

ULTRASTRUCTURAL STUDY OF *PIRHEMOCYTON* VIRUS IN LIZARD ERYTHROCYTES

A. P. ALVES DE MATOS*, I. PAPERNA**

SUMMARY

Ultrastructural study of *Pirhemocyton* infection in the lizard *Agama impalearis*, and the geckoes *Tarentola mauritanica*, *Ptyodactylus hasselquistii*, *Gehyra australis* and *Heteronotia binoei* confirmed is viral nature. Despite the apparent structural similarity, being all icosahedral iridovirus-like, virions from the diverse saurian hosts differed in size, nucleoid morphology and cytopathic effects on the erythrocytes of their respective hosts. Virions from gecko infections were altogether larger than those found in *A. impa-*

learis. In the latter there was also no vacuole formation. The gecko infections are very different from one another in the pattern of their cytoplasmic membranes and the nature of their vacuoles. In *P. hasselquistii* virions became associated with the vacuole binding membrane. In the other geckoes the vacuole was an unbound inclusion of osmiophilic substance, very different from that found in the vacuole of *P. hasselquistii*.

RÉSUMÉ : Étude ultrastructurale du virus *Pirhemocyton* des érythrocytes de lézards.

L'étude ultrastructurale de l'infection par *Pirhemocyton* du lézard *Agama impalearis* et des geckos *Tarentola mauritanica*, *Ptyodactylus hasselquistii*, *Gehyra australis* et *Heteronotia binoei* a permis de confirmer sa nature virale.

Malgré leur apparente similitude structurale, tous ayant le type iridovirus icosaédrique, les virions provenant de sauriens différents se différencient par leur taille, la morphologie nucléoïde et l'effet cytopathogène sur les érythrocytes.

Les infections des geckos diffèrent de façon importante l'une de l'autre par la morphologie de leurs membranes et la nature de leur vacuole. Chez *P. hasselquistii* les virions s'associent à la membrane limitante de la vacuole. Chez les autres geckos la vacuole est une inclusion de substance osmiophile non limitée par une membrane et très différente de celle qui est observée dans la vacuole de *P. hasselquistii*.

INTRODUCTION

The viral nature of an inclusion named *Pirhemocyton* and found in erythrocytes of a wide variety of reptiles from all continents, has been demonstrated from only one host to date, *Gehyra variegata* (Stehbens and Johnston, 1966). The viral nature of a similar looking inclusion, named *Toddia*, from frogs has already been demonstrated from 3 host species (Bernard *et al.*, 1968; Sousa and Weigl, 1976; Desser and Barta, 1984; Gruia-Gray *et al.*, 1989). Several

studies have also demonstrated the viral nature of similar erythrocytic inclusions in fish (Walker and Sherburne, 1977; Smail, 1982). All these viruses have icosahedral, intracytoplasmic, iridovirus-like particles. Infections are known to differ by the cytopathic changes they induce, and in the cytoplasmic structures formed in the erythrocytes — a vacuole (= albuminoid body) in *Pirhemocyton* (Stehbens and Johnston, 1966), a crystalloid body in *Toddia* (Sousa and Weigl, 1976), and none of these in viral erythrocytic necrosis (VEN) of fish (Walker and Sherburne, 1977). The purpose of this study was to confirm the viral nature of *Pirhemocyton* from a wider range of hosts and from diverse geographic regions. Our second objective was to study the ultrastructural nature of the many manifestations of infection in the different reptilian host erythrocytes as disclosed by our recent light microscopic investigations of *Pirhemocyton* and *Toddia* (Paperna and Alves de Matos, submitted).

* Electron Microscopy Unit, Pathologic Anatomy Department, Curry Cabral Infectious Diseases Hospital, and Structural Biology and Development Section of the Department of Zoology and Anthropology, Lisbon University, Lisbon, Portugal.

** Department of Animal Sciences, Faculty of Agriculture of the Hebrew University of Jerusalem, Rehovot 76-100, Israel.

Accepté le : 22 septembre 1992.

