

## ASPECTS OF CLASSIFICATION OF HEMIPTERA HEMOCYTES FROM SIX TRIATOMINE SPECIES

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*The objective of this work was to characterize, and compare different morphological types of hemocytes of Rhodnius prolixus, Rhodnius robustus, Rhodnius neglectus, Triatoma infestans, Panstrongylus megistus, and Dipetalogaster maximus. This information provides the basis for studying the cellular immune systems of these insects. Seven morphological hemocyte types were identified by phase-contrast microscopy: prohemocytes, plasmotocytes, granular cells, cystocytes, oenocytoids, adipohemocytes and giant cells. All seven types of hemocytes are not present in every species. For example, adipohemocytes and oenocytoids were not observed in P. megistus and P. infestans, and giant cells were rarely found in any of the species studied. The hemocytes of Rhodnius and Dipetalogaster are more similar to each other than those from Triatoma and Panstrongylus which in turn closely resemble each other. Emphasis is placed on methodological problems arising in this work which are discussed in detail.*

Key words: hemocytes – Hemiptera – triatomine – *Rhodnius* – *Triatoma* – *Panstrongylus* – *Dipetalogaster*

A classification for *Rhodnius* hemocytes was proposed by Wigglesworth (1933). He characterized four types of hemocytes in the hemolymph of *Rhodnius* nymphs, and in 1955 he identified two additional cell types. Jones (1962, 1965) described the hemocytes of *R. prolixus* in a very detailed study using phase contrast microscopy but included only a few original photomicrographs of his observations. Recently, Lai-Fook (1968, 1970) investigated the fine structure of the *R. prolixus* hemocytes and although identifying five cell types failed to recognize the granular cells. Finally, Barracco et al. (1987) observed five types of hemocytes in the hemolymph of *Panstrongylus megistus*, although they could only identify the prohemocytes and plasmotocytes unequivocally. Unfor-

tunately, the classification schemes provided by these authors have led to some controversy and no comparative study has yet been conducted to provide a unified classification of triatomine blood cell types. Such a scheme is essential in order to provide a basis for studies on vector/parasite interactions involving these cells. The aim of this paper is therefore to characterize and compare different morphological types of hemocytes in various triatomine species. In addition, problems involved in both classifying and stabilizing these cells for microscopical examination are discussed, and the drawbacks of the various methods used assessed.

### MATERIALS AND METHODS

Adults and fourth and fifth-instar nymphs of male and female *R. prolixus*, *R. robustus*, *R. neglectus*, *T. infestans*, *P. megistus*, and *D. maximus* were used throughout the experiments. The insects were fed on pigeons every ten days and maintained at 28 °C and 70% RH. They were bled seven to ten days after feeding by applying gentle pressure to the abdomen with a fine pair of forceps following amputation of one or more legs. Small drops of hemolymph were carefully collected onto three sterile microscopy slides: on slide containing pure

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hemolymph, the second hemolymph mixed with one small drop of anticoagulant solution (0.01M ethylenediamine tetra-acetic acid, 0.1M glucose, 0.062M sodium chloride, 0.03M trisodium citrate, 0.026M citric acid, pH 4.6, 370 mOsm) and the last one hemolymph mixed with one drop of insect saline plus phenylthiourea (1-2 small crystals in 0.17M NaCl). For fixed monolayers the hemolymph was taken up into 20  $\mu$ l pipettes previously rinsed with 0.1M sodium phosphate buffer in 0.17M NaCl with few crystals of phenylthiourea. The hemolymph was expelled on to glass slides and the hemocytes allowed to attach for 1 h at room temperature, rinsed in phosphate buffer before fixation in 2.5% glutaraldehyde in 0.1M cacodylate buffer for 1 h at room temperature. The monolayers were finally rinsed in phosphate buffer-saline, and examined under microscopic. Preparations were observed and photographed under phase contrast optics of a Zeiss Photomicroscope III and photographed with Pan F (Ilford) or Pan X (Kodak) films. All slides, coverslips and containers were heat-treated (180 °C for 4 h) to remove endotoxin and either examined immediately without coverslips or else mounted with small pieces of cover glasses, depending upon the amount of blood involved. All solutions were made up in endotoxin-free water (double glass distilled) and filter sterilised (0.22  $\mu$ m) before use.

## RESULTS

For an adequate interpretation of our results, we made some important preliminary observations which demonstrated that: (i) some of the hemocyte types of different species of triatomines were highly unstable and fragile in non-sterile conditions; (ii) the hemocytes sometimes appeared differently according to the diluent used; (iii) differences observed between the hemocytes of the various species might reflect the nutritional and/or developmental conditions of the insects so that these parameters must be carefully controlled; (iv) caution must be applied to the interpretation of previous reports in which techniques derived from vertebrate hematology have been utilised; (v) finally, some types of hemocytes rapidly transformed during observation under the microscope or after hemolymph sampling, and these factors could easily lead to erroneous interpretation of the hemocyte morphology.

Therefore, we decided to observe hemocytes in whole, undiluted hemolymph, or with saline or without an anticoagulant or phenylthiourea, in an attempt to stabilize the cells. Usually one or more of these hemocyte treatments successfully stabilized the cells for observation.

