

MORPHOLOGICAL CHARACTERIZATION OF THE HEMOCYTES OF THE PULMONATE SNAIL *BIOMPHALARIA TENAGOPHILA*

MARGHERITA ANNA BARRACCO; ANA ANGÉLICA STEIL & ROGÉRIO GARGIONI

Departamento de Biologia, Universidade Federal de Santa Catarina, Caixa Postal 476, 88040-900 Florianópolis, SC, Brasil

The blood cells of the pulmonate snail Biomphalaria tenagophila, an important transmitter of the trematode Schistosoma mansoni in Brazil, were examined by light and transmission electron microscopy (TEM). Two hemocyte types were identified: hyalinocytes and granulocytes. Hyalinocytes are small young (immature), poorly spreading cells, which have a high nucleocytoplasmic ratio and are especially rich in free ribosomes. They do not appear to contain lysosome-like bodies and represent less than 10% of the circulating hemocytes. Granulocytes are larger hemocytes which readily spread on glass surface and which strongly react to the Gomori substrate, indicating the enzyme acid phosphatase usually found in lysosomes. Ultra-structurally, they contain a well-developed rough endoplasmic reticulum, dictyosomes and some lysosome-like dense bodies. Granulocytes do not exhibit a characteristic granular aspect and the few granules observed in the cytoplasm should correspond to a lysosome system. They were named granulocytes instead of amoebocytes to use the same terminology adopted for Biomphalaria glabrata in order to make easier comparative studies.

This is a preface study for more specific investigations on the functional activities of the blood cells of B. tenagophila and their interactions with the trematode parasite.

Key words: *Biomphalaria tenagophila* – gastropod – snail – mollusc

Pulmonate snails of the genus *Biomphalaria* are known to be intermediate hosts for the trematode *Schistosoma mansoni*, the most well-studied of the blood flukes infecting humans. Among the different species, some strains of *B. glabrata* have demonstrated to be highly susceptible to trematode infection (Lie, 1982). The degree of compatibility of the snail and the parasite is essential for the trematode transmission in nature. As stated by Loker & Bayne (1986) the *B. glabrata*-*S. mansoni* system has become the most intensively studied model for investigating the cellular and molecular basis of snail susceptibility and resistance to trematode infectivity. In south and southeast of Brazil, the freshwater snail *B. tenagophila* has a great importance in the epidemiology of schistosomiasis (Paraense, 1975) although it is less susceptible to trematode infection than *B. glabrata* (Magalhães, 1979; Dias et al., 1987).

In Santa Catarina state, south of Brazil, *B. tenagophila* was the unique transmitter of *S. mansoni* as yet described (Bernardini & Machado, 1981; Malek, 1985). Very recently, the other trematode transmitter, *B. straminea*, was also detected in Santa Catarina state (Ferrari & Hofmann, 1992) but all the described foci of schistosomiasis in this locality are maintained by *B. tenagophila* (Bernardini & Machado, 1981). *B. glabrata* species does not seem to occur in this region.

Since various kinds of freshwater snails are known to be intermediate hosts of helminthic parasites in humans and domestic animals, the relationships between snail-parasite are essential to the understanding of the biology of parasites in host snails and to develop methods for the control of snails in endemic areas. As the trematode *S. mansoni* develops in the snail hemocoel, the immunobiology of the trematode-snail relationships is strongly related to the circulating cells in the hemolymph or hemocytes, and to humoral factors within the snail plasma (Lie, 1982; Loker & Bayne, 1986;

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van der Knaap & Loker, 1990). Therefore, studies on the hemocytes and serum factors of the intermediate host have been particularly carried out in *B. glabrata* where the circulating cells have been characterized morphologically (Harris, 1975; Cheng, 1975; Cheng & Auld, 1977; Joky et al., 1983) and also through enzyme and surface marker techniques, using lectins and monoclonal antibodies (for references see review of Sminia & van der Knaap, 1986). As far as we know, investigations on the hemocytes of *B. tenagophila* were never reported.

The purpose of this paper is to describe the numbers and the morphological characteristics of the circulating cells of the freshwater snail *B. tenagophila*, the unique trematode transmitter which maintains foci of schistosomiasis in Santa Catarina state. Such descriptions are a preface to more specific studies concerned with their functions as related to internal defense against the trematode parasite.