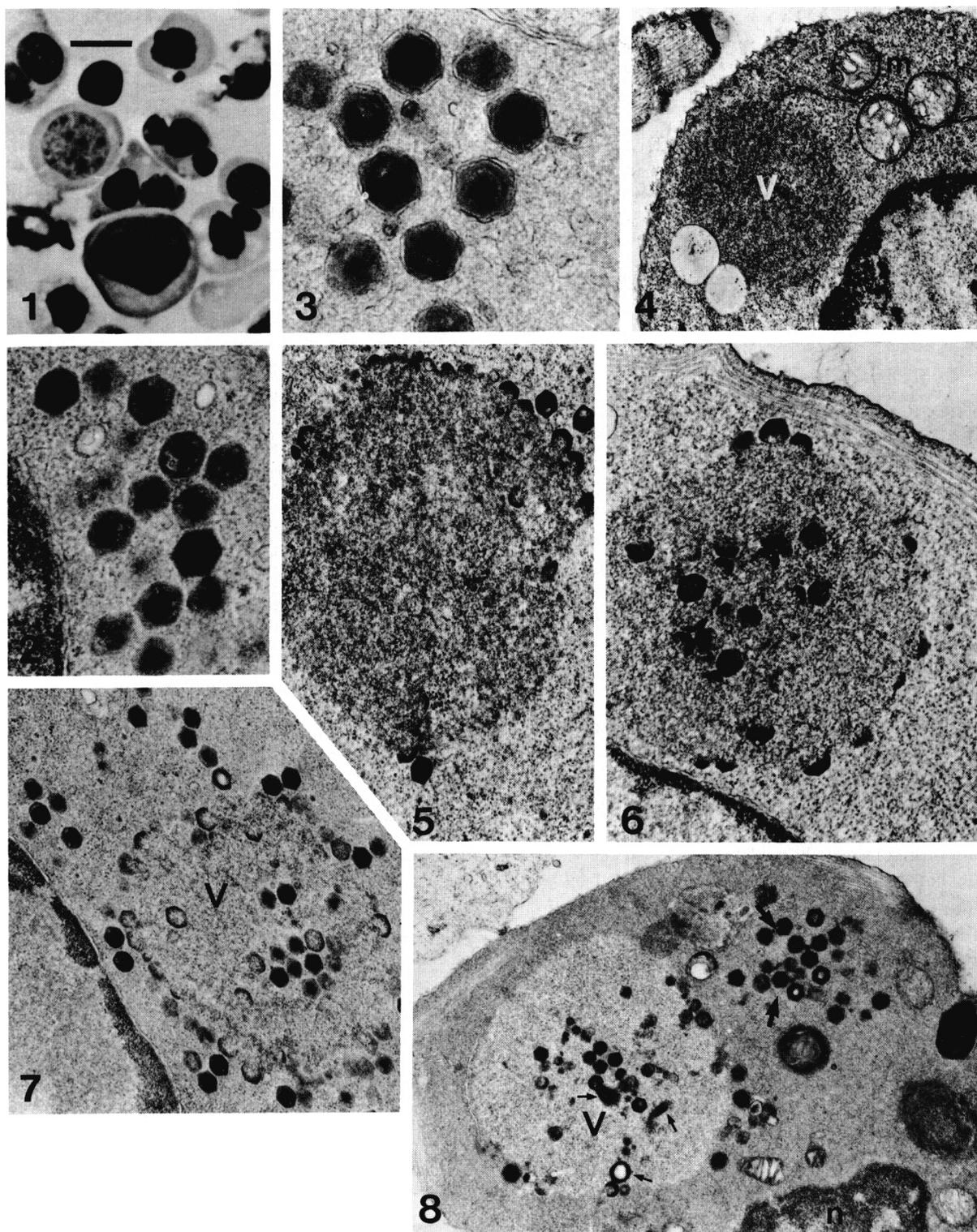


PLANCHE I.

FIG. 1. — Light microscopic view of a Giemsa stained blood smear from *Pirhemocytion* infected *A. impalearis*; bar: 10 μ m.

FIGS. 2-8. — Electron microscopic view of virus-infected erythrocytes of *A. impalearis*: 2. Virions, void of membranes ($\times 34,700$); 3. Virions enclosed by two membranes ($\times 41,200$); 4. Initial virosome (V) (m, erythrocyte's mitochondria) ($\times 14,300$); 5. Capsids emerging at the virosome periphery ($\times 21,900$); 6. Virions forming both within the virosome and at its periphery ($\times 26,000$); 7. Virions spreading from the virosome into the cytoplasm ($\times 17,000$); 8. View of an infected immature erythrocyte (n, host nucleus; V, virosome; bold arrow, intracytoplasmic virions; fine arrow, defective particles) ($\times 13,800$).

MATERIALS AND METHODS

For transmission electron microscope (TEM) studies, blood was collected from a clipped toe into a glass capillary, was allowed to coagulate and was then fixed in glutaraldehyde (2.5 % in 0.1 M, pH 7.4 cacodylate buffer) for 24 hours at 4° C. Pieces of blood vessels, lung and liver were also fixed as above. The material was post-fixed in osmium tetroxide (1 % in the same buffer) for 1 hour at room temp. After rinsing in the cacodylate buffer and further in distilled water, the material was stained en-bloc in aqueous solution of Uranyl acetate (2.5 %) for 3 hours in room temp., rinsed in distilled water and dehydrated in graded ethanols and embedded in Epon. Thin sections cut on a LKB III microtome with diamond or glass knife were stained on grid with uranyl acetate and lead citrate and examined with a Phillips CM10 or JEOL 100S TEM.

RESULTS

VIRUSES FROM *Agama impalearis* ERYTHROCYTES

A naturally infected lizard, collected in southern Morocco, was obtained from Pr. Rioux, and succumbed to the infection 20 days later. A blood sample for TEM study was taken one day before the lizard's death. Hematological examination of this sample revealed infection in all of the mature, and in > 40 % of the immature erythrocytes, which together comprised 72 % of the erythrocyte count (Fig. 1).

Virion size was 190-219 nm ($n = 10$). The capsid was icosahedral (hexagonal and pentagonal profiles). Some virions had a dense nucleoid which was either spherical or rectangular. The inside (core) of the other particles was filled with a substance of variable density (Figs. 2, 3). Generally, no membranes could be detected around the virions (Fig. 2), but in some cells, virions are enveloped by two membranes (Fig. 3).

The initial virosome inclusion was seen as a spherical, densely granular mass with no virions (Fig. 4). The first particles appeared as incomplete capsids, either at the periphery (Fig. 5) or inside the virosome (Figs. 6, 7). The viral particles matured and became icosahedral virions, which moved away from the virosome into the cytoplasm (Figs. 7, 8). Large, old virosomes had a lower granular density than the surrounding cytoplasm (Fig. 8). Most of the virions remained in the cytoplasm, but a few could also be found in the nucleus (Fig. 9). However, no virosomes could be found in the nucleus. Virosomes sometimes exhibited aberrant structures such as small vesicles (Fig. 10) and dense material (Fig. 11).

The cytoplasm of the infected cells contained mitochondria, Golgi apparatuses and membranes, characteristic to immature erythrocytes. In some erythrocytes, the cytoplasm also contained membrane-bound crystals (Fig. 12). Many cells contained myelin-like structures, suggesting progressive degradation (Fig. 13).