Taking these facts into consideration, hemocytes in the various species of triatomines (*R. prolixus*, *R. robustus*, *R. neglectus*, *T. infestans*, *P. megistus*, and *D. maximus*) studied could broadly be classified morphologically and from their behaviour *in vitro* into seven cell types by phase-contrast microscopy. These hemocyte categories are prohemocytes (PRs), plasmatocytes (PLs), granular cells (GRs), cystocytes (CYs), oenocytoids (OEs), adipohemocytes (ADs) and giant cells (GCs). However, all seven types of hemocytes are not present in each species (Table). For example, adipohemocytes and oenocytoids were not observed in *P. megistus* and *T. Infestans*, and giant cells were rarely found in any of the species.

*Prohemocytes (PRs)* – PRs were present in variable numbers in all the insects examined (Table). In the three *Rhodnius* species, they were small, agranular, mostly round, stable cells, sometimes forming small pseudopods (Figs 1, 2). In contrast to Jones (1965) observations, PRs never broke down *in vitro*. In some cases PRs were observed in mitosis (Fig. 3). In *T. infestans*, the PRs were similar to those of the *Rhodnius* spp. but were more common with higher numbers forming pseudopods and all intermediates were present between PRs, agranular PLs and granular PLs. In *P. megistus* and *D. maximus*, the PRs were very similar to the former species described and were particularly common in nymphs.

*Plasmatocytes (PLs)* – PLs were found in all species of insects examined and were, together with the granular cells (GRs), the commonest hemocytes in the hemipterans studied. In the three *Rhodnius* species, the PLs were variable in form as described by Jones (1965). They were often spindle-shaped cells as in Fig. 4 or rounded and flattened *in vitro* with vacuolar plus granular inclusions (Figs 4-6). The latter were typical and led to the formation of the characteristic flattened PLs as observed in glutaraldehyde-fixed monolayers (Fig. 7). Sometimes the PLs were unstable *in vitro* with bright granules/vacuoles and resembled cystocytes (Fig. 6). In *P. megistus* and *T. infestans*,

TABLE  
Showing incidence and characteristics of main hemocyte types found in triatomines

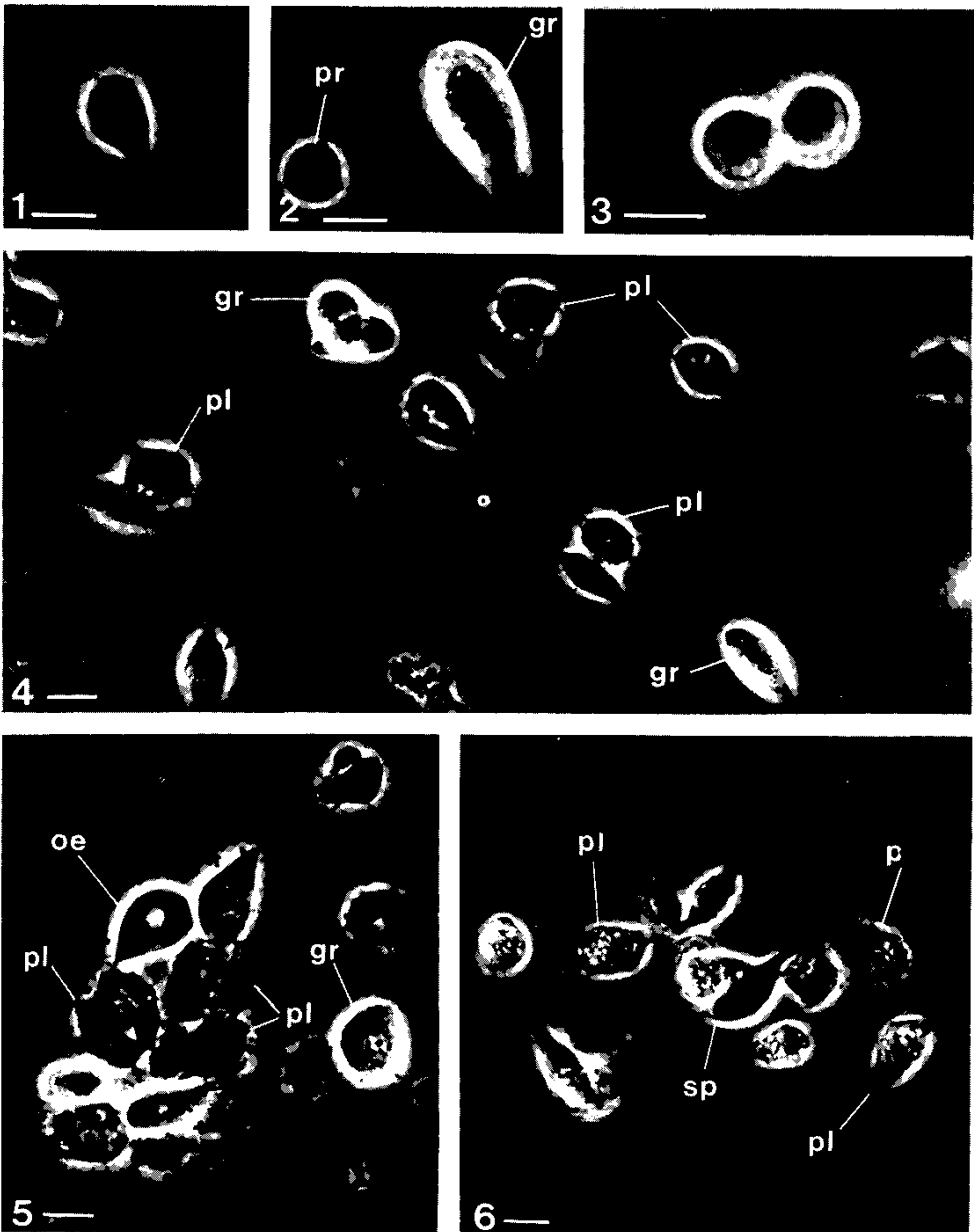
Species	Cell type								
	PRs	PLs	GRs	CYs	OEs	ADs	GCs	SPs	Coagulation
<i>R. prolixus</i>	+	+	+	+	+	not seen	+	= PLs + RGs	+
	stable	variable	stable	unstable			rare	+ OEs	occasional
	mitotic	common	common					common	fibrous
<i>R. robustus</i>	mitotic	common	common	unstable	+	not seen	rate	common	fibrous
<i>R. neglectus</i>	mitotic	common	common	unstable	+	rare	not seen	common	fibrous
<i>T. infestans</i>	+	great	common	unstable	not seen	not seen	+	= PLs + GRs	fibrous
	common	size					rare	few	
		range,							
		granular							
		spread							
		rapidly							
<i>P. megistus</i>	+	+	+	+	not seen	not seen	not seen	= GRs	fibrous
	mitotic	variable						few	
		common							
		granular							
		spread							
		rapidly							
<i>D. maximus</i>	+	+	+	+	occasional	not seen	not seen	common	fibrous
	common	less							
	in nymphs	granular							

