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Electron microscopic study on the development of *Babesia ovis* (Piroplasmia) in the salivary glands of the vector tick *Rhipicephalus bursa**

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Summary

The formation of *Babesia ovis* sporozoites in salivary gland cells of the vector tick *Rhipicephalus bursa* was studied by electron microscopy. The kinetes of *B. ovis* were found lying intracellularly on the second day after infestation (a.i.) of the ticks. The parasites enlarged rapidly losing all features of the motile form. Invaginations of the cell membrane initiated a fragmentation of this developmental stage. On the third day a.i. the parasite (measuring up to 40×25 μm) was divided into numerous single membrane-bounded cytomeres, each provided with at least one lobed nucleus. On the fourth day a.i. sporozoite differentiation started at the periphery of the cytomeres, indicated by the appearance of several pellicle-bounded, exogenous protrusions into each of which a small portion of the nucleus was incorporated. Since the cytomeres lay very close together this differentiation occurred more by segmentation than by budding. Rhoptries and the so-called spherical body appeared in this developmental phase. Finally, the isolated, immature sporozoites lay in a granular matrix which contained remnants of the host cell cytoplasm. On the fifth day a.i. the sporozoites were fully developed, typically pear-shaped (2.8×1.2 μm) and provided with all characteristic structures of the invasive form. – This reproduction was compared to similar processes in other species of the Piroplasmia and the Haemosporina.

Key words: Babesia ovis; Piroplasmia; *Rhipicephalus bursa;* ultrastructure.

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Introduction

The piroplasm *Babesia ovis* multiplies in the erythrocytes of sheep and goat, occasionally leading to death within ten days. The parasite is transmitted by the tick *Rhipicephalus bursa*. The development of *B. ovis* in the vector tick has been object of several light and electron microscopical investigations (reviewed by Friedhoff, 1981). Recently the ultrastructure of the kinete and its differentiation in the ovarian tissue was described (Weber, 1980; Moltmann et al., in press). In the present study the asexual reproduction of *B. ovis* in the cells of the salivary glands which leads to the formation of infectious stages is described. This development is compared to similar processes in other *Babesia* and *Theileria* species.

Materials and Methods

Two strains of the two-host tick *Rhipicephalus bursa* were crossbred. One strain, Ankara 1959, was infected with *Babesia ovis* and has been maintained in Hannover since 1959. The other strain, Ankara 1948, was not infected, and has been maintained in the laboratory since 1948. The strain of *B. ovis*, Hannover 1959, was transmitted through 33 tick generations since 1959. Besides transovarial transmission, that persisted through all generations, alimentary infection occurred when the female ticks engorged on infected sheep. These infections were always tick-borne; never was a sheep infected by blood inoculation (Friedhoff and Smith, 1981).

The ticks transmitted *B. ovis* in the adult stage only (Friedhoff and Smith, 1981). The larval progeny of the female ticks that had developed a heavy infection of the hemolymph 5 days after repletion at 28° C was fed on rabbits. The engorged nymphs were incubated at 28° C and 80 to 90% relative humidity until moulting. The newly hatched adults were kept at room temperature and 80 to 90% r.h. up to 53 days after repletion in the nymphal stage.

The adult ticks were fed on rabbits for 1 to 5 days before dissection. The salivary glands of the ticks were removed while being submersed in cold 5% (v/v) glutaraldehyde in 0.1 M cacodylate buffer, pH 7.3. The tissue was fixed for at least 48 h and then repeatedly rinsed in cacodylate buffer. The preparations were treated for 2 h in 2% (w/v) OsO_4, dehydrated in a series of ethanols, and embedded in Araldite (Ciba-Geigy). Ultrathin sections were cut on a Reichert OMU 3, mounted on copper grids and stained with an alcoholic solution of uranyl acetate for 30 min, followed by lead citrate for 10 min (Reynolds, 1963). The sections were examined in a Zeiss electron microscope EM 9 S2.