VIRUSES FROM *Tarentola mauritanica* ERYTHROCYTES

Study material was obtained from two naturally infected *T. mauritanica* geckoes caught near Banyuls-sur-Mer, SW France. Hematological examination of samples taken for TEM revealed > 95 % infection in the mature erythrocytes of both geckoes (Fig. 14). Immature cells comprised 33 to 43 % of the total count, but less than 1 % of these were infected.

Virion size was 260-292 nm ($n = 10$). Capsids were icosahedral (hexagonal and pentagonal profiles). The core was of heterogeneous density, with a flocculent texture. The virions were always enclosed by two membranes (Fig. 15).

The virosome was a round-spherical fibrillar mass of lower density than the host cytoplasm (Fig. 16). It may contain scattered membranes (Fig. 17). Virions matured and emerged from the virosome into the cytoplasm (Figs. 17-20). Some were found associated to the nuclear membranes (Figs. 19, 21, 22), others to the cytoplasmic membranes (Figs. 18, 19).

The vacuole usually occurred in association with the virosome. It was comprised of a homogeneous, unbound globule containing either an osmiophilic substance (Fig. 16), or a lucent substance (Figs. 17, 18). All observed infected cells were mature erythrocytes with no signs of degeneration.

VIRUSES FROM *Ptyodactylus hasselquistii*

Naturally infected *P. hasselquistii* geckoes (Fig. 23) were collected in the Lower Jordan valley, CisJordan. For ultrastructural studies, blood samples were taken from five infected geckoes at different stages of erythrocytic infection (from 28 to 100 %). Ultrastructurally studied material (Figs. 24-32) therefore included early infections in which the initial stages of virosome formation were predominant, before the development of the vacuole (Figs. 26-28), and late infections where a large vacuole containing virions was forming (Figs. 23, 25, 29-32).

Virions size was 230-253 nm ($n = 10$). Capsids were icosahedral (hexagonal and pentagonal profiles). The nucleoid had an outer dense band, and the most complete-looking ones had a central whorl-like configuration. Others had a center of variable density or were homogeneously granular (incomplete forms) (Figs. 24, 25).

Early rounded virosomes were distinguished from the rest of the cytoplasm by their more sparse contents (Figs. 26-28). Viral capsids developed from the virosome (Fig. 26). In the process of viral formation, aberrant elongate particles also appeared (Fig. 27). Some, including presumably young or inactive virosomes, were outlined with furrows, apparently endoplasmic reticulum cisternae (Fig. 28).

The vacuole was membrane-bound and appeared adjacent to the virosome (Fig. 29). It was filled with a reticulate homogeneous (Figs. 25, 29) or flocculent substance (Figs. 30, 31). In many vacuoles the center became