the PLs had a huge variation in size from 10-40  $\mu\text{m}$  in diameter (Figs 8-10) and contained more granular inclusions than the *Rhodnius* spp., some of which were very large in *T. infestans* (Figs 8, 9). The PLs of these two species also spread more rapidly than those of *Rhodnius*. In addition, *T. infestans* contained small PLs intermediate in size between PRs and immature PLs (Fig. 8). The PLs of *D. maximus* had less size range than *Triatoma* and fewer granules than *Triatoma* and *Panstrangylus*, so that they were very much like those of *Rhodnius* and did not spread quickly *in vitro*. The PLs appeared to degranulate sometimes *in vitro* and could be related to the CYs.

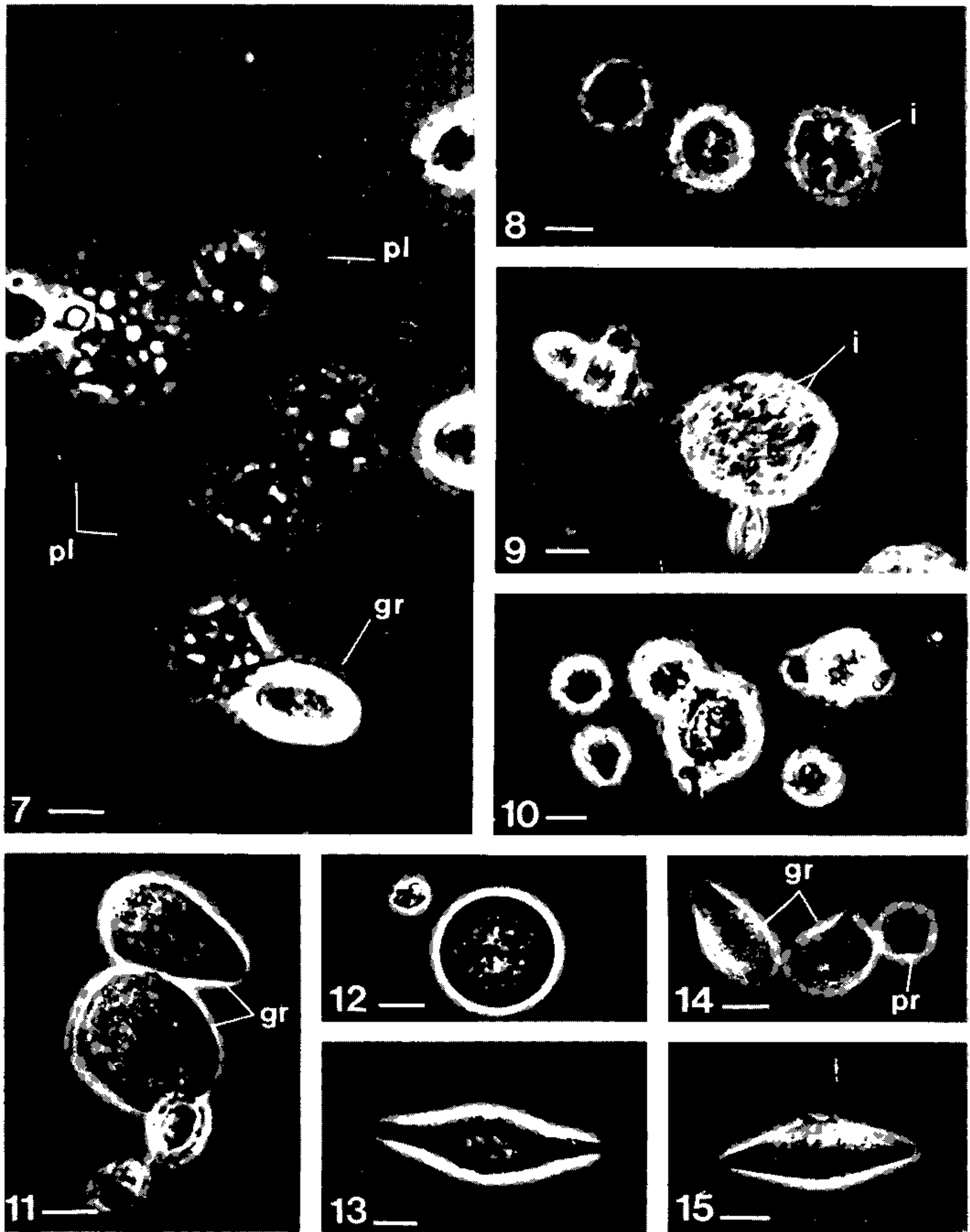
**Granular cells (GRs)** – GRs were observed in all the insect species studied in which they were very similar in structure (Figs 11-14, 39). They were usually round to spindle-shaped, stable cells, 15-30  $\mu\text{m}$  in diameter and enclosed large numbers of small granules which were rarely observed to be discharged from the cells. Usually the GRs retained their regular outline but occasionally a small pseudopod or extension was formed (Fig. 15). It is apparent that some, but not all, of the so-called spindle cells de-