Results

Single kinetes of *Babesia ovis* were found in non-secreting cells of the salivary glands of the adult ticks on the second day after infestation (a.i.). The parasites were situated in the host cell cytoplasm without being enclosed in a parasitophorous vacuole (Fig. 1). The spherical kinete measured about 3.5 μm in diameter and was bordered by a single membrane underneath which some remnants of the inner membranous layer of the former pellicle occurred. The cell was provided with an ovoid nucleus and several cisternae of the endoplasmic reticulum. Sections of micronemes were distributed at the margin of the

cytoplasm. The parasites enlarged rapidly, reaching a diameter of about 5.0 μm on the second day a.i. Within these stages cisternae of the endoplasmic reticulum accumulated at the cell periphery. Subsequently tube-like invaginations of the cell membrane initiated a fragmentation of this developmental stage (Figs. 2, 10a).

On the third day a.i. the fragmentation process had advanced considerably. The large fission body (Figs. 3, 10b; measuring a maximum of 40×25 μm) was divided into numerous cytomeres, leaving clefts in between. These clefts were filled by the host cell cytoplasm as identified by the occurrence of host cell mitochondria and osmiophilic endoplasmic reticulum (Fig. 4). The cytomeres were of varying size and shape, bounded by a single membrane, and were each provided with at least one lobed nucleus (Fig. 3). In larger cytomeres the nucleus was found to divide into smaller portions. Often small, intranuclear spindle apparatus occurred in lobes of the nucleus, stretching over a distance of about 0.2 μm from one side of the nuclear envelope to the other (Fig. 4). The most advanced developmental stage on the third day a.i. revealed a highly fissured body with numerous cytomeres.

On the fourth day a.i. differentiation of sporozoites started more or less simultaneously at numerous places (Figs. 6, 7, 10c). The limiting membrane of the cytomeres was underlined by an additional 2-membrane system at places where a nucleus lay near to the cytomere boundary, thus giving rise to a typical coccidian pellicle. A small portion of the nucleus, containing a short spindle apparatus, protruded towards the newly formed pellicle (Fig. 6). Two osmiophilic globules ($\varnothing$ about 0.3 μm), probably representing rhoptry precursors, appeared on both sides of the nuclear protrusion (Fig. 7). Several of these differentiating structures were formed within a single cytomere, all deriving their nuclear material from one maternal nucleus.

Since the cytomeres lay very close to each other the following sporozoite differentiation occurred more by segmentation than by budding (Fig. 5). Nearly all of the cytomere-cytoplasm was distributed to the developing parasites. Thus sporozoites in all stages of development were found lying in a granular matrix, which contained remnants of the cytomere and the host cell cytoplasm (Fig. 9). The apical complex of the sporozoites was provided with a polar ring and up to 5 rhoptries, whereas the inner layer of the pellicle was not yet continuous (Fig. 8). The so-called spherical body (Friedhoff et al., 1972) appeared in this stage of development in the vicinity of the nucleus (Fig. 8). It was a single-membrane bounded, vacuolar structure ($\varnothing$ about 0.8 μm) containing a fine granular material with some condensed, osmiophilic regions. Finally a large number of sporozoites (up to 600 were counted in a single ultrathin section) lay in a granular matrix (Figs. 9, 10d). The differentiated sporozoites were typically pear-shaped (2.8×1.2 μm) and surrounded by a 3-membrane pellicle. Besides a nucleus and a mitochondrion, a spherical body was prominent, lying anterior to the nucleus in the broadest part of the cell.

Abbreviations used in the figures

AT = Acinus tissue
BM = Basal membrane of the acinus
CL = Cleft between the cytomeres
CY = Cytomere of parasitic origin
ER = Endoplasmic reticulum of the parasite
ERH = Endoplasmic reticulum of the host cell
HC = Host cell
IVM = Invaginations of the outer membrane of the parasitic cell
MI = Mitochondrion of the parasite
MH = Mitochondrion of the host cell
MN = Micromenes
N = Nucleus of the parasite
NH = Nucleus of the host cell
PE = Pellicle
R = Rhoptries
SB = Spherical body
SP = Spindle apparatus
SPO = Sporozoites

Figs. 1–9. Electron micrographs of *Babesia ovis* within the salivary glands of adult ticks (*Rhipicephalus bursa*).

Fig. 1. Section through a kinete which lies directly within the cytoplasm of the host cell. Remnants of the pellicle (PE) and micronemes (MN) still occur. (2. day a.i.) ×24,000.