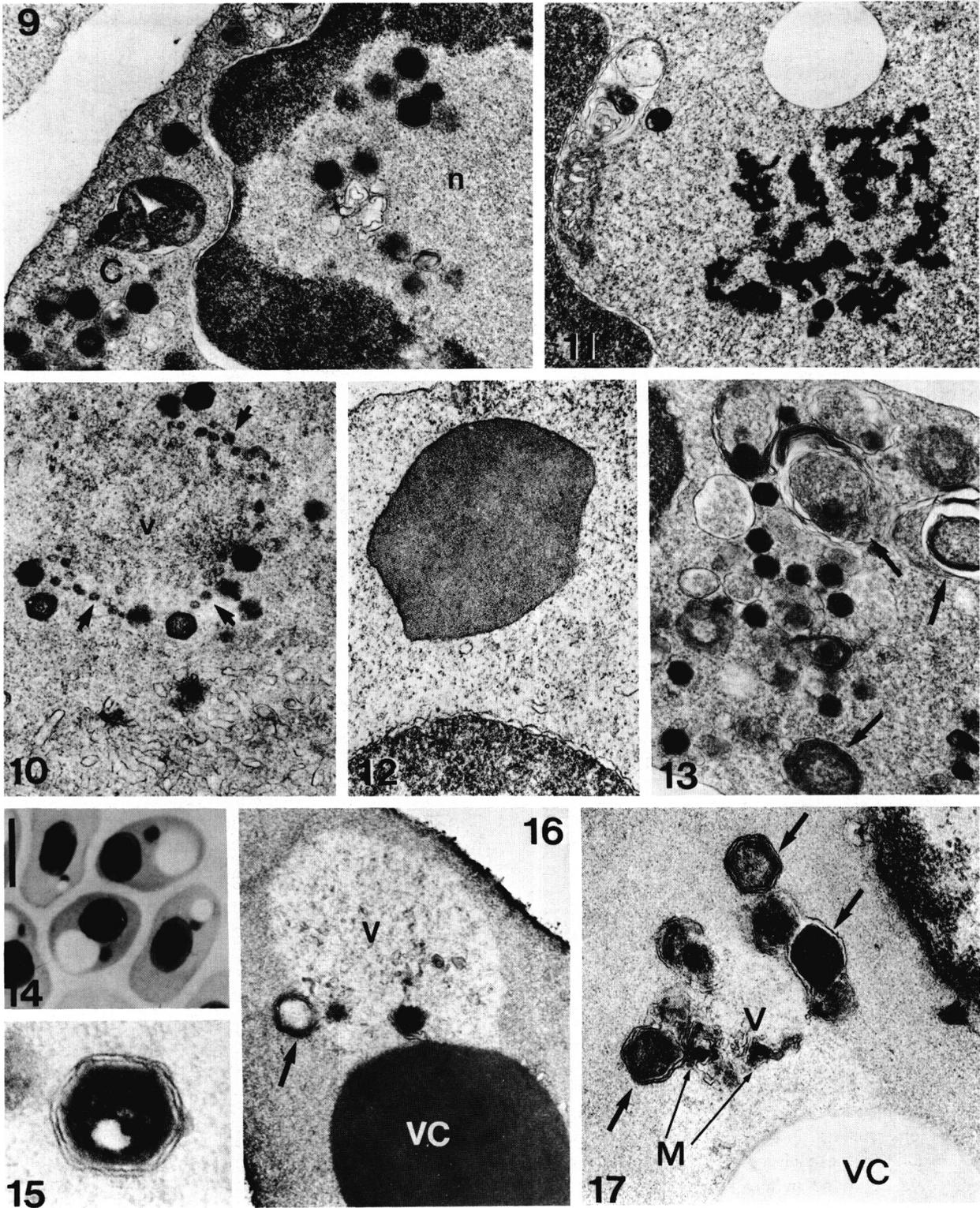


PLANCHE II.

FIGS. 9-13. — 9. Intracytoplasmic (C) and intranuclear (n) virions ($\times 25,000$); 10. Aberrant structures - vesicles (arrows) in the virosome (V) ($\times 28,800$); 11. Strands of dense material in the virosome ($\times 24,400$); 12. Membrane-bound crystal in the cytoplasm ($\times 33,000$); 13. Cytoplasm with myelin-like structures (arrows) ($\times 22,500$).

FIG. 14. — Light microscopic view of a Giemsa stained blood smear from *Pirhemocytan*-infected *T. mauritanica*; bar: 10 μ m.

FIGS 15-17. — Electron microscopic view of virus-infected *T. mauritanica* erythrocytes: 15. Membrane-enclosed virion ($\times 76,000$); 16. Virosome (V) adjacent to a vacuole with dense matrix (VC) ($\times 17,200$); 17. Virosome (V) with virions (arrows) and membranes (M) adjacent to an electron lucent vacuole (VC) ($\times 35,000$).

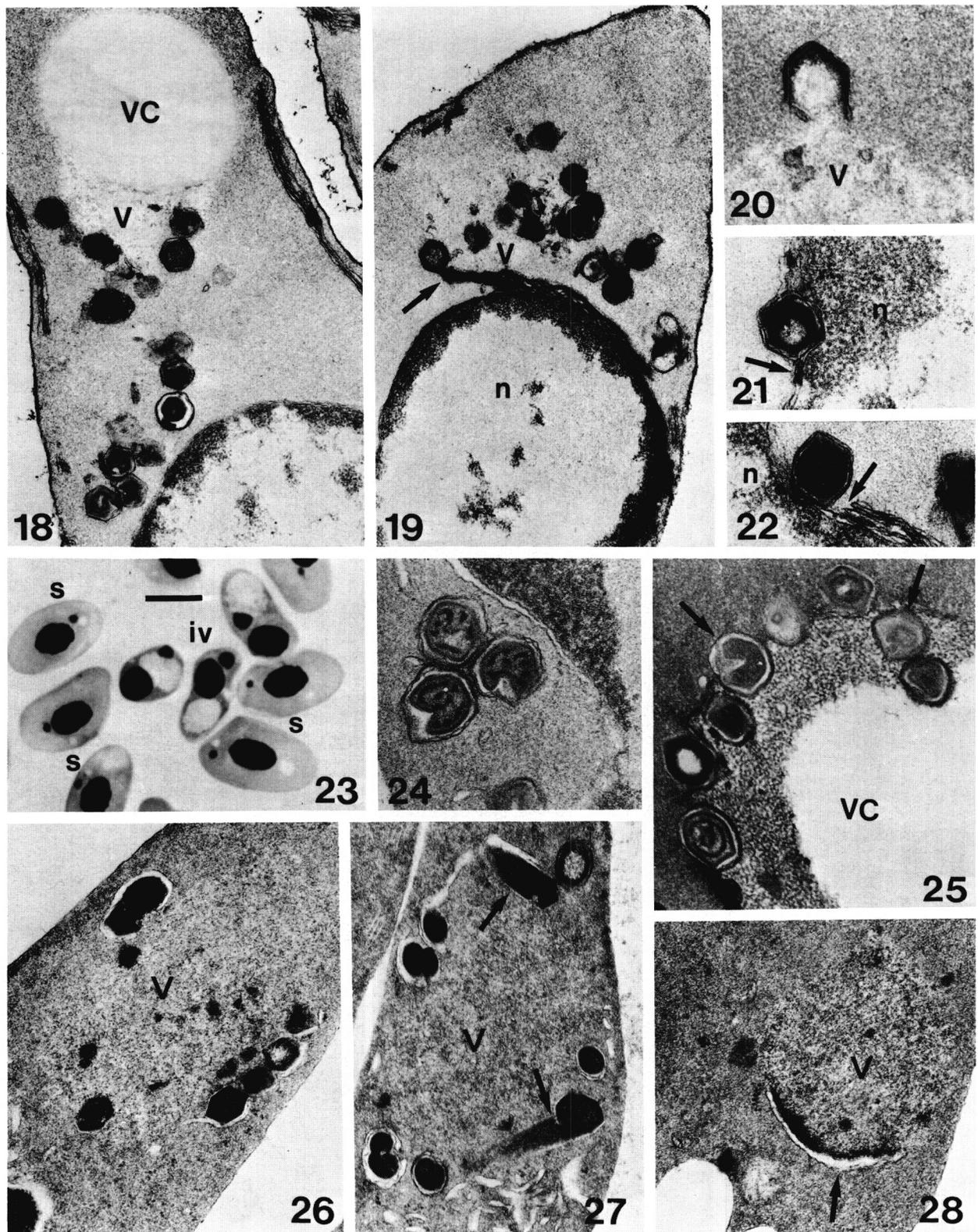


PLANCHE III.

