	Microgranular amoebocytes	Granular amebocytes	Granular leucocytes
Granulocytes with large granules (GL)	large granules (1–2 μm)	?		Macrogranular amoebocytes		
Vesicle-containing cells (VC)	vesicles (0.5 μm) SER/Golgi complex	Granular cells type 3 (g3)?				
Fibrous material-containing cells (FM)	fibrous material in vesicles RER/Golgi complex	Granular cells type 2 (g2)?				
Vacuolated cells type 1 (VA1)	one large vacuole RER	Vacuolated cells type 1 (v1) and type 3 (v3)?	Vesicular cells		Signet ring cells	Vacuolated cells
Vacuolated cells type 2 (VA2)	several vacuoles central nucleus	Vacuolated cells type 2 (v2) and/or type 4 (v4)?		Morula cells?	Compartment cells	
Basophilic cells (BA)	vesicular RER Golgi complex	Granular cells type 2 (g2)?				
Lymphoid cells (LY)	poor organella	Lymphoid cells (ly)				Lymphocytes

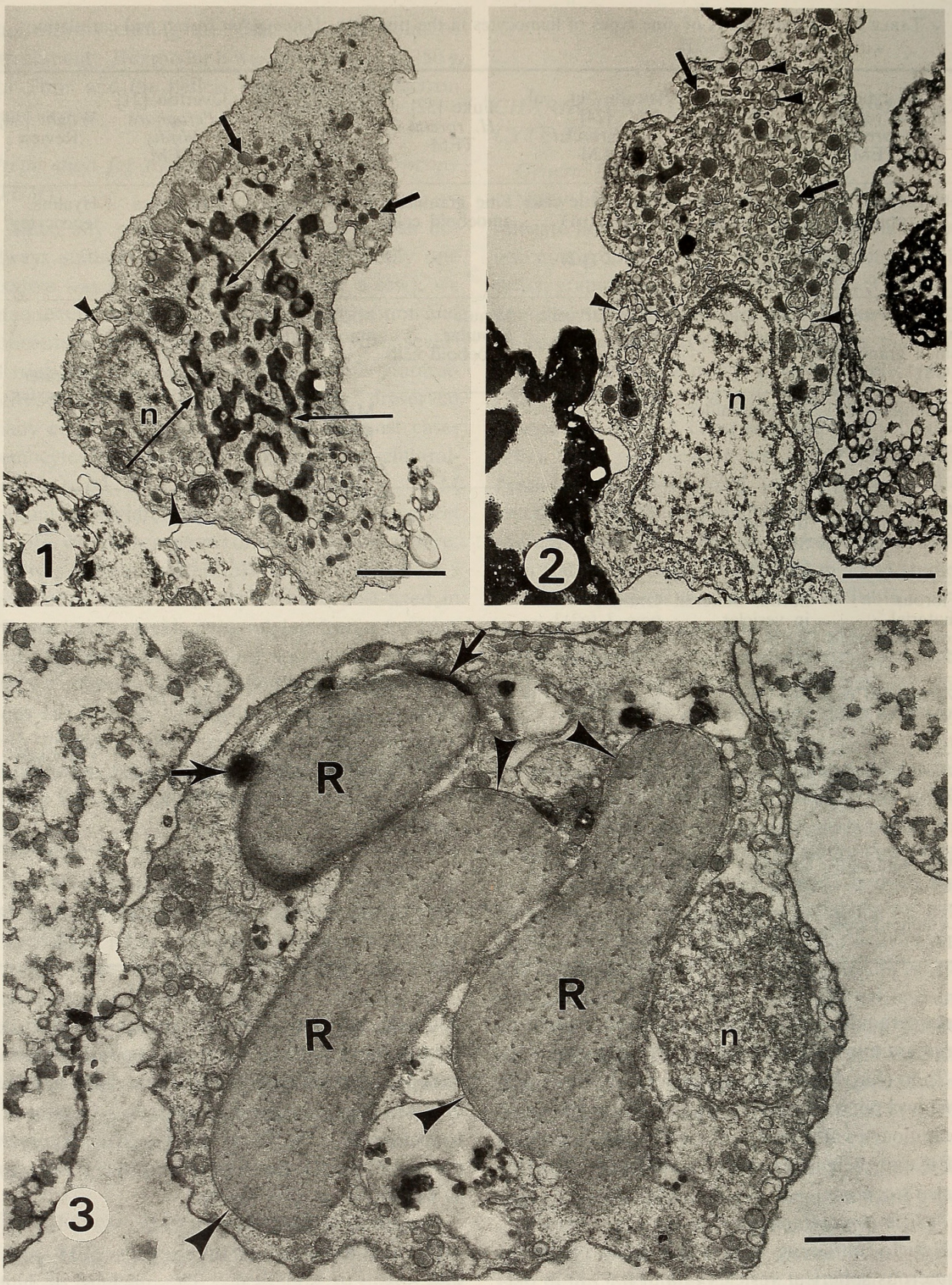
large granules (GL cells) contained numerous large granules whose diameter was approximately 1–2 μm (Fig. 5), but the volume which they occupied was not as large as it was in vacuolated cells. Granulocytes also contained small vesicles, slender RER and their nuclei were located near the cytoplasm's center.