scribed by other authors include the GR cell type. GRs are readily identified and with the PLs are the most common hemocyte type, often together comprising 80-95% of the blood cells present.

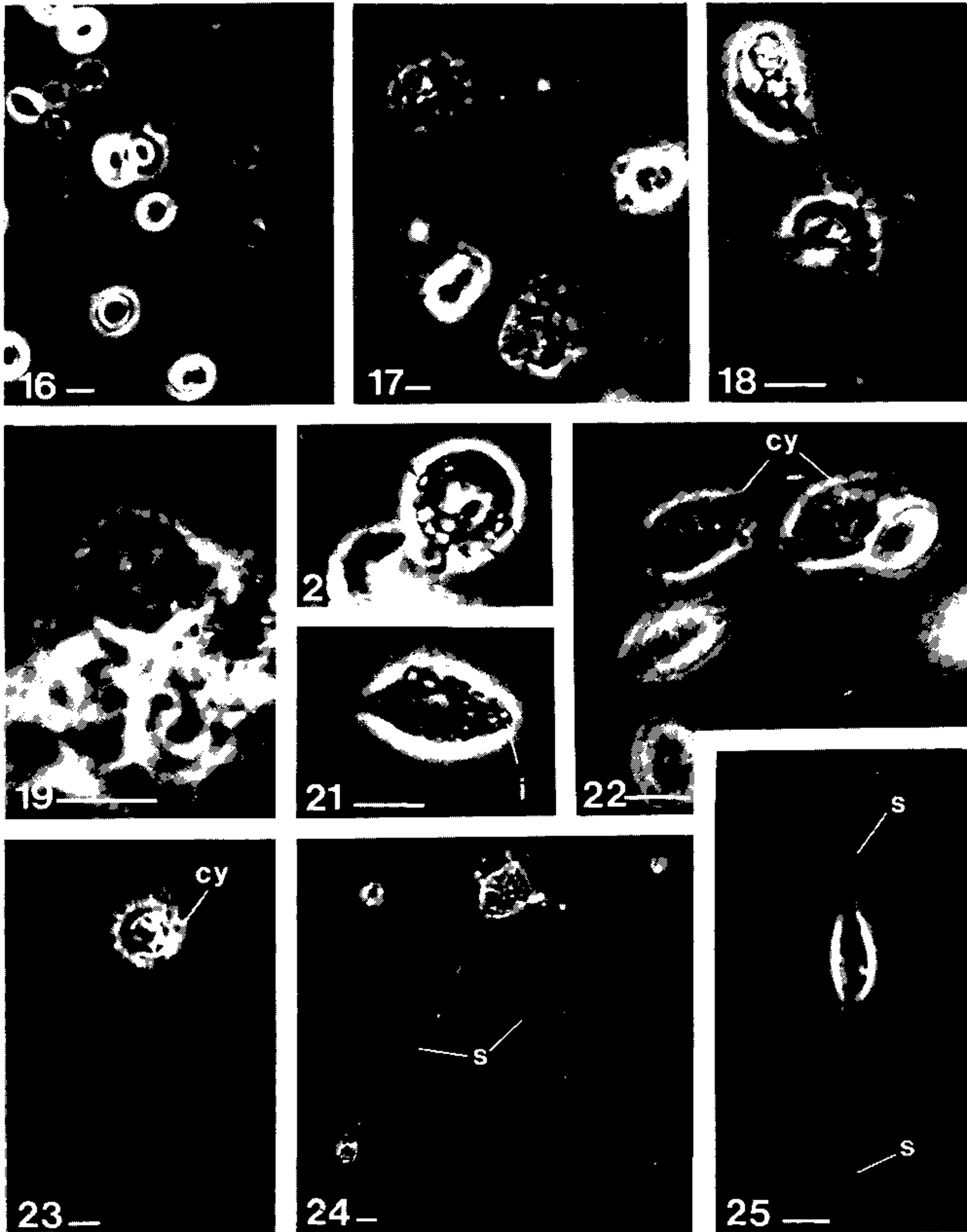
**Cystocytes (CYs)** – CYs were very fragile cells as compared with PRs, GRs and PLs. In unstable conditions, these cell types broke down and the only indication of their presence was a few naked nuclei and cell debris (Figs 16-19). They were thus very easy to overlook unless the hemolymph was examined using a variety of methods. It was impossible to estimate the number of CYs present in a blood sample because of their instability and possible relatedness to the PL cell type (Figs 21, 22). CYs were distinct from GCs as they were usually smaller, fewer in number and contained large granules, which were phase bright, surrounding a large nucleus (Figs 20-22). We also observed that CYs could be involved in formation of "islets of coagulation" as described by Grégoire (1955) (Fig. 23). Coagulation thus does occur in triatomines but it may usually be limited to the formation of a few extracellular strands which originate from entrapped CYs (Figs 24, 25).