FIGS. 18-22. — 18. Infected mature erythrocytes with a virosome (V) adjacent to a vacuole with lucent contents (VC), and virions (arrows), emerging from the virosome and free in the cytoplasm ($\times 20,000$); 19. Virosome and virions, one associated to the nuclear membrane (arrow) (n, erythrocyte's nucleus) ($\times 17,900$); 20. Virion emerging from the virosome (V) ($\times 35,700$); 21, 22. Enlarged view of virions associated with the nuclear membrane (arrows; n, host cell nucleus) ($\times 38,700$).

FIG. 23. — Light microscopic view of a Giemsa stained blood smear from *Pirhemocytan* infected *P. hasselquistii*. Infected erythrocytes contain either no, or very small vacuoles (s), or very large vacuoles (iv); bar = 10 μ m.

FIGS. 24-32. — Electron microscopic view of virus infected erythrocytes of *P. hasselquistii*: 24. Virions in the cytoplasm ($\times 53,400$); 25. Virions (arrow) fringing a vacuole, note reticulated content of the vacuole (VC) ($\times 45,000$); 26-28. Virosomes (V) in erythrocytes at an early stage of infection, still without a vacuole: 26. With fringing capsids ($\times 31,200$); 27. With aberrant elongate particles (arrows) ($\times 31,200$); 28. Without particles, accompanied by furrow-like cisternae (arrow) ($\times 31,200$).

empty (Figs. 25, 29, 32). The vacuole became fringed with complete, incomplete and aberrant virions, associated with the vacuole-binding membranes (Figs. 25, 29-32). Virion-fringed vacuoles also developed in the nucleus of some cells (Fig. 31). Additional, smaller vacuoles sometimes occurred next to virosomes which were already associated with a large vacuole (Figs. 29, 32). Virions could also be found free in the cytoplasm (Fig. 24).

VIRUSES FROM *Gehyra australis*

Infection developed in a *Gehyra australis* gecko from Townsville, northeast Queensland, Australia, after four months in captivity (in Israel) (Fig. 33). This infection superimposed itself on existing natural *Haemoproteus gehyrae* infection (see Paperna and Landau, 1991).

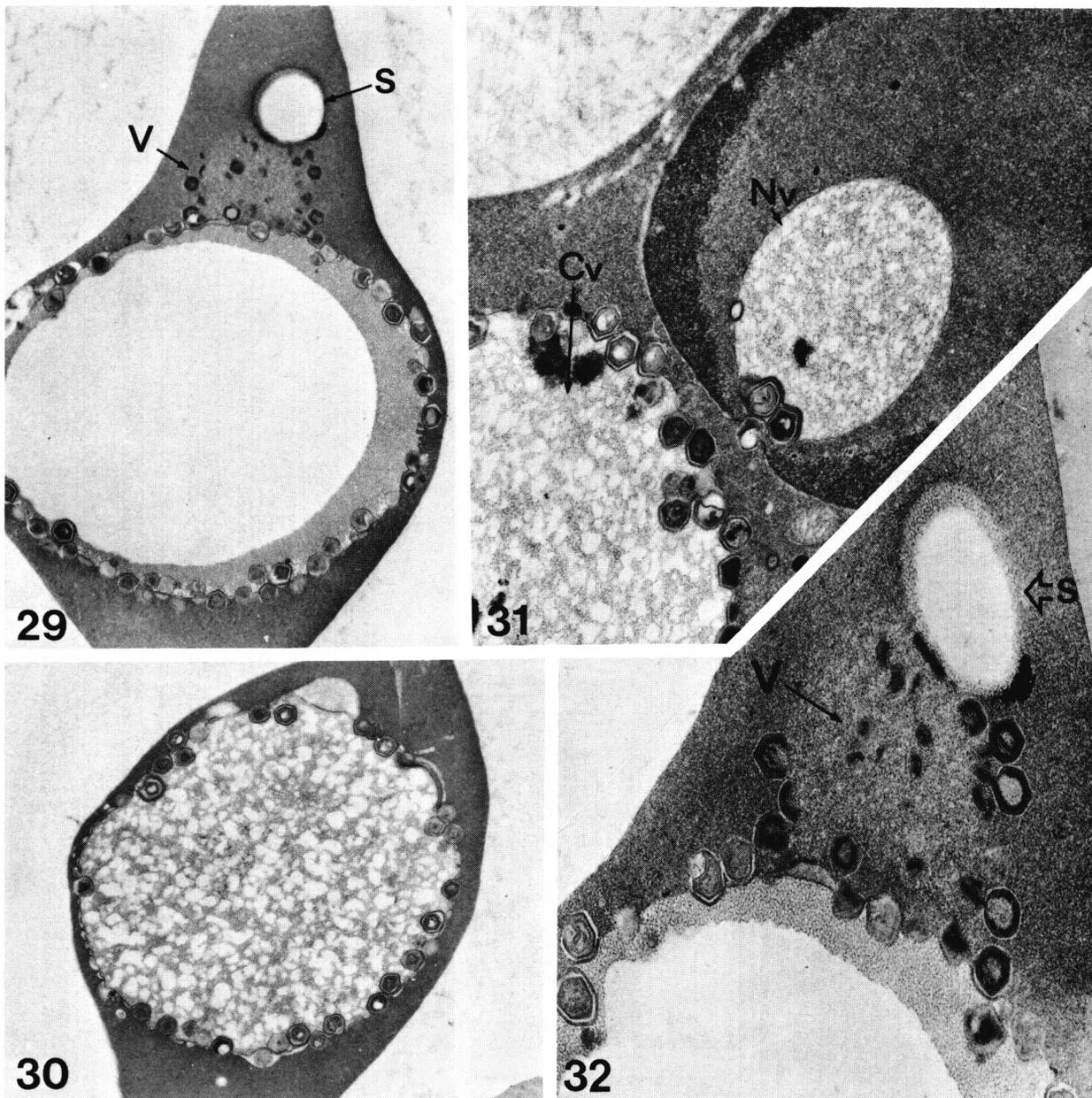


PLANCHE IV.