Vesicle-containing cells

Vesicle-containing cells (VC cells) possessed numerous spherical vesicles (about 0.5 μm in diameter) filled with material of low electron density (Figs. 6, 7). Many of the spherical vesicles seemed to lose their contents during fixation and dehydration. The cytoplasm had long, flexible and rod shaped mitochondria, abundant smooth-surfaced endoplasmic reticulum (SER) but a small amount of RER. The Golgi complex was well developed. Immature VC cells (figure not shown) had numerous globular RER and a prominent Golgi complex as well as the specific vesicles.

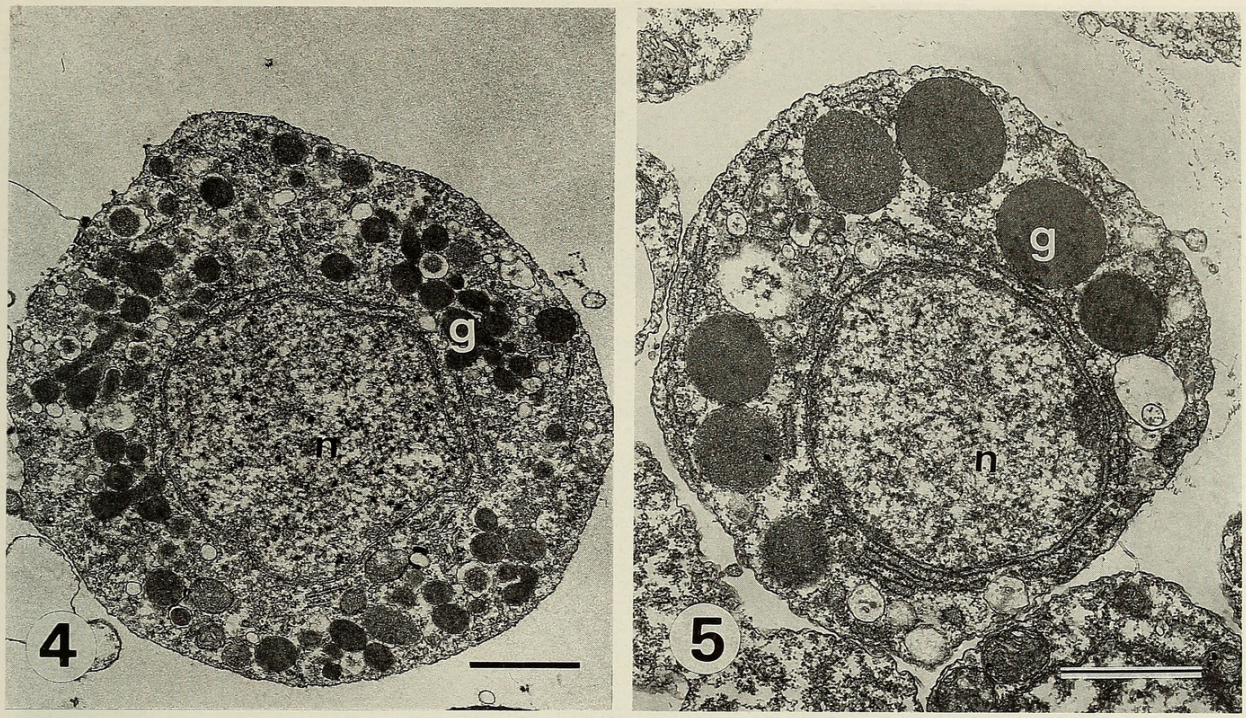
Fibrous material-containing cells

Fibrous material-containing cells (FM cells) were oval or spherical. The nucleus of high electron-density occupied a central position and it often appeared to be compressed by tightly packed cytoplasmic vesicles. These tightly-packed vesicles



FIGS. 1, 2. Phagocytes (or macrophages; PH cells) with small vesicles (arrow heads) and granules (thick arrows). Note electron dense anastomosing canalicular structure (thin arrows) in a phagocyte of Fig. 1. n, nucleus. scale bar, 1 μm.