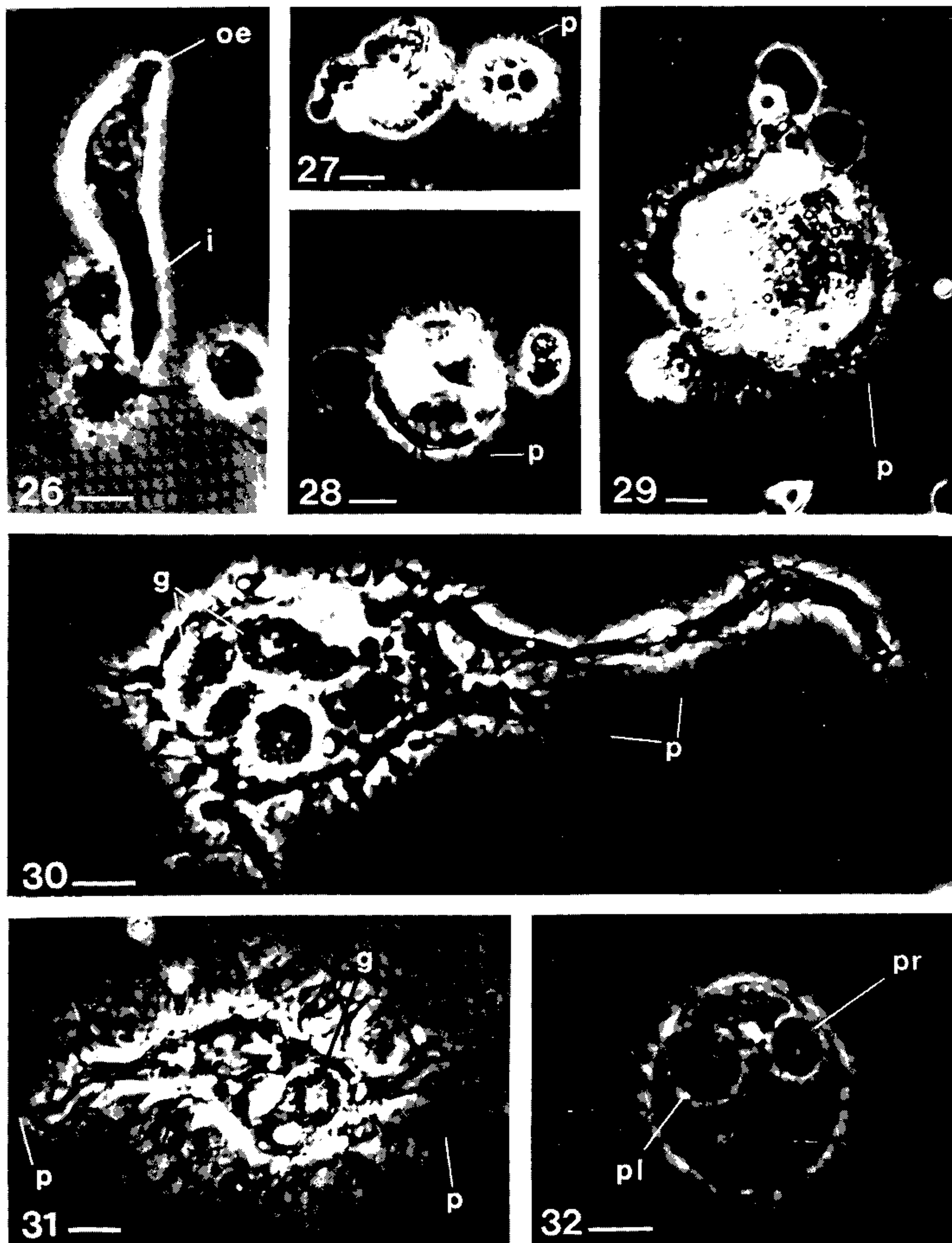
Phase contrast micrographs of different hemocyte types from six triatomine species. Scale bars = 10  $\mu\text{m}$ . Fig. 1: prohemocyte of *Triatoma infestans*. Note small pseudopod (arrow). Anticoagulant plus coverslip. Fig. 2: showing relative sizes of prohemocyte (pr) and granular cell (gr) from *T. infestans*. Pure hemolymph plus coverslip. Fig. 3: mitotic prohemocytes of *Panstrongylus megistus*. Anticoagulant plus coverslip. Fig. 4: plasmotocytes (pl) and granular cells (gr) of *Rhodnius prolixus*. The plasmotocytes attach firmly to the glass with pseudopods and some are spindle-shaped (unlabelled arrows). The granular cells remain rounded and do not usually form pseudopods. Pure hemolymph plus coverslip. Fig. 5: plasmotocytes (pl), granular cells (gr) and oenocytoid (oe) of *R. prolixus*. The plasmotocytes have flattened *in vitro* and contain both vacuolar and granular inclusions. Pure hemolymph plus coverslip. Fig. 6: as Fig. 5 but note bright granules in plasmotocytes (pl) so that they resemble cystocytes. The spindle cell (sp) shown may well belong to the oenocytoid category.