FIGS. 29-32. — Advanced-stage erythrocyte infection with a vacuole: 29. Erythrocyte with large vacuole, an adjacent virosome (V) and additional small vacuole (S) ($\times 12,800$); 30. Virions-fringed vacuole with flocculent substance ($\times 12,800$); 31. Virions-fringed cytoplasmic (Cv) and nuclear (Nv) vacuoles, both with flocculent substance ($\times 26,000$); 32. Enlarged view of particle-forming virosome (V), large, and new, small (S), vacuoles, both with peripherally distributed reticulated contents ($\times 33,000$).

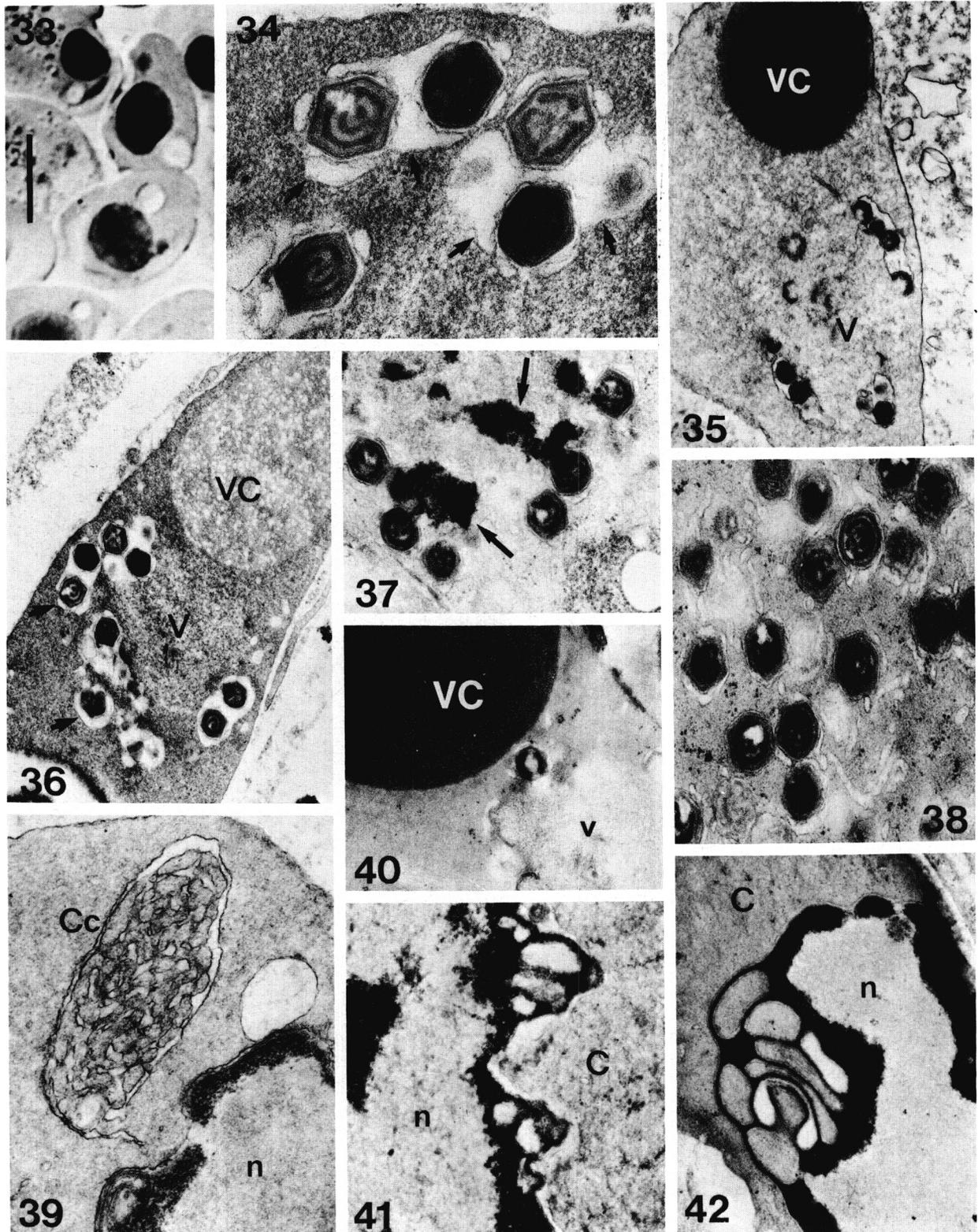


PLANCHE V.

FIG. 33. — View by light microscopy, with Nomarski interference, of Giemsa stained *G. australis* Pirhemocytin-infected erythrocytes (bar = 10 μ m).

FIGS. 34-42. — Electron microscopic view of virus-infected *G. australis* erythrocytes: 34. Virions within membranous bound spaces ($\times 60,250$); 35. Infected erythrocyte showing a virions-fringed virosome (V) and electron dense vacuole (VC) ($\times 20,700$); 36. Erythrocyte with virosome (V), virions (arrows) and vacuole containing foamy matrix (VC) ($\times 20,400$); 37. Aberrant virion formation (arrows) ($\times 29,300$); 38. Virions associated to membranes in the cytoplasm ($\times 38,800$); 39. Intracytoplasmic, virus-free membranous complex (Cc) ($\times 27,900$); 40. Enlarged view of electron-dense vacuole (v, virosome) ($\times 23,960$); 41, 42. Nuclei (n) of infected erythrocytes with peripheral convolutions and vesiculations (C, cytoplasm) ($\times 28,000$ and $\times 24,000$).