FIG. 3. A phagocyte engulfed three RRBC (R) in its phagosomes. Three hours after intracoelomic injection of RRBC. Arrow heads, phagosome membrane; Arrows, electron dense substances; n, nucleus. scale bar, 1 μm.



FIGS. 4, 5. Granulocyte with small granules (GS; Fig. 4) and granulocyte with large granules (GL; Fig. 5). g, granules; n, nucleus. scale bar, 1 μm.

filled with fibrous material were the most prominent feature (Figs. 8, 9, 10). Rather immature cell types contained large amounts of vesicular or spherical RER (Fig. 8). High electron-density of their cytoplasmic matrix was a characteristic feature of mature cells (Figs. 9, 10). Vesicles varied in size, shape and were often fused together. Certain vesicular structures in the cytoplasmic periphery appeared to be open. A prominent Golgi complex with dense contents, electron dense bodies and small vesicles were also observed in the cytoplasm (Figs. 9, 10).

Vacuolated cells

Vacuolated cells type 1 (VA1 cells) had an eccentric nucleus and a large vacuole which usually contained electron dense material (Fig. 11), while the vacuolar content often appeared to be lost during preparation (Fig. 12). Small vesicles with or without electron-dense substances were observed. RER was well developed in some of these cell type (Fig. 12). Vacuolated cells type 2 (VA2 cells) contained variable numbers of large vacuoles which occupied most of the cell's volume (Fig. 13). The content of vacuoles appeared homogeneous in certain cells but heterogeneous in others. The nucleus occupied a central position.

Basophilic cells

Basophilic cells (BA cells) has numerous distended RER (Fig. 14) and a centrally located nucleus. Spherical or ellipsoidal mitochondria and well developed Golgi complex were usually present.

Lymphoid cells

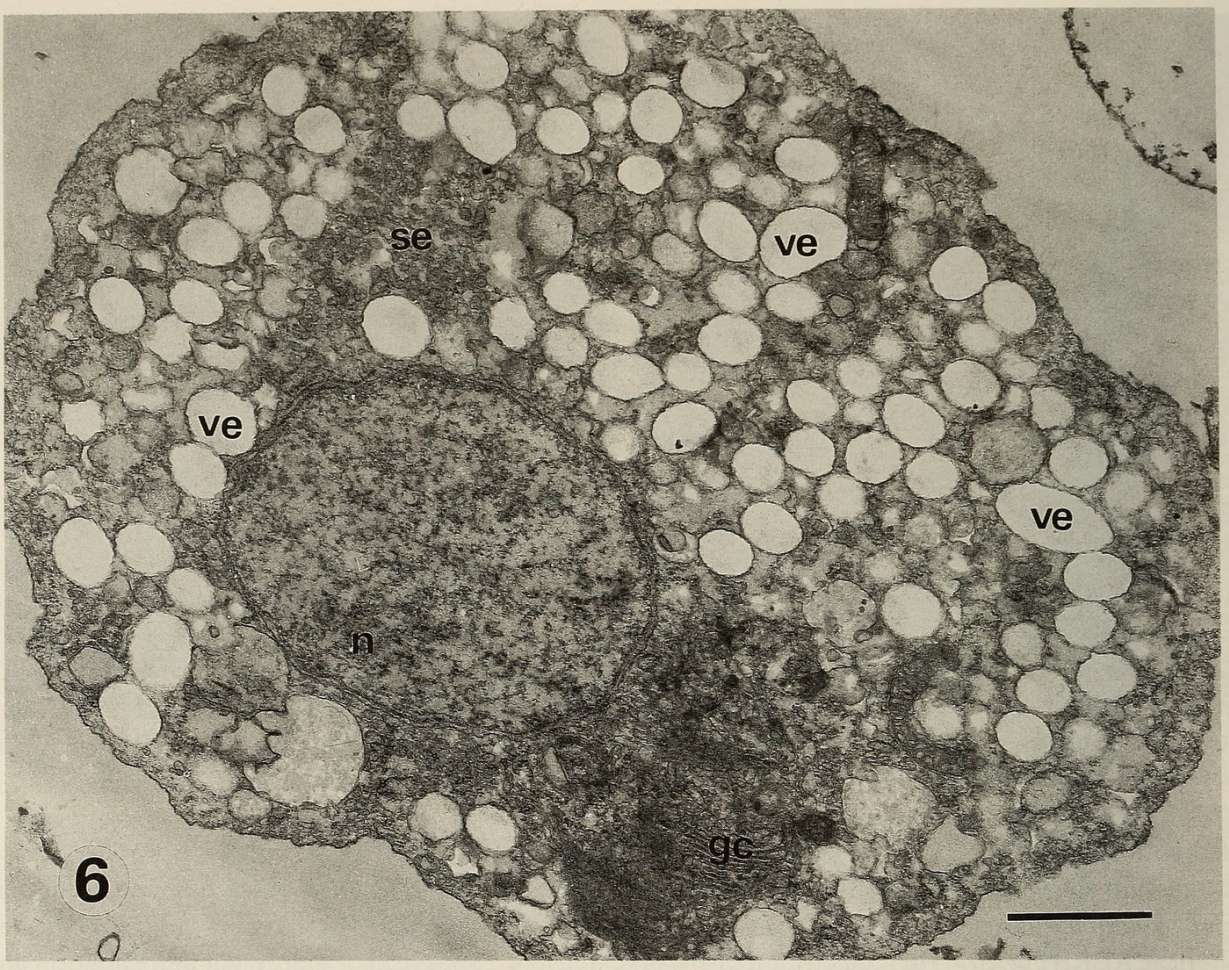
Lymphoid cells (LY cells) were small round or oval (figure not shown). We observed only a small quantity of cytoplasmic organelles without a characteristic component. Sparse organelles consisted of a few spherical mitochondria, a few short and slender RER, and clusters of free ribosomes.