MATERIALS AND METHODS

The pulmonate snail *B. tenagophila* was collected in different slow-flowing streams and ponds in the locality of São Francisco do Sul, north of Santa Catarina state, where foci of schistosomiasis were detected. The animals were maintained and cultivated in the Department of Microbiology and Parasitology of the Federal University of Santa Catarina. The animals were kept in aquaria at 25 °C and fed with fresh lettuce *ad libitum*.

Hemolymph of adult specimens was collected directly from the heart cavity, by inserting a 26-gauge needle attached to a 1 ml syringe, in the cardiac region. The animals were placed in distilled water for at least 2 hr, before bleeding.

Observations with the phase contrast microscope (PCM) – Fresh hemolymph samples, unfixed or fixed with 1% glutaraldehyde in 0.1M sodium cacodylate, pH 7.2, were spread in thin films between a slide and a coverslip and observed under PCM.

Giemsa stained smears – Blood smears were air dried, fixed with methanol for 10 min and stained with Giemsa in phosphate buffer (1:3), 0.1M, pH 7.2 for 20-30 min. After washing in running water the blood smears were observed under the light microscope.

In all the following histochemical stainings, hemolymph smears were fixed in 10% formalcalcium pH 7.2 for 20-30 min at room temperature (20 °C) and washed thoroughly with running water.

Gomori method for acid phosphatase – After fixation, blood smears were incubated with Gomori substrate for 30 min at 37 °C (Bancroft & Stevens, 1982). Smears were then rinsed with distilled water, immersed in a weak (1%) solution of ammonium sulfide for 1-2 min, washed again with running water and mounted. In control experiments, blood smears were incubated without the enzyme substrate, sodium β -glycerophosphate.

Sudan black B for lipids – Fixed blood smears were treated with 50% ethanol for 3 min and immersed in a Sudan black B solution in 70% ethanol (Bancroft & Stevens, 1982). The smears were then immersed in 50% ethanol washed with distilled water and mounted.

Periodic Acid – Schiff (PAS) for carbohydrates – Hemolymph fixed smears were incubated with 1% periodic acid, washed with distilled water and treated with the Schiff reagent for 30 min (Bancroft & Stevens, 1982). The smears were then, washed for 3 min in each of three consecutive sulfurous water bathes (10 ml of HCl 1N in 210 ml of NaHSO₃ 0.05%), left with running water for about 30 min and mounted.

Alcian blue pH 1.0 for sulfated glucosaminoglycans and sulfomucins – After fixation, blood smears were washed with running water for 2 hr and stained with 1% Alcian blue in 0.1N hydrochloric acid, pH 1.0 for 18 hr at room temperature. The smears were, then, dehydrated in two changes of absolute ethanol, cleared in xylene and mounted (Ashhurst, 1979).

Alcian blue pH 2.6 for sialic acid residues and acid glucosaminoglycans – Blood smears were fixed and washed as above and stained with 1% Alcian blue in 3% acetic acid pH 2.6 for 18 hr at room temperature. The smears were then washed for 5 min with distilled water, rapidly dehydrated, cleared with xylene and mounted (Ashhurst, 1979).

Transmission electron microscopy (TEM) – The collected hemolymph was immediately placed in a 3% glutaraldehyde solution in 0.1M

sodium cacodylate pH 7.2 and fixed for 1 hr at 4 °C. Hemocyte pellets obtained by centrifugation (100 g) were washed in the same cacodylate buffer and post-fixed with 0.1% osmium tetroxide in cacodylate buffer for 30 min at 4 °C. Blood cell pellets were then washed with a 0.1M NaCl solution and contrasted with 1% uranyl acetate for 15-18 hr at 4 °C. After routine dehydration in graded ethanol solutions, the blood cells were embedded in Spurr resin. Ultra-thin sections were mounted on copper grids, contrasted with lead citrate and examined with a ZEISS-Electron Microscope operated at 60 kV.

Total hemocyte counts (THC) – The number of hemocytes was determined from undiluted fresh hemolymph, by using a Neubauer chamber and a similar procedure to that used for white blood cell counts. The THC was also estimated in 10 animals which were heat-treated before blood collection. For this, the snails were maintained on hot water vapours (60 °C) for 5 min.

Differential hemocyte counts (DHC) – This was carried out by counting the different hemocyte types and calculating their relative percentages in Giemsa stained smears or in fresh hemolymph smears examined in PCM. At least 200 cells were counted from each animal.

RESULTS

Two hemocyte types are identified in the hemolymph of the freshwater snail *B. tenagophila* through light and transmission electron microscopy (TEM). Their numbers in circulation are shown in Table I. Degenerated cells are often seen in circulation.