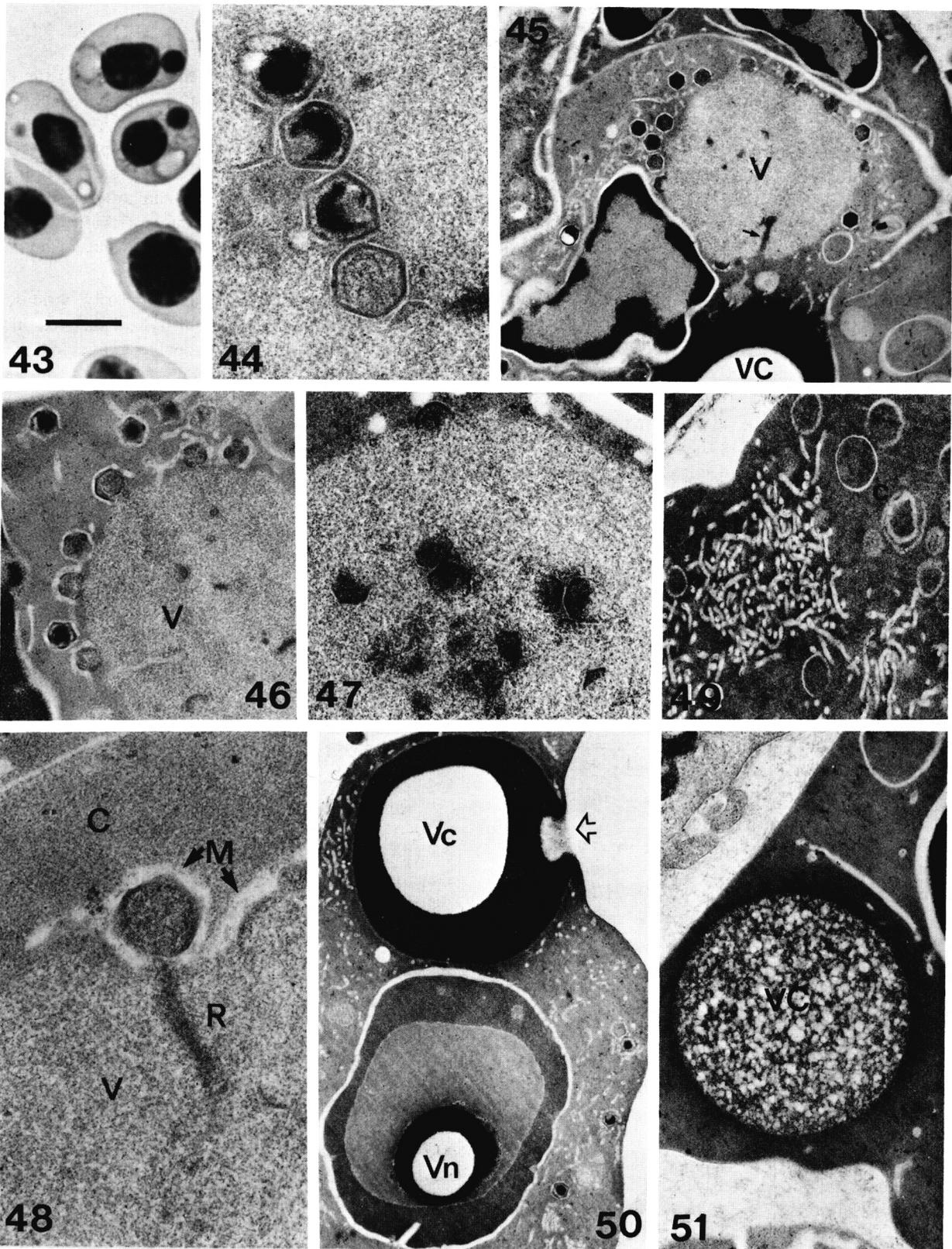


PLANCHE VI.

FIG. 43. — Light microscopic view of a Giemsa stained blood smear from *Pirhemocytion*-infected *H. binoei* (bar = 10 μ m).
 FIGS. 44-51. — Ultrastructural view of virus-infected *H. binoei* erythrocytes: 44. Virions ($\times 48,700$); 45. Infected erythrocyte showing virus-fringed virosome (V) and a vacuole (VC) (arrow, virion rootlet — see Fig. 48) ($\times 10,900$); 46. Virosome (V) with peripherally formed virions ($\times 19,230$); 47. Virosome with centrally formed virions; 48. Virion with rootlet (R) extending from the virosome (V) into the cytoplasm (C) (M, membrane shadows) ($\times 50,000$); 49. Erythrocyte cytoplasm loaded with tubular (t) and circular flattened (c) membranes ($\times 15,300$); 50. Intracytoplasmic (Vc) and intranuclear (Vn) vacuoles ($\times 11,800$); 51. Foamy intracytoplasmic vacuole (VC) ($\times 20,400$).

Virions size was 262-274 nm ($n = 10$). Capsids were icosahedral (hexagonal and pentagonal profiles). The nucleoid in complete particles was limited by a dense band, and its core was of variable consistency, but often formed an additional central whorl (Fig. 34).

Virosomes were distinguishable from the cytoplasm either by being denser or sparser. Virions were formed both at the periphery and inside the virosome (Figs. 35, 36), sometimes accompanied by aberrant viral structures (Fig. 37). Membranes became associated with the virions as they emerged from the virosome and escaped into the surrounding cytoplasm (Figs. 35, 36, 38). Some of the observed membrane complexes did not contain viruses (Fig. 39).