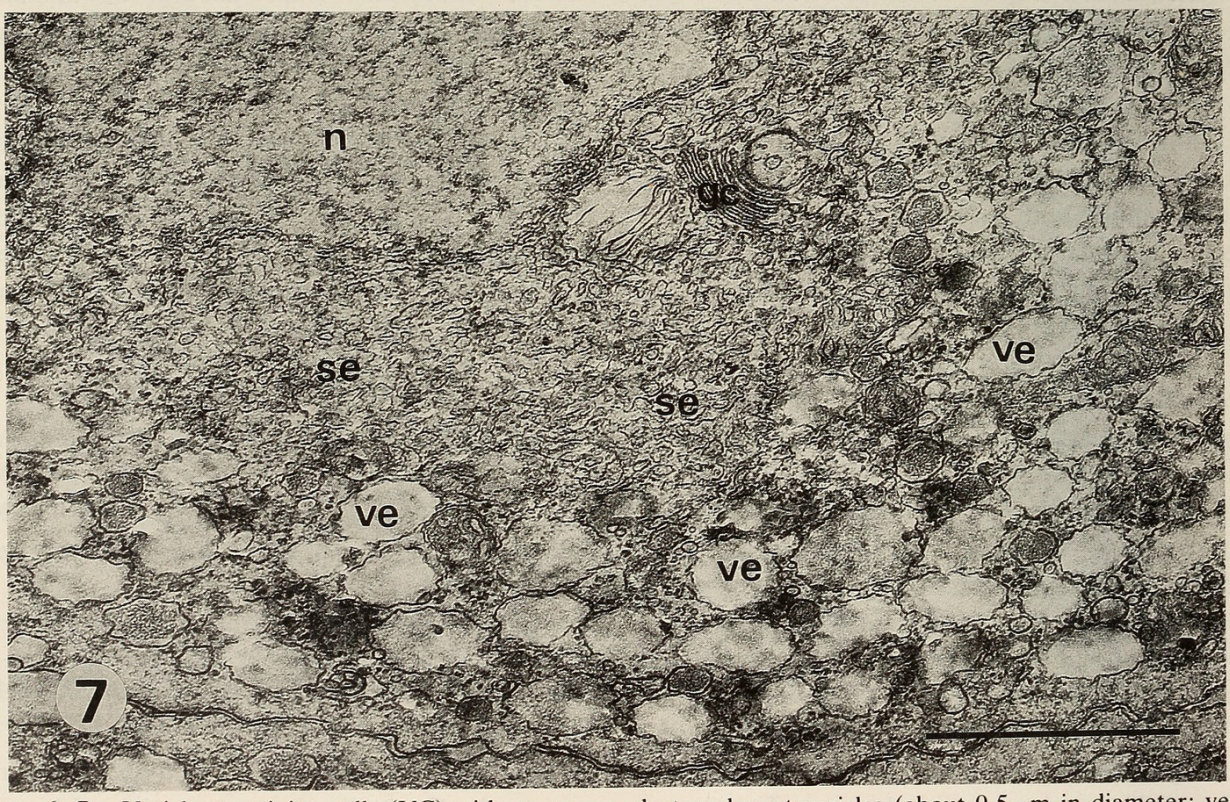
HEMOCYTES IN YOUNG TUNICATE

All adult hemocyte types were also found in the coelom of young tunicates, 8–10 days after hatching. The fibrous material-containing cells (FM cells) seemed to be more abundant in younger tunicates than in adults. Vacuolated cells type 2 (VA 2 cells) were observed in the larval tunic.