Hyalinocytes – Small spherical hemocytes (5-8 µm) with a large nucleus surrounded by a thin cytoplasm layer (Figs 1, 2). They are easily identified by their high nucleocytoplasmic ratio (ca. 0.70) (Figs 1B, 2B). In fresh blood smears observed on the phase contrast microscope (PCM), they adhere to the glass surface but do not spread appreciably. Thus, they are usually stable cells and filopodia and pseudopodia are not commonly seen on their surface. Through TEM they appear as immature young cells, whose cytoplasm is especially rich on free ribosomes (Figs 1A, 1C). They can also contain scattered mitochondria, and a poorly developed RER and dictyosomes (Figs 1A, 2A). Lysosome-like bodies are not usually seen. Hyalinocytes are scarcely reactive to the histochemical stainings used (Table II). They represent less than 10% of the hemocytes in circulation (Table I).

Granulocytes – Larger and polymorphic hemocytes (7-15 µm) (Figs 3, 4, 5) which readily spread on glass, extending numerous thin filopodia when observed on fresh blood smears (Figs 5A, 5B, 5C). They have a relatively larger cytoplasm. Some granulocytes appear morphologically more stable and more basophilic when stained by the Giemsa (Fig. 4B). When observed through TEM, the cytoplasm layer contains loose ribosomes, RER, well developed Golgi bodies, mitochondria and some lysosome-like dense bodies (Figs 3C, 4A, 4D, 5D). Granulocytes are strongly reactive to the Gomori substrate which indicates the presence of acid phosphatase, usually found in lysosomes (Table II). Granulocytes appear to contain carbohydrates and a scarce content of acid glucosaminoglycans since they are positive to PAS and are moderately stained by the Alcian blue (pH 2.6). It is the major hemocyte type in

TABLE I

Total (THC) and differential (DHC) hemocyte counts were determined in the hemolymph of adults of *Biomphalaria tenagophila*. Ten animals were used in each THC analysis. Fifteen animals were used for DHC

Total Hemocyte Count (THC)					
Untreated animals			Heat-treated animals		
Mean	SD	Range	Mean	SD	Range
252	± 130	108 - 552	836	± 127	630 - 1,214
Differential hemocyte counts (DHC)					
Number of cells		Hyalinocytes (%)		Granulocytes (%)	
2,915		9.1		90.9	