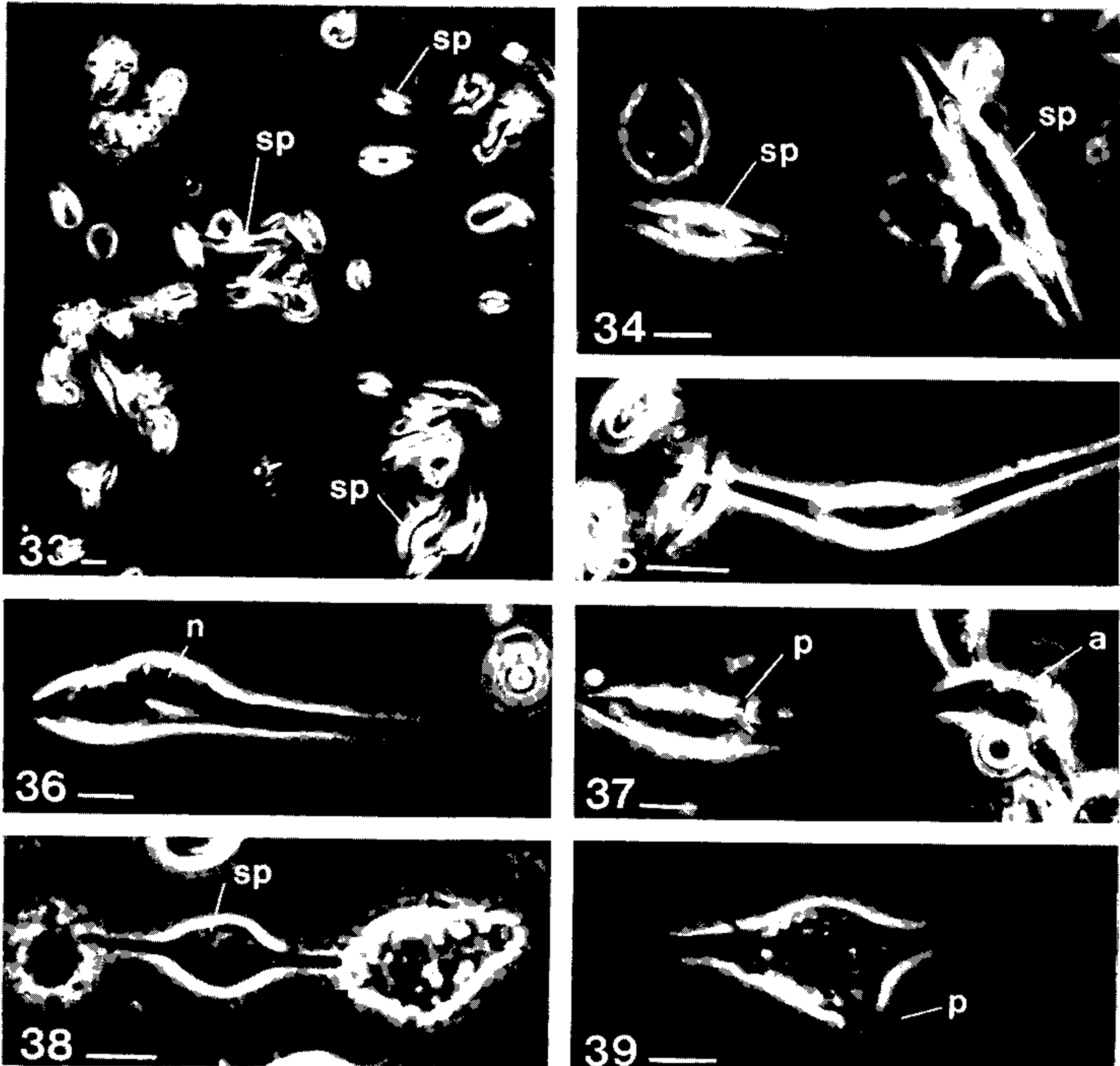
Phase contrast micrographs of different hemocyte types from six triatomine species. Scale bars = 10  $\mu$ m. Fig. 7: glutaraldehyde-fixed monolayer of *Rhodnius prolixus* hemocytes showing extensively flattened plasmatocytes (pl) with vacuolar and granular inclusions. Granular cells (gr). Cells attached for 1 h *in vitro* before fixation. Fig. 8: showing range in size of plasmatocytes of *Triatoma infestans* from small prohemocyte-like forms with no inclusions (unlabelled arrow) to large form with extensive pseudopods and granular inclusions (i). Pure hemolymph plus coverslip. Fig. 9: as Fig. 8 showing very large plasmatocyte with numerous inclusions (i). Fig. 10: range of plasmatocytes of different sizes in *P. megistus*. Anticoagulant plus coverslip. Fig. 11: two large granular cells (gr) of *R. robustus*. Note numerous small inclusions and lack of pseudopod formation. Anticoagulant with no coverslip. Fig. 12: granular cell from *Dipetalogaster maximus*. Note numerous granules and stability of this cell type *in vitro*. Anticoagulant plus coverslip. Fig. 13: as Fig. 12 but note spindle-shape of this granular cell. Fig. 14: two granular cells (gr) and a prohemocyte (pr) of *T. infestans*. Pure hemolymph plus coverslip. Fig. 15: granular cell of *D. maximus* which is both spindle-shaped and forming a pseudopod (unlabelled arrow). Phenylthiourea plus coverslip.