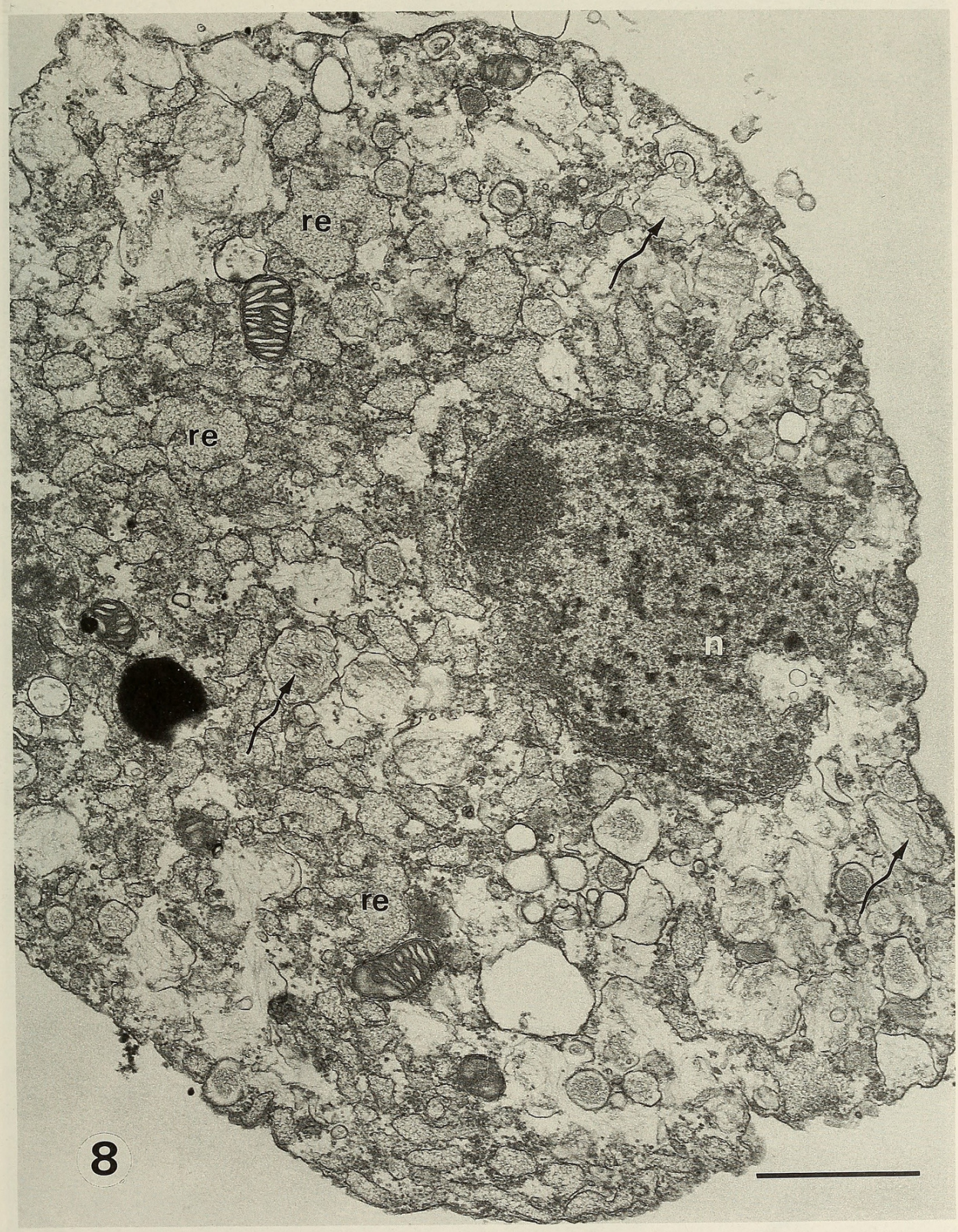
EFFECT OF FIXATIVE'S pH ON PRESERVATION OF HEMOCYTE FINE STRUCTURE

Four different pHs, 6.4, 7.4, 8.0 and 8.5 were examined. Generally the preservation of hemo-