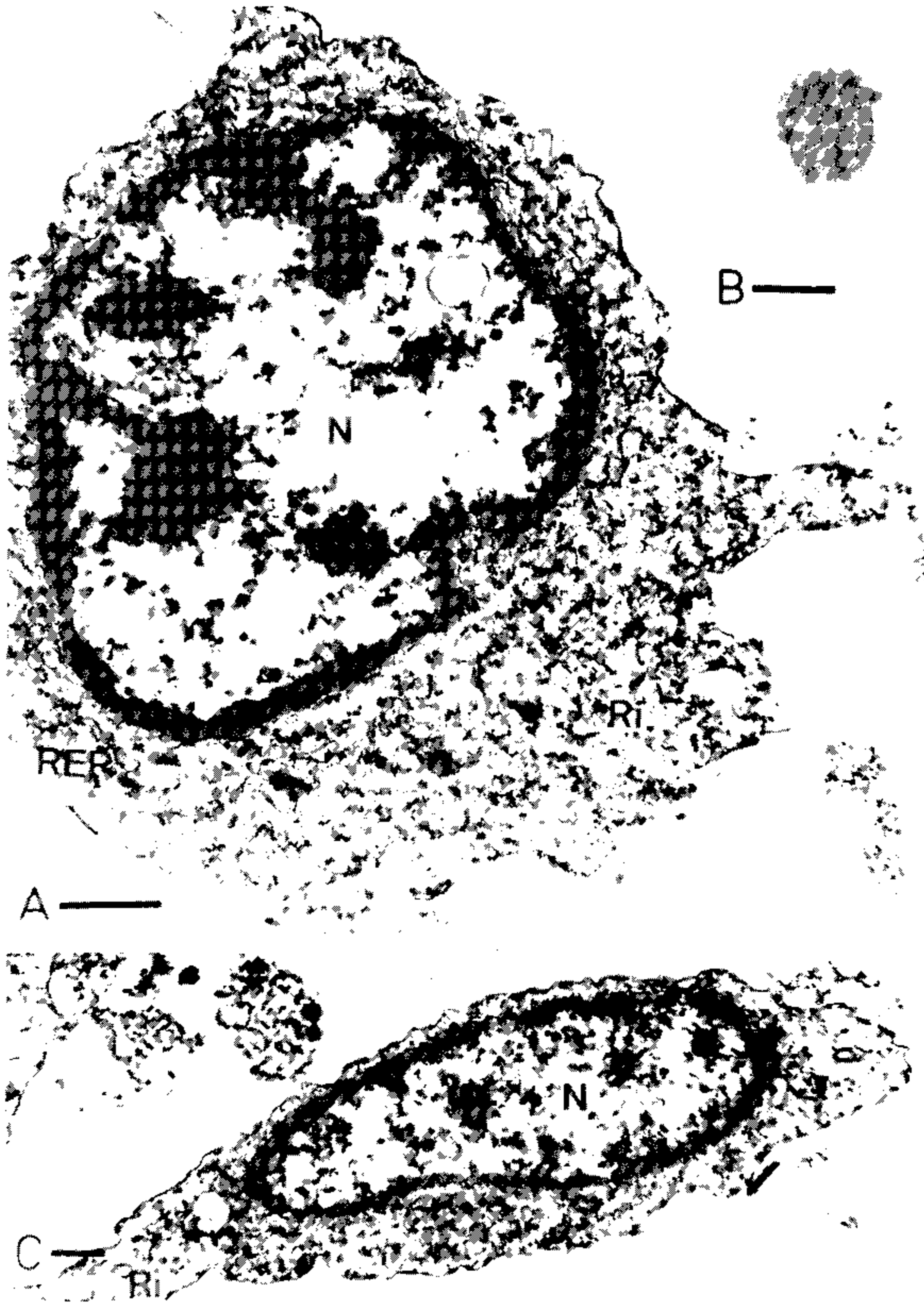


Fig. 1: hyalinocytes of *Biomphalaria tenagophila*. A and C: electron micrographs. Note presence of abundant free ribosomes. B: hyalinocyte stained by the Giemsa. N: nucleus; RER: rough endoplasmic reticulum; Ri: ribosomes. In A and C, bar = 1 μ m; in B, bar = 5 μ m.

circulation, representing more than 90% of the circulating cells (Table I).

Degenerated cells – These are often observed in the hemolymph of some animals. They can be easily identified by a disrupted cytoplasm and the characteristic chromatin masses in the nucleus (Figs 6A, 6B, 6C). They are presumably not originated by an inadequate fixation, since they are already present on fresh

blood preparations immediately examined under PCM. They are also found on fixed blood smears stained by the Giemsa (Fig. 6B) and on TEM preparations (Figs 6A, 6C), besides the other hemocyte types which appear considerably well-preserved. They may possibly reflect some particular physiological conditions of the snails. In some animals, the degenerated cells can reach more than 10% of the circulating hemocytes.