Phase contrast micrographs of different hemocyte types from six triatomine species. Scale bars = 10  $\mu\text{m}$ . Fig. 16: pure hemolymph from *Rhodnius neglectus* showing unstable nature of many of the hemocytes which have broken down to form cell ghosts and much debris (arrows). No coverslip. Fig. 17: pure hemolymph from *Panstrongylus megistus*. Note cell debris (arrows) indicative of rapid cell breakdown *in vitro*. With coverslip. Fig. 18: pure hemolymph from *Dipetalogaster maximus* showing two broken down cells and discharged granules (arrows). With coverslip. Fig. 19: pure hemolymph from *R. prolixus* showing swollen cell with peripherally located granules (arrow). With coverslip. Fig. 20: cystocyte cell type from *R. robustus*. Note central nucleus and peripherally located bright granules (arrows). Anticoagulant plus coverslip. Fig. 21: cell from *D. maximus* resembling both a plasmatocyte (with pseudopod arrowed) and a cystocyte with large nucleus and bright granules (i). Glutaraldehyde-fixed plus coverslip. Fig. 22: hemocytes from *Triatoma infestans* two of which are cystocytes (cy) attached to strands of coagulation (unlabelled arrows). Pure hemolymph plus coverslip. Fig. 23: presumed "islet of coagulation" formed around a cystocyte (cy). This type of coagulum is rarely seen and may have resulted from drying out of the preparation which was of pure hemolymph with no coverslip. Fig. 24: typical type of coagulation seen in triatomines and formed by extracellular strands (s) entrapping cell debris. *P. megistus* pure hemolymph plus coverslip. Fig. 25: coagulation strands (s) extending from a cell which is difficult to identify in this preparation. *D. maximus* hemolymph with phenylthiourea plus coverslip.



Phase contrast micrographs of different hemocyte types from six triatomine species. Scale bars = 10  $\mu$ m. Fig. 26: large spindle-shaped oenocytoid (oe) or *Rhodnius prolixus*. Note eccentrically-located nucleus and elongated inclusions (i). Glutaraldehyde-fixed plus coverslip. Figs 27-29: adipohemocytes of *R. neglectus*. Note extensive pseudopod formation (p) and numerous inclusions. All diluted with anticoagulant plus coverslip. Fig. 30: giant cells from *R. robustus*. Note extensive pseudopods (p) and ingestion of granule-containing cells (g) and other cell types. Anticoagulant plus coverslip. Fig. 31: giant cell from *R. robustus* showing large pseudopods (p) and ingested granule-containing cell (g). Anticoagulant plus coverslip. Fig. 32: giant cell from *Triatoma infestans* enclosing prohemocyte (pr) – and plasmatocyte (pl) – like cells. Anticoagulant plus coverslip.



Phase contrast micrographs of different hemocyte types from six triatomine species. Scale bars = 10  $\mu\text{m}$ . Fig. 33: low power micrograph of hemocytes from *Rhodnius neglectus* showing presence of numerous spindle-shaped (sp) cells in anticoagulant solution. No coverslip. Fig. 34: two spindle cells (sp) from *R. prolixus*. Saline plus coverslip. Fig. 35: very elongated spindle cell from *R. robustus*. This cell is so large that it may represent a separate cell type rather than an unusual form of granular cell or plasmatocyte. Anticoagulant plus coverslip. Fig. 36: two spindle cells from *R. robustus*. The eccentric nucleus (n) and inclusions may place these cells in the oenocytoid category of cells. Anticoagulant plus coverslip. Fig. 37: large spindle cell from *R. prolixus* which has formed a small pseudopod (p) and has flattened and attached to the glass at one of the cell (a) Saline plus coverslip. Fig. 38: spindle cell (sp) from *R. prolixus*. Glutaraldehyde-fixed plus coverslip. Fig. 39: spindle-shaped granular cell from *D. maximus* attaching to glass with pseudopods (p). Anticoagulant plus coverslip.

**Oenocytoids (OEs)** – OEs were observed mainly in the *Rhodnius* spp. and were not found or rarely seen in the other three species (Table). Some of them were round or oval, fairly stable cells, with clear and homogenous cytoplasm rarely containing inclusions and not forming pseudopods (Fig. 5). There was no difference between this rounded type of OE found in any of the triatomine species. Sometimes, the OEs were large, spindle-shaped cells,

with an eccentric nucleus, containing one or more dense nucleoli and a cytoplasm filled with both clear and phase-dark, elongated inclusions (Fig. 26).

**Adipohemocytes (ADs)** – ADs were rarely present in most species studied (Table). When present, they were small to large (20-60  $\mu\text{m}$  in diameter), fairly fragile, spherical cells and sometimes resembled large PLs with pseudopods



which ingested large, refractile fat droplets (Figs 27-29). Occasionally they more indistinguishable from typical circulating fat body cells and may also be related to the GCs described below.