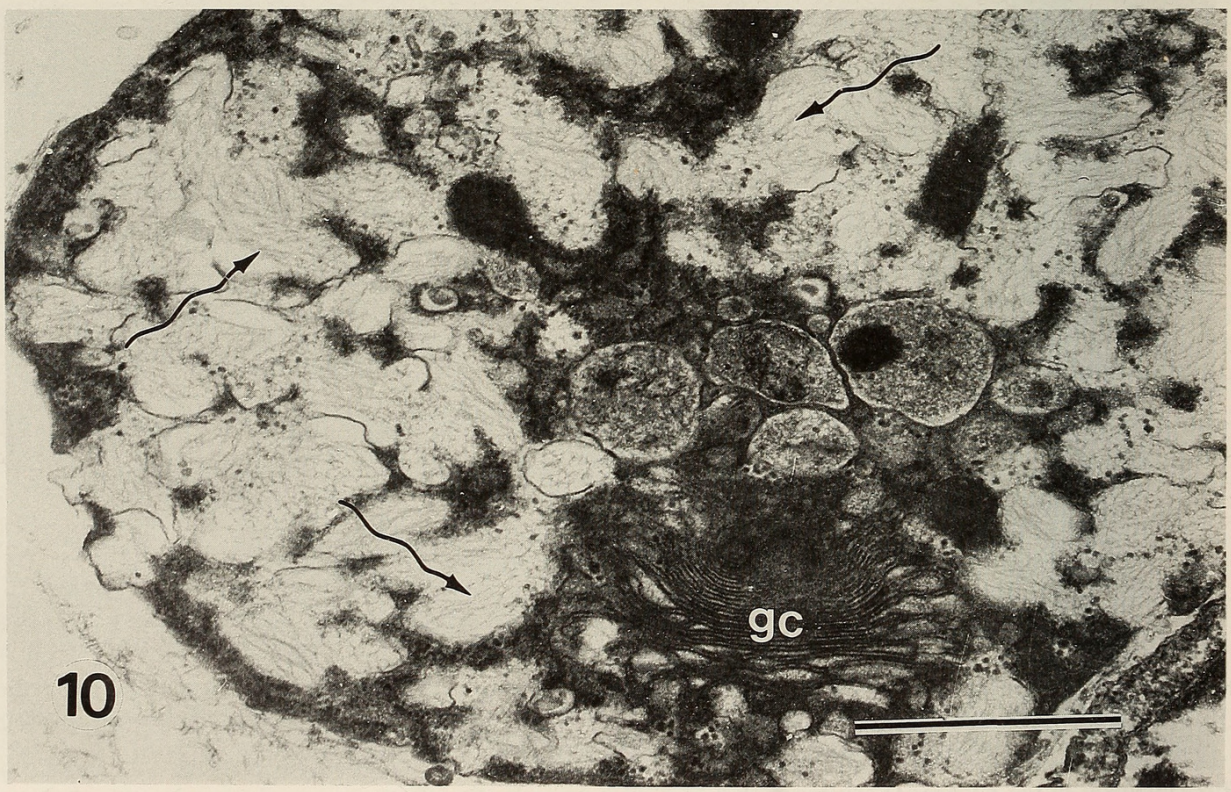


FIGS. 6, 7. Vesicle-containing cells (VC) with numerous electron lucent vesicles (about 0.5 μm in diameter; ve). Note many smooth-surfaced endoplasmic reticulum (se) and prominent Golgi complex (gc). scale bar, 1 μm.



FIGS. 8–10. Fibrous-material containing cells (FM). Well developed rough-endoplasmic reticulum (re) in rather immature FM cell (Fig. 8). Note fibrous-material (arrows) in vesicles, prominent Golgi complex (gc), electron dense hyaloplasm and nucleoplasm. n, nucleus. scale bar, 1 μm.