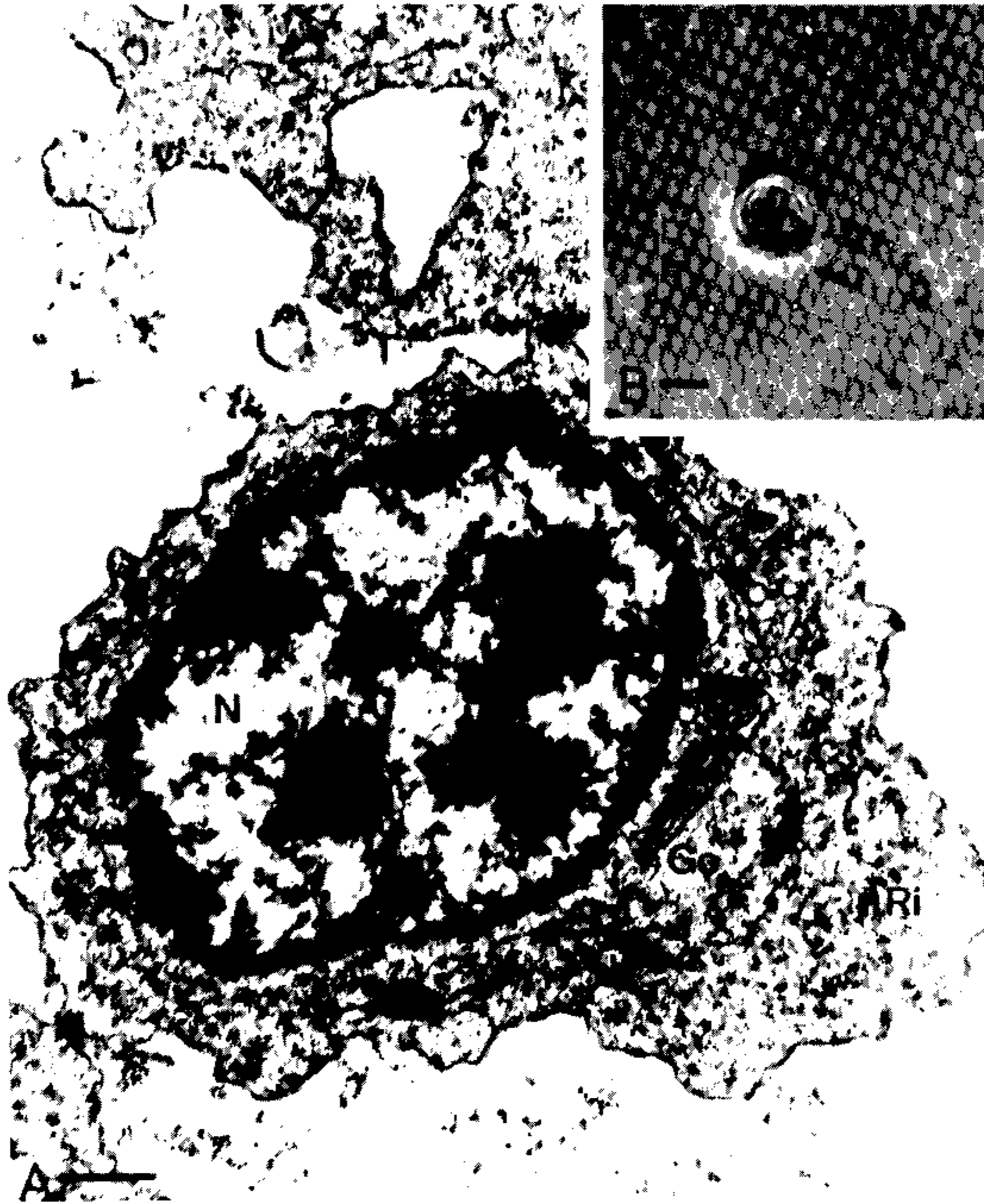


Fig. 2: hyalinocytes of *Biomphalaria tenagophila*. A: electron micrograph. B: hyalinocyte examined on the phase contrast microscope. Go: Golgi body; N: nucleus. In A, bar = 1 μ m; in B, bar = 5 μ m.

TABLE II

Histochemical stains. The blood cells of *Biomphalaria tenagophila* were treated with different histochemical stains (see Materials and Methods for details). Five animals were used in each test. The numbers 0-3 represent the intensity of reaction or staining. 0-negative staining; 1-weak; 2-moderate; 3-strong

Histochemical test	Hyalinocyte	Granulocyte
Gomori method	0-1	1-3
Sudan black B	0	0
Periodic acid + Schiff (PAS)	0-1	1-3
Alcian blue pH = 2.6	0	0-2
Alcian blue pH = 1.0	0	0

DISCUSSION

The two hemocyte types distinguished in the blood of *B. tenagophila* are comparable to

the descriptions of similar cells in *B. glabrata* (Harris, 1975; Cheng, 1975; Cheng & Auld, 1977; Schoenberg & Cheng, 1980; 1981). As in *B. glabrata* (Cheng, 1975), the hyalinocytes of *B. tenagophila* are spherical cells which attach to the glass surface but do not spread or flatten appreciably. They have a high nucleocytoplasmic ratio and the cytoplasm appears poorly differentiated under the transmission electron microscope (TEM). Lysosome-like granules do not seem to occur in hyalinocytes which is also corroborated by their weak reactivity to the Gomori substrate for the enzyme acid phosphatase. This appears to be also true for the hyalinocytes of *B. glabrata* (Harris & Cheng, 1975) and the snail *Bulinus guernei*, transmitter of the trematode *Schistosoma hematobium* (Krupa et al., 1977). The number of hemocytes in circulation (THC) has been established in several gastropods. However, it



Fig. 3: granulocytes of *Biomphalaria tenagophila*. A and B: granulocytes stained by the Giemsa. C: electron micrograph. Note the extended filopodia (arrowheads). N: nucleus; RER: rough endoplasmic reticulum; Ri: ribosomes. In A and B, bar = 5 μm ; in C, bar = 2 μm .