Fig. 2. Periphery of an early developmental stage showing an invagination of the outer cell membrane (IVM). (2. day a.i.) ×20,000.

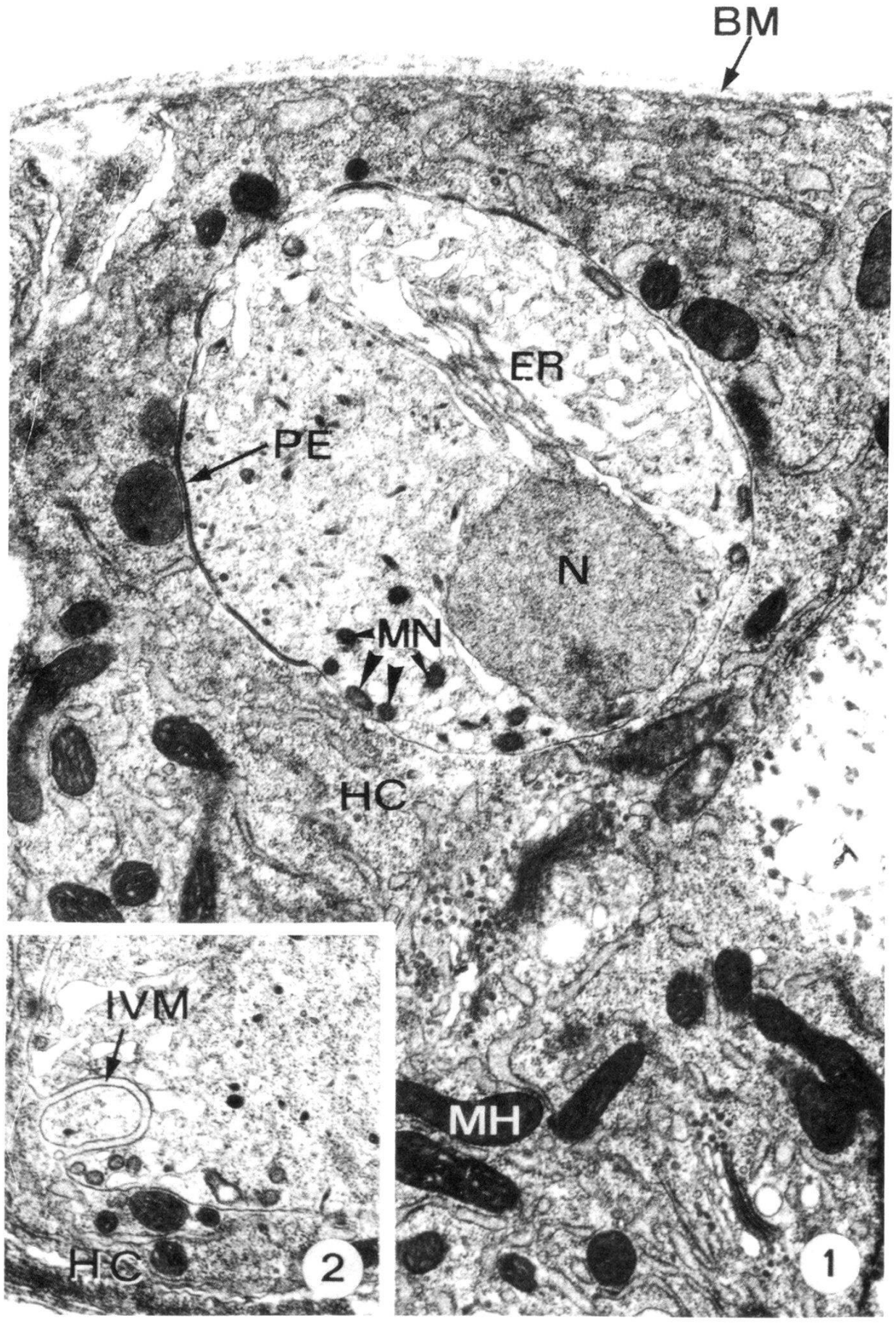
Figs. 3–4. Sections through fissured developmental stages. The cytoplasm is divided into numerous cytomeres (CY) leaving clefts (CL) in between. These clefts are filled with host cell cytoplasm, host cell mitochondria (MH) and endoplasmic reticulum (ERH). Small spindle apparatus (SP) occur inside the lobulated nucleus of each cytomere. (3. day a.i.) 3. ×20,000; 4. ×40,500.