n
gc
9


gc
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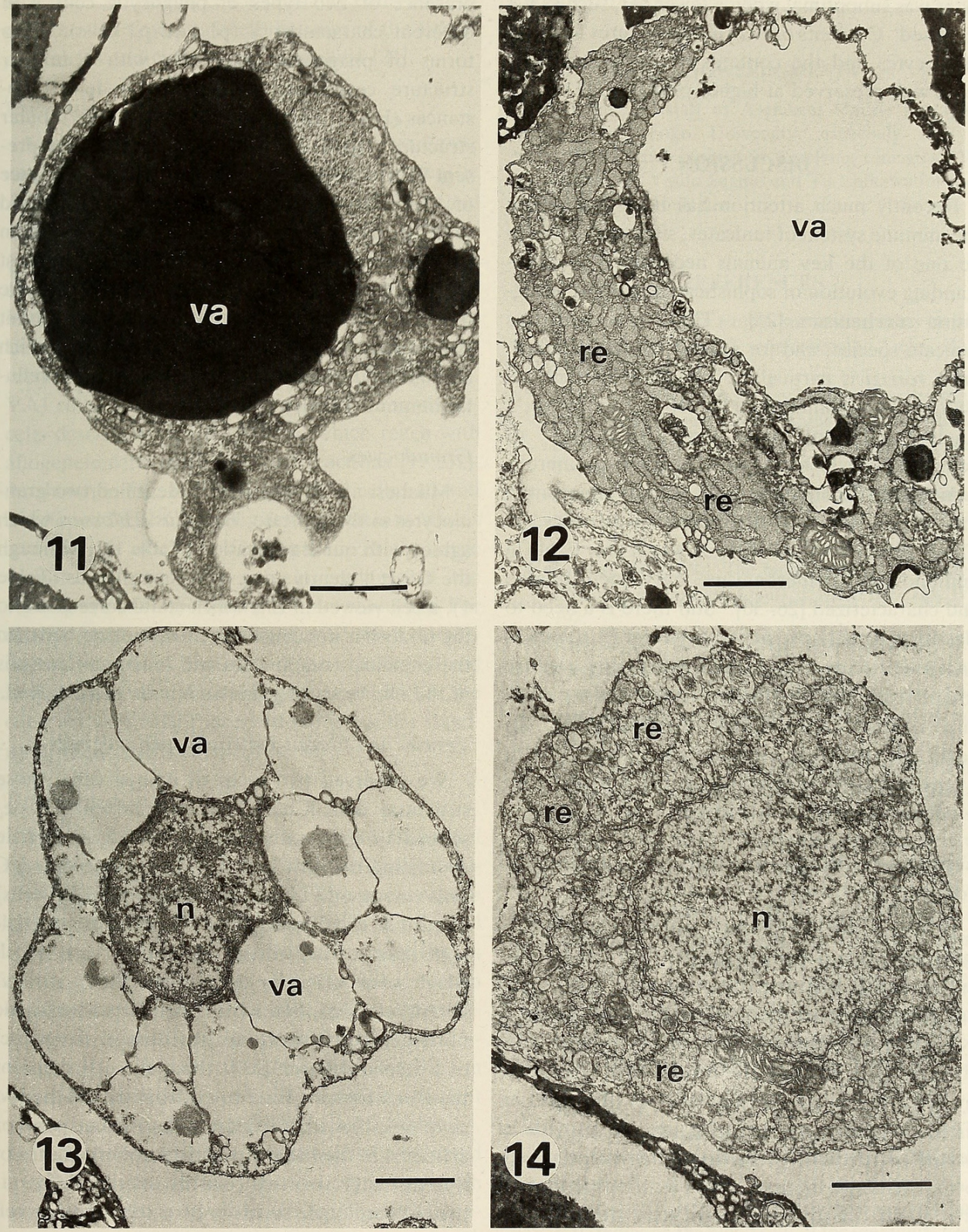

Figs. 11, 12. Vacuolated cells type 1 (VA1) with a single large vacuole (va). Vacuolar content of the cell in Fig. 11 preserved well with high pH fixative. The cell in Fig. 12 contains well developed rough-endoplasmic reticulum (re). scale bar, 1 μm.

Fig. 13. Vacuolated cell type 2 (VA2) with several large vacuoles (va) and central nucleus (n). scale bar, 1 μm.

Fig. 14. Basophilic cell (BA) with numerous dilated rough-endoplasmic reticulum (re). scale bar, 1 μm.

cytes was suboptimal when a lower pH, such as 6.4 was used. Contents of vesicular structures found in phagocytes and the contents of vacuolated cells were well preserved at higher pHs (Fig. 11).

DISCUSSION

Recently much attention has been devoted to the immune system of tunicates, since they seem to be one of the key animals necessary for understanding evolution of sophisticated immune recognition mechanisms [25]. There are numerous tunicate species, and for several reasons, *Halocynthia roretzi* is particularly an ideal experimental animal for immunological investigations. First, a single individual has large quantities of hemolymph which in turn contains numerous hemocytes. Second, and perhaps most importantly this species can be obtained throughout the year. Third, certain humoral factors, which play a role in immuno-defense mechanisms, have been isolated and characterized [26–30]. Still there is a need to classify and characterize hemocytes of *H. roretzi* as one basic study essential to reveal the cellular components of the immune system and to minimize confusion concerning functional cells. To begin this approach, we investigated hemocytes in a previous study by light microscopy [24] and here we describe their ultrastructure.