varies greatly from species to species and within a species, according to the method and the site used to extract hemolymph and also to the physiological conditions of snails (see review of Sminia, 1981). In *B. tenagophila*, the THC varies from 100 to 550 hemocytes/ μl . In heat treated animals this number increases more than 3 times (630 to 830 hemocytes/ μl). These numbers are comparable to the THC of *B. glabrata* (100 to 1,000 cells/ μl) (Jeong & Hencyman, 1976; Cheng & Auld, 1977; Stumpf & Gilbertson, 1978; Noda & Loker, 1989), *Helix pomatia* (Bayne, 1974) and *Lymnea stagnalis*

(500 to 4,000 hemocytes/ μl) (Sminia, 1972). The relative numbers of hyalinocytes and granulocytes (approximately 10% and 90% respectively) in the blood of *B. tenagophila* are similar to the described in *B. glabrata* (Schoenberg & Cheng, 1980, 1981; Noda & Loker, 1989). Granulocytes appear more differentiated than hyalinocytes and have a relatively larger cytoplasm containing more membrane systems. They readily spread in contact with glass and extend numerous filopodia when observed on fresh blood smears. They contain lysosome-like dense bodies that strongly react with the

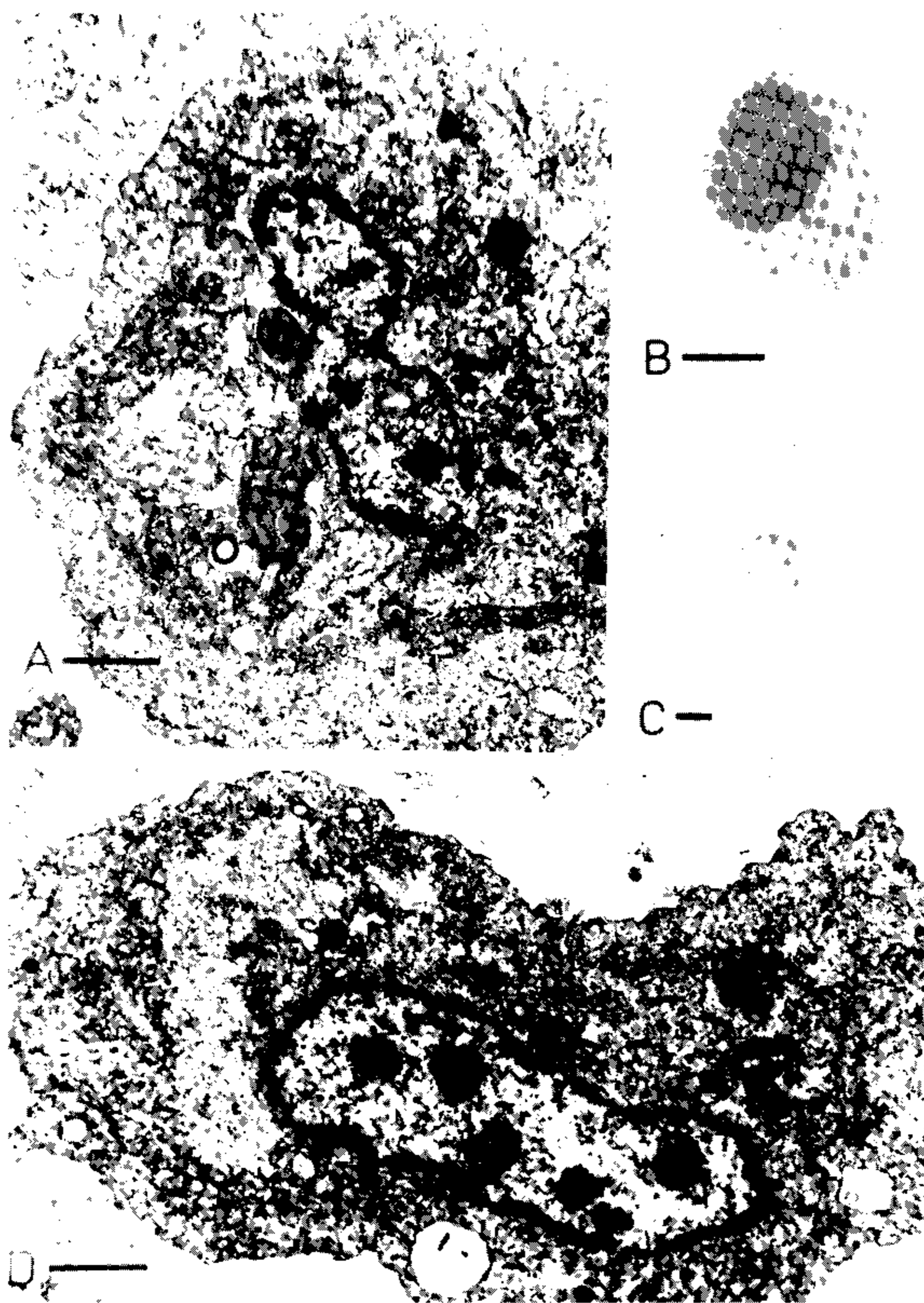


Fig. 4: granulocytes of *Biomphalaria tenagophila*. A and D: electron micrographs. Note the presence of several dense granules which presumably correspond to lysosomes (arrows). B: granulocyte stained by the Giemsa; some small granules can be observed. C: altered granulocyte observed on the phase contrast microscope. N: nucleus; RER: rough endoplasmic reticulum; Ri: ribosomes. In A and D, bar = 1 μ m; in B and C, bar = 5 μ m.

Gomori substrate which indicates the lysosome enzyme, acid phosphatase. This is also described in the granulocytes of *B. glabrata* (Harris & Cheng, 1975; Cheng & Garrabrant, 1977) and of other snails, as in *L. stagnalis* (Sminia, 1972), *Planorbis corneus* (Ottaviani et al., 1986) and *Viviparus ater* (Franchini & Ottaviani, 1990). We cannot assume, however, that the granulocytes of *B. tenagophila* exhibit a typical cytoplasmic granulation when observed on fresh blood smears, as described in