Fig. 5. Segmentation of the cytomeres into sporozoites in the course of which all cytoplasm is distributed to the parasites. Spherical bodies (SB) appear in this stage. (4. day a.i.) ×25,000.

Figs. 6–7. Initial sporozoite differentiation at the cytomere boundary. A small portion of the nucleus containing a spindle apparatus (SP) protrudes towards the newly formed pellicle (PE). Precursors of rhoptries (R) appear on both sides of the nucleus. (4. day a.i.) 6. ×40,000; 7. ×40,000.

Fig. 8. Section through a nearly mature sporozoite. The pellicle (PE) does not yet completely surround the parasitic cell. (4. day a.i.) ×35,000.

Fig. 9. Section through an acinus filled with numerous sporozoites (SPO). The nucleus of the host cell (NH) and the acinus tissue (AT) are degenerating. (5. day a.i.) ×5,000.


BM
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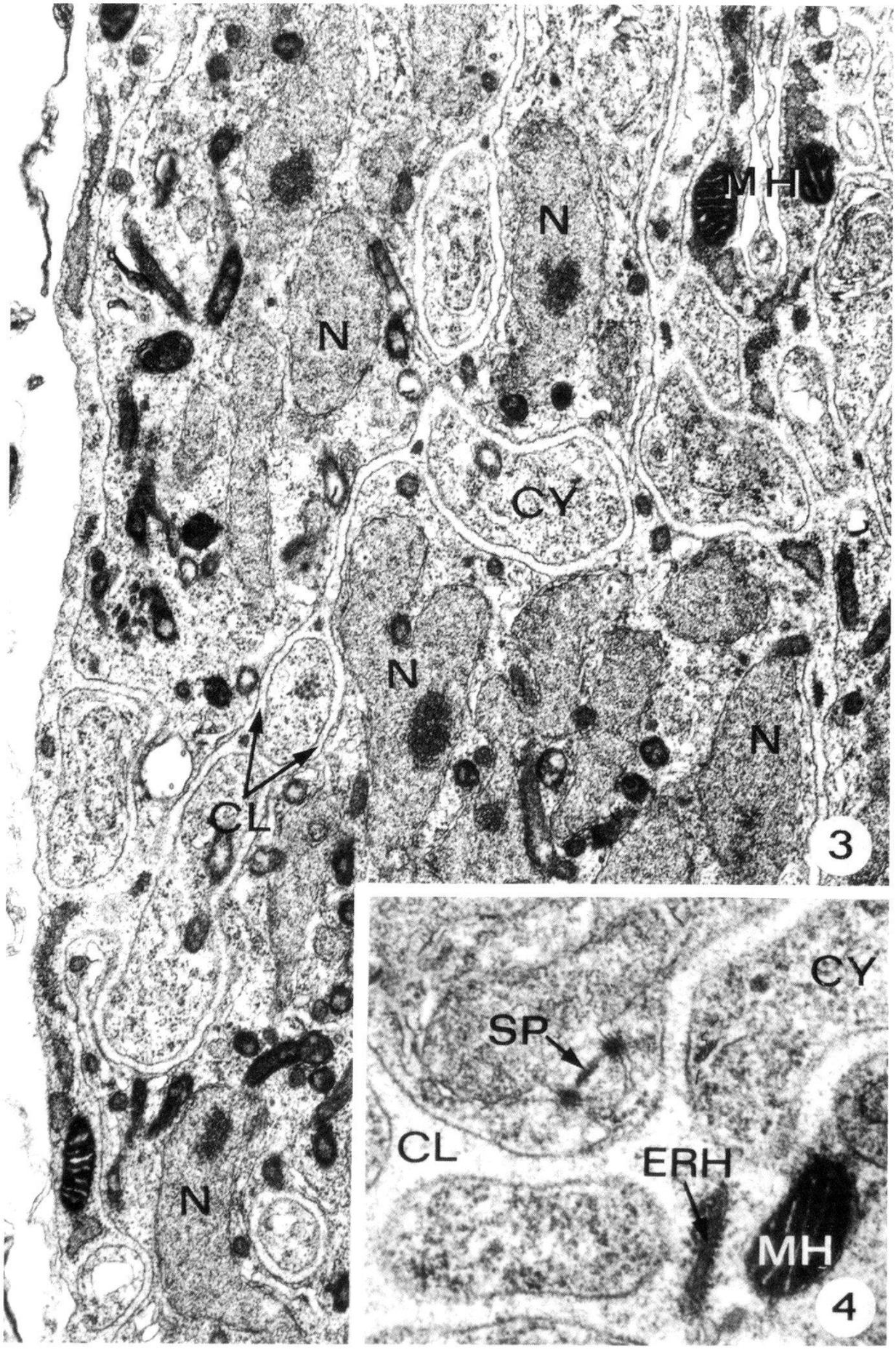
1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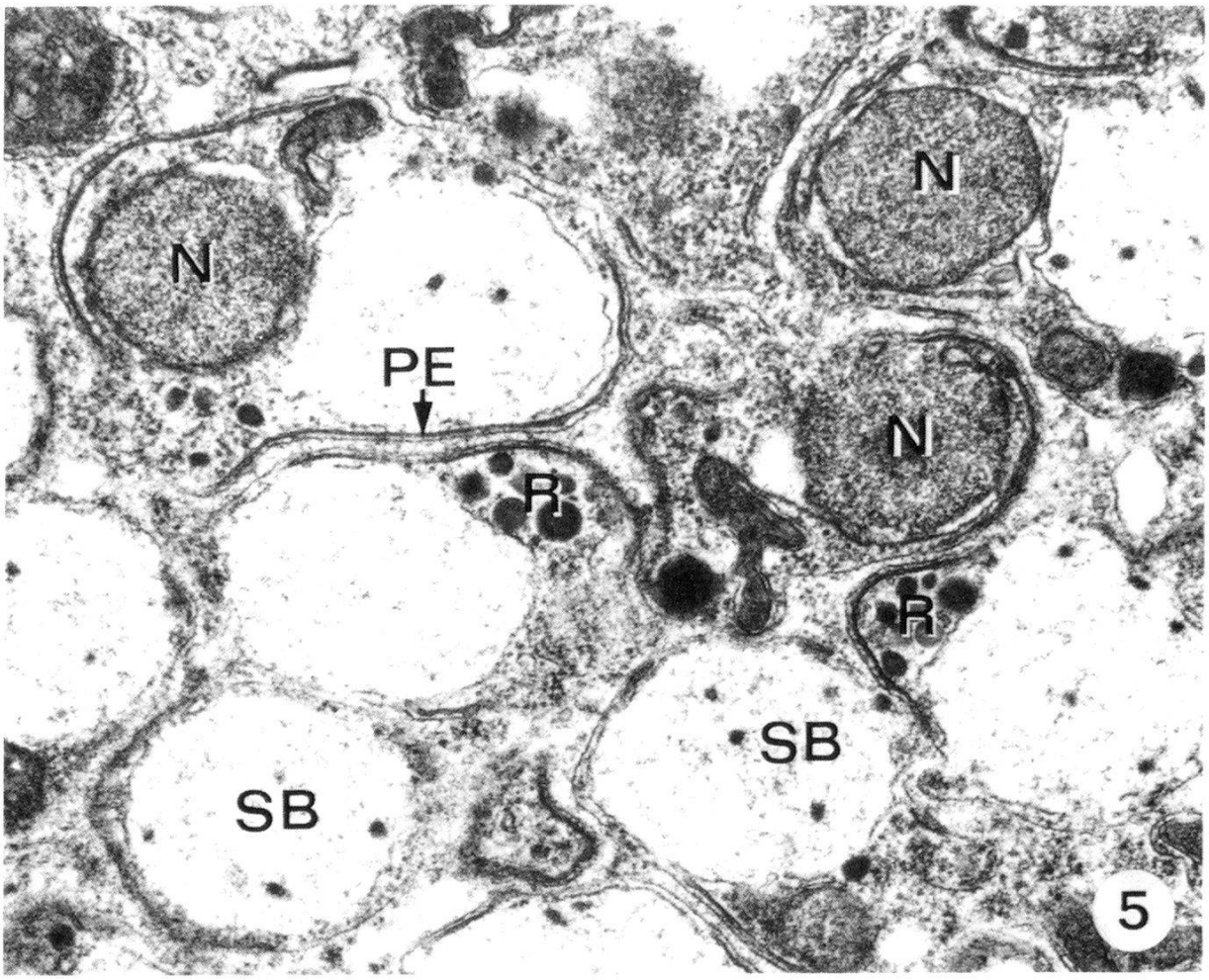
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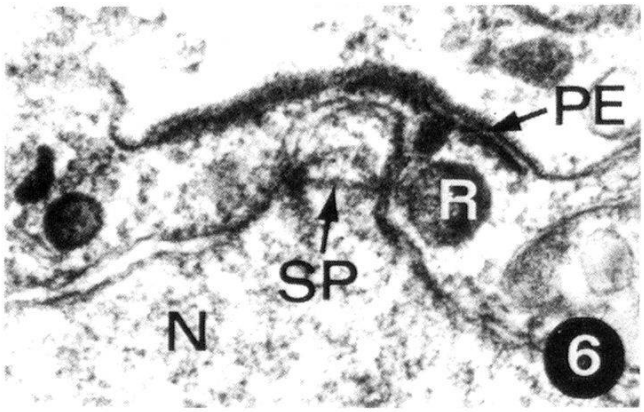
SB
5