Correlation with the previously-reported classification

In the previous LM study, we classified hemocytes into ten groups: Phagocytes type 1 and 2 (p1- and p2-cells), granular cells type 1, 2, 3 (g1-, g2- and g3-cells), vacuolated cells type 1, 2, 3 and 4 (v1-, v2-, v3- and v4-cells) and lymphoid cells (ly-cells) [24]. Due to limited power of resolution by LM and limited information from thin slices of TEM materials, we are only able to provide a partial correlation. Nevertheless, considering our own results and those of others we propose four or five major cell groups; phagocytes, granulocytes, vesicle- and fibrous material-containing cells, and vacuolated cells (Table 1).

Phagocytes or macrophages

In our previous LM study [24] we described the presence of two types of phagocytic cells with different characteristics (p1 and p2 cells). Two forms of phagocytic cells, one with a tubular structure containing highly electron-dense substances (Fig. 1) and the other without the tubular structure (Fig. 2), were also identified in the present TEM observation. The biological significance of this ultrastructural difference is obscure and thus it is difficult to present a definite correlation with the results of LM classification [24]. The most important finding is that both cell forms have pseudopodia, lysosomal granules and exhibit strong phagocytic activity, characteristics which strengthen their important role in front line, cellular immuno-defense mechanisms.

Granulocytes

Milanesi and Burighel [23] identified two granulocytes in the tunicate, *Botryllus schlossori* which agrees with our classification (Table 1). Although the GS cell clearly corresponds to g1-cells (Table 1), a cell viewed in LM which could correspond to the GL cell is not conclusive. Differences between the chemical components and functional analysis of the two types of granules remain to be solved.

Vesicle- and fibrous material-containing cells

We observed two types of unique cells whose structural details have not been adequately described in the past. One is the 0.5 μm vesicle containing cell (VC cells). We assume that VC cells correspond to granular cells type 3 (g3-cells) of our LM classification [24]. The fibrous material-containing cell (FM cell) is another novel cell which we describe for the first time in *H. roretzi.* Glomerulocytes, which contained intracytoplasmic fibrous material, were described in the hemocoel of a styelid ascidian [31]. Since the cell shape of the glomerulocyte is different from that of the FM cell, disk-like in the glomerulocyte but oval or spherical in the FM cell and distibution patterns of fibrous material in two cells are entirely different, the glomerulocyte seems to be another unique cell type in certain ascidians. Although structural differences between these two cells are emphasized above, we cannot rule out the possibility that contents of fibrous materials from these two cells are the same nor are they similar in their charac-

ters. By LM the FM cell is probably equivalent to granular cell type 2 (g2-cell) because of the size of its vesicles and cytoplasmic basophilia [24].

Vacuolated cells

Vacuolated cells were classified into two types, one had a single, large vacuole with an eccentric nucleus (vacuolated cells type 1, VA1 cells) and the other had several vacuoles with central nuclei (vacuolated cells type 2, VA2 cells). VA1 cells are apparently the same type as signet ring cells and VA2 cells are the compartment cells according to Overton [21]. For these two types Wright [20] recommended the term vacuolated cells (Table 1). VA1 cells also seem to correspond to the vesicular-cells described by Fuke (1979), which reach with allogeneic cells during contact reactions [17, 32]. As shown in Fig. 12 some of the VA1 cells had well developed RER suggesting active protein synthesis and storage of synthesized protein in the large vacuole. Perhaps the protein contents of the vacuole are excreted during contact reactions. For clarity, preservation of vacuolar contents largely depends on pH of fixatives. When cells were fixed in a solution of higher pH, such as pH 8–8.5, the contents were well preserved reflecting the stability of their chemical components.

Differentiation of hemocytes

We have classified the hemocytes of *H. roretzi* into nine groups and each of them had ultrastructurally distinguishable features. However, we are unable to dismiss the possibility that certain cell types may merely represent differentiation stages of the other hemocytes. For example, certain basophilic cells (BA cells) may be considered as immature because of transitional forms which contain large amounts of RER together with 0.5 μm vesicles or vacuoles. Now we have only insufficient evidence to delineate cell differentiation pathways. Thus further experimentation should be conducted to solve this important question. One approach would be to do extensive investigations of hemopoietic tissues, homolymph, and hemocytes in young tunicates. These may provide information necessary for understanding developmental cell lineages especially if compared with adult stages that have been combined with successful *in vivo* and *in vitro* assays [33–35].

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