B. glabrata (Cheng, 1975; Cheng & Auld, 1977). Few granules can occasionally be distinguished around the nuclear periphery. Through TEM, some dense cytoplasmic bodies are effectively seen in the granulocytes of *B. tenagophila*, but a typical granular aspect, as reported in *Pomacea canaliculata* (Shozawa & Suto, 1990) or in *Mytilus edulis* (Moore & Lowe, 1977), is not observed. These dense bodies should presumably correspond to a lysosome system which is corroborated by the



Fig. 5: granulocytes of *Biomphalaria tenagophila*. A to C: granulocytes examined on the phase contrast microscope. Note the spreading aspect and the thin filopodia. D: electron micrograph. Note the well-developed Golgi body; the cytoplasm does not exhibit characteristic granules. Go: Golgi body; Mi: mitochondria; N: nucleus; RER: rough endoplasmic reticulum; Ri: ribosomes. In A to C, bar = 5 μm ; in D, bar = 1 μm .

strong reactivity of these cells to the Gomori substrate. Numerous lysosome-like structures, which contain enzymes typically associated with lysosomes were also described in *B. glabrata* (Harris & Cheng, 1975; Sminia & Barendsen, 1980; Morona et al., 1984). Therefore, we also named them granulocytes, due to the presence of such lysosome granules and to the nomenclature usually adopted for *B. glabrata* (Cheng, 1975; Harris, 1975; Jeong & Heyneman, 1976; Cheng & Auld, 1977) as well as for other

snails as *B. guernei* (Krupa et al., 1977) and *P. canaliculata* (Shozawa & Suto, 1990). However, it would have been more appropriate to denominate them amoebocytes, since they can be especially recognized by their readily spreading behaviour instead of a typical cytoplasmic granulation.

In the other freshwater snail, *L. stagnalis*, Sminia et al. (1983) described one basic type of hemocyte which would pass through a dif-