PE
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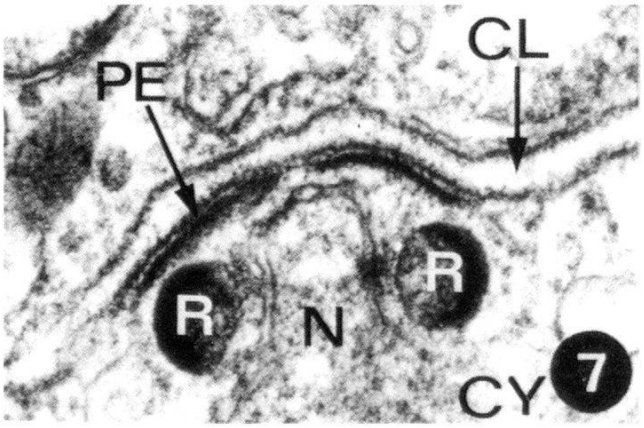
6


CL
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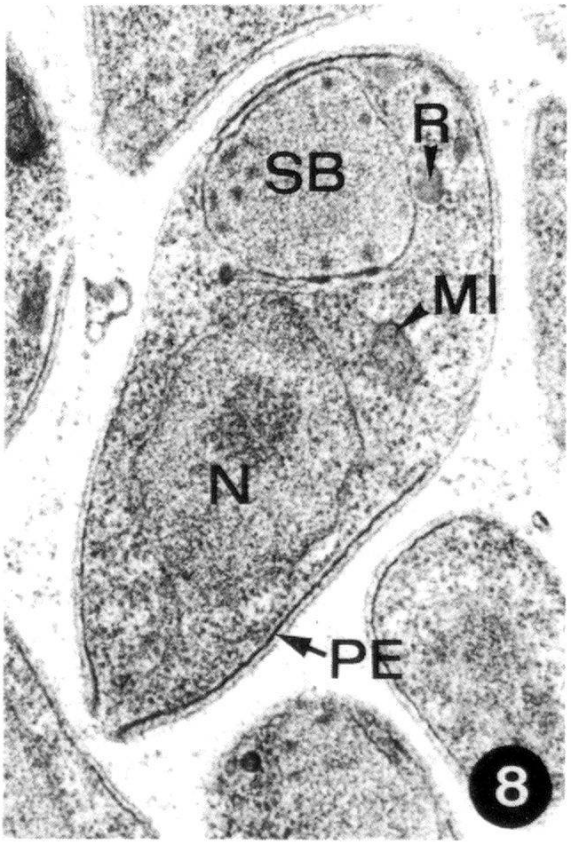
CY
7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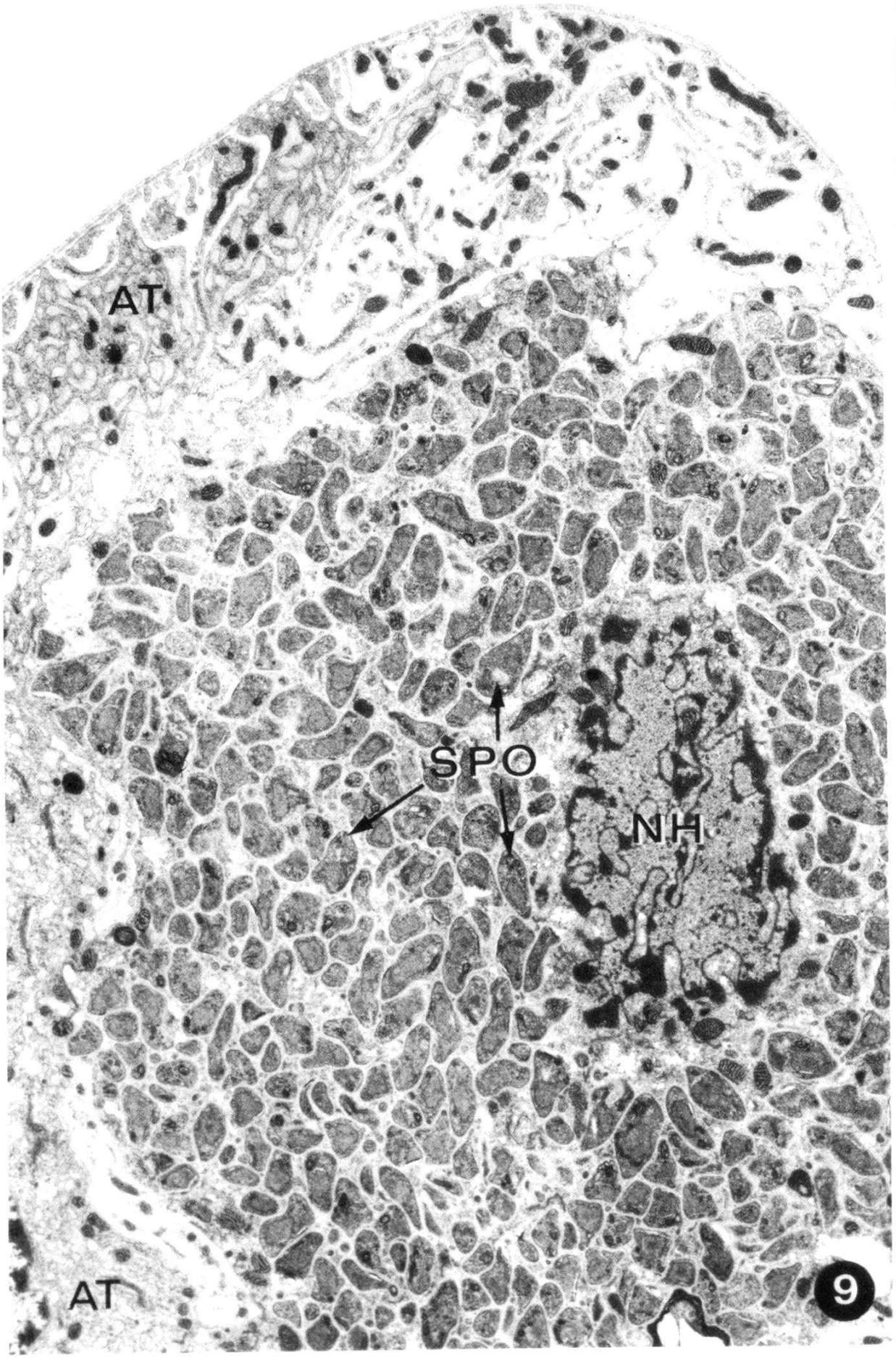
8