*Giant cells (GCs)* – GCs were occasionally seen as described by Jones (1965) in *R. prolixus* as granulocytophagous cells. They were enormous cells up to 100  $\mu\text{m}$  in diameter, stable *in vitro* and rapidly forming numerous pseudopods for attachment to the slides (Figs 30-32). They enclosed intact GRs or CYs and probably other cell types which they have presumably phagocytosed. GCs were confined mainly to the *Rhodnius* spp. and *T. infestans* although their rarity in these species may not preclude their occurrence very occasionally at the correct developmental stage in the other triatomines. They could well be related to large PLs and represent a giant phagocytic cell type released from internal tissues and involved in ingestion of old cells and/or debris. Alternatively, they may be formed by a fusion of PLs although multinuclei were not observed.

*Other cells* – Many spindle cells (SPs) were also observed in all the hemipterans studied but especially so in the three *Rhodnius* spp. (Figs 33-38). However, many of the hemocytes described under this heading are believed to be unusual forms of GRs, PLs and/or OEs (Figs 13, 15, 26, 36-39). Sometimes, as in Fig. 35, in which the SP is enormously elongated to ca. 85  $\mu\text{m}$ , this may not be the case and SPs could be a separate hemocyte category. Assuming this concept, fewer SPs were observed in *T. infestans* and *P. megistus* than in *Rhodnius* spp. and *D. maximus* where they were commonly found. Another problem encountered with the SP cell type was the apparent increase in incidence of these cells in the presence of the anticoagulant solution (Fig. 33), so that many of them may well be artifacts of the manipulation method used.

#### DISCUSSION

All authors who have studied triatomine blood cells have encountered problems in establishing a convenient classification for the hemocytes. In *R. prolixus*, for example, Wigglesworth (1933, 1955) described four hemocyte types (proleukocytes, oenocytoids, amoebocytes and lipocytes) and in 1955 he reclassified the hemocytes to include plasmotocytes and

adipocytes. Jones (1965) presented a more complete classification of *R. prolixus* hemocytes, using different developmental stages of insects and various experimental techniques, but he did not describe cystocytes. Based on his description, he probably confused cystocytes with some unstable GRs or PLs. Lai-Fook (1970) described five types of hemocytes in *R. prolixus* based on Jones's classification. Apparently, however, she may have misinterpreted the identity of the granulocytophagous cells (giant cells), described in this present paper and by Jones (1965), since her description of these cells "filled with granules" seems more appropriate for the granular cell type which she did not recognize as a separate hemocyte category. Barraco et al. (1987) showed that in *P. megistus* only prohemocytes and plasmotocytes could clearly be identified. They pointed out that all others hemocyte types could have a more ambiguous classification.

In this present investigation, the comparative study of hemocytes from six different triatomine species supports most, but not all, of the classification scheme established earlier by Jones (1965) on the basis of light microscopic, phase contrast and histochemical studies. The identify of PRs, PLs, GRs, OEs, and ADs is generally accepted and the descriptions of their morphology given by Jones (1965) were in good agreement with our observations. There was, however, one major difference between Jones (1965) classification scheme in *Rhodnius* and our present findings with six triatomine species. We clearly identify as a separate category the CY cell type. Jones (1965) described degenerating PRs with small, round, cartwheel-like nuclei and a cytoplasm with fine rapidly moving particles. In addition, Jones (1965) observed "granular hemocytes" degenerating of lysing shortly after withdrawal of the hemolymph and again with cartwheel-like nuclei and many rapidly moving cytoplasmic granules. Such descriptions are characteristic for degranulating and degenerating CYs (e. g. Grégoire, 1955; Price & Ratcliffe, 1974) and clearly indicate the difficulties and precautions that are necessary in order to identify these cells. Thus, the use of non-sterile glassware and solutions, and simply smearing the cells for staining will cause the desintegration of these cells. Care should also be exercised over the use of coverslips which again in the present study have been shown to cause the loss of cells. Without coverslip, however, rapid evaporation

from the slide will occur and this in turn will introduce its own artifacts into the hemolymph preparations. Thus, multiple techniques are required in order to stabilise these CY cells and even the use of anticoagulant solution may introduce artifacts such as an increase in the incidence of spindle-shaped cells.

The present paper also describes hemolymph coagulation in the triatomines studied which again contrasts the observations of Wigglesworth (1937), Jones (1965) and Lai-Fook (1970). We clearly observed hemolymph coagulation in all six triatomines examined although it was usually limited to the formation of a few fibrous extension and attached granules from individual or small groups of cells. Occasionally, we observed that a typical "islet of coagulation" was formed around cystocyte-like cells. No hemolymph gelation occurred, however, of the sort described in some other insect species (Grégoire, 1970).

Finally, regarding the GCs, these were rarely found in *Rhodnius* spp. and *T. infestans* and never observed in the others triatomine species. Usually, these hemocytes have been seen engulfing intact GRs or other cell types. Although Jones (1965) termed these types of hemocytes as granulocytophagous cells in *R. prolixus*, we preferred the name of giant cells as other cell types besides GRs may well be ingested by these cells. At present, however, the function(s) of this cell type as well as the GRs and OE in triatomines have yet to be precisely defined.

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