The vacuole was unbound. Its content was usually homogeneous with variable density, either having a reticular texture which sometimes becomes foamy (Fig. 36), or, being of a homogeneous osmiophilic density (Figs. 35, 40). During the process of infection, alterations appeared in the marginal nuclear chromatin, whereby it became peripherally condensed with convolutions and vesiculations (Figs. 41, 42).

VIRUSES FROM *Heteronotia binoei*

This study was made on blood obtained from three naturally infected geckoes from Townsville, NE Queensland, Australia (Fig. 43).

Virion size was 247-273 nm ($n = 10$). Capsids were icosahedral (hexagonal and pentagonal profiles). The nucleoid was dense and did not always completely fill the entire center of the particle (Fig. 44).

The virosome was round, homogeneously granular, and less dense than the cytoplasm (Fig. 45). Virions matured and emerged at the periphery (Fig. 46) but were also found inside the virosome (Fig. 47). Some peripherally developing virions extended rootlets into the virosome (Fig. 48). Viruses appeared to be associated with membranes (Figs. 46, 48). However, in part of the material these membranes were damaged during processing and only their shadows were left behind (Fig. 48). Two types of membrane configuration seemed to occur: a tubular type, which developed in closer association with the virosome and may also extend to the nucleus, and peripheral, circular-flattened cisternae (Fig. 49). Virions were preferentially associated with the tubular components.

The vacuole was round, consisting of an electron-dense homogeneous substance (Fig. 50) which sometimes turned foamy (Fig. 51). The vacuole center was often empty. Nuclei also contained vacuoles (Fig. 50).

DISCUSSION

The ultrastructural study of *Pirhemocytion* infection in the above studied saurian reptiles clearly confirms its viral

nature, as demonstrated by Stehbens and Johnston (1966) in *Pirhemocytion* of *Gehyra variegata*. These etiological agents have an iridovirus-like morphology, similar to other erythrocyte-infecting viruses found in amphibians (*Toddia*; Sousa and Weigl, 1976; Gruia-Gray *et al.*, 1989) and fish (VEN; Smail, 1982). Despite the similarity between *Pirhemocytion* and iridoviruses, conclusive classification cannot be made in the absence of further biochemical data (Willis, 1990).

Despite the apparent structural similarity, virions from the diverse saurian hosts studied here differed in size, nucleoid morphology and cytopathic effects on the erythrocytes of their respective hosts.

Virion size has been claimed to be an important taxonomic criterion for the classification of iridoviruses (Willis, 1990). Differences in virion size among geckoes are not very distinct, but they are altogether larger than those found in *A. impalearis*. In the latter there is also no vacuole formation and the virus is therefore suggested to be different from those in gecko infections.

The gecko infections are very different from one another in the nature of their vacuoles and in the pattern of their cytoplasmic membranes. Virions from the *H. binoei* infection differed from the other geckoes in their nucleoid morphology. Virions of *P. hasselquistii* were the smallest among the geckoes and in this gecko's erythrocytes there was also a unique vacuole-membrane association. Virion sizes reported by Stehbens and Johnston (1966) from *G. variegata* (200-230 nm) are somewhat below our measurements for virions from geckoes. Such variation could result from differences in fixation or embedding media.

The so-called albuminoid body mentioned in early light microscopic descriptions (Chatton and Blanc, 1914) was identified by Stehbens and Johnston (1966) as a vacuole in their study of *G. variegata*. Similar structures were observed in *T. mauritanica*, *G. australis* and *H. binoei* in the present study. They were found not to be membrane-bound and to be osmiophilic with variable electron density, which suggests lipid contents. Only in *P. hasselquistii* was the vacuole membrane-bound, containing material of a different nature.

A well-developed system of cytoplasmic membranes was observed in all geckoes, but not in *A. impalearis*. Virions were associated with these membranes, and also in particular with the membranes binding the vacuole in *P. hasselquistii*. Although the origin of these membranes is unknown, their formation could be related to the synthesis of viral unit membranes which occur in iridovirus virosomes to from the virion capsids (Devauchelle *et al.*, 1985).

Stehbens and Johnston (1966) followed Mackerras (1961) in naming their described viral infection from *G. variegata* *Pirhemocytion tarentolae*. Despite of similarities between virions from these geckoes and those found in *T. mauritanica*, the type host of *P. tarentolae*, as well as the pre-

sence of a similar unbound vacuole, in light of the great diversity shown among *Pirhemocyton* infections of geckoes, we would hesitate to regard viruses of the Australian *G. variegata* and those of the Mediterranean *T. mauritana* as one and the same.

Acknowledgements. — The authors thank Pr. Eduardo CRESPO of the Department of Zoology and Anthropology, Lisbon University and Dr. Celeste CAMPOS of the Pathologic Anatomy Department, Curry Cabral Infectious Diseases Hospital, Lisbon for their help and support; Dr. Moura NUNES of the Cancer Institute Lisbon, for providing technical facilities and Pr. RIOUX of the Laboratoire d'Écologie médicale et de Pathologie parasitaire, Faculté de Médecine, Montpellier, for providing me with *Pirhemocyton* infected *A. impalearis* and blood smear slides from *Pirhemocyton* infected *T. mauritana*.

We wish also to thank Dr. Bruce COPEMAN of the graduate School for Tropical Veterinary Sciences at James Cook University of North Queensland for his hospitality and support of our field work in Australia and lastly, Dr. I. LANDAU Laboratoire de Zoologie (Vers) associé du CNRS et Laboratoire de Protozoologie et Parasitologie comparée, Muséum National d'Histoire naturelle, Paris for support, laboratory facilities and good advice.

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