AT
SPO
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AT
9

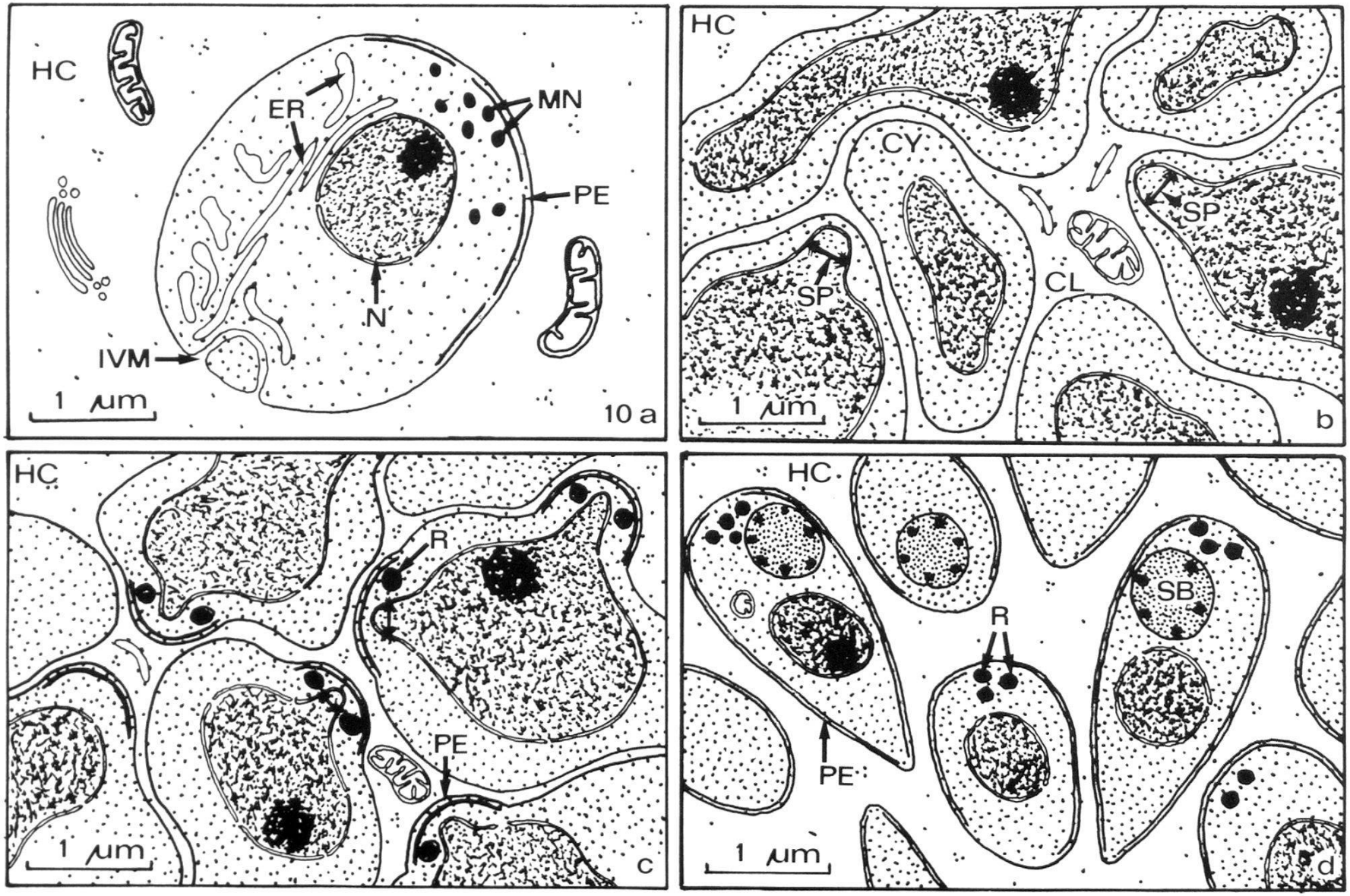

Fig. 10. Diagrammatic representation of the formation of sporozoites of *Babesia ovis* in the salivary gland cells of *Rhipicephalus bursa*. a) The intracellular kinete with beginning invaginations of the cell membrane. b) The growing parasite is fragmented into numerous cytomeres. c) Sporozoite differentiation starts at the periphery of the cytomeres as exogenous protrusions. d) Nearly mature sporozoites lying in a granular matrix

Developmental stages of *B. ovis* were found only in non-secreting cells at the periphery of the salivary alveoli. During the rapid growth of the parasite, the host cell was considerably enlarged. Its cytoplasm eventually degenerated to a flocculent matrix, only containing some osmiophilic mitochondria and the crenated host cell nucleus (Fig. 9). The infection of an alveolus with *B. ovis* was not considered