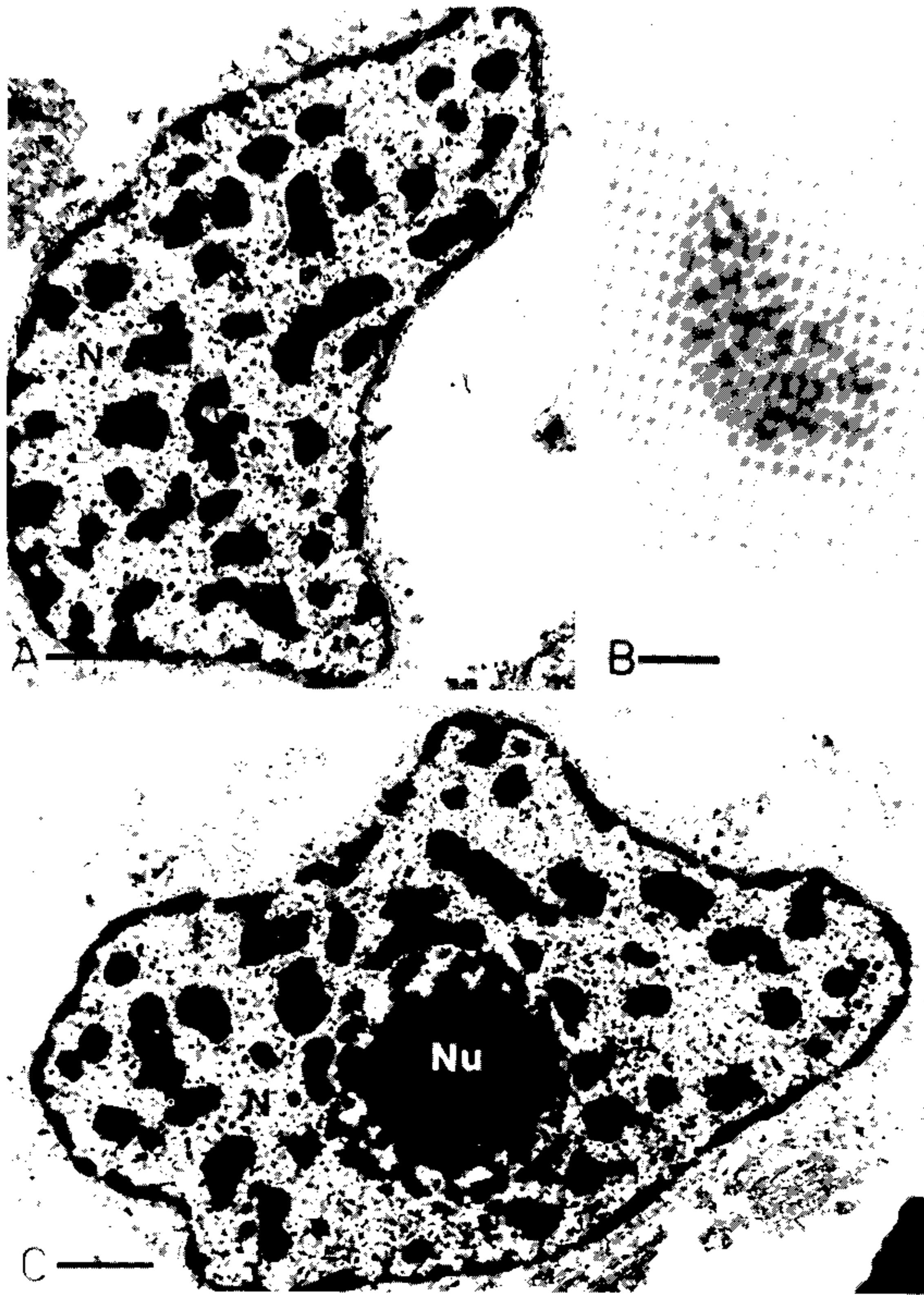


Fig. 6: degenerated cells found in the blood of some *Biomphalaria tenagophila* snails. A and C: electron micrographs. Note the disrupted cytoplasm and the characteristic masses of condensed chromatin. B: degenerated cell stained by the Giemsa. N: nucleus; Nu: nucleolus. In A and C, bar = 1 μ m; in B, bar = 5 μ m.

ferentiation series from round (young) cells to readily spreading (mature) cells. The different developmental stages of hemocytes would vary in functional capacities but only quantitatively, not qualitatively. Thus, spreading hemocytes show a higher phagocytic capacity than the round hemocytes (Sminia & van der Knaap, 1986). On the other hand, investigations on surface membrane characteristic, especially in *B. glabrata*, using lectins and monoclonal antibodies, appear to indicate that several populations of hemocytes would effectively exist

(for references, see reviews of van der Knaap & Loker, 1990; Adema et al., 1991) each one with its own function and progenitor cell. Since we carried out only morphological observations in *B. tenagophila*, we cannot assume that hyalinocytes and granulocytes are different maturation stages of the same hemocyte type or are distinct cell populations with different functions and origins. Granulocytes are referred as actively phagocytic (Jeong & Heyneman, 1976; Sminia & Barendsen, 1980; van der Knaap & Loker, 1990) and are the most in-

volved in destruction of trematode larvae (Cheng & Garrabrant, 1977; Loker et al., 1982; Jeong et al., 1984; Loker & Bayne, 1986). The morphological data obtained in this study with *B. tenagophila* are a preface to more specific investigations on the functional activities of the hemocytes that are presumably involved with the snail susceptibility to trematode infection. As stated above the snail *B. tenagophila* assumes great importance in the epidemiology of schistosomiasis in Santa Catarina state, since the different foci of the disease in this locality are maintained by this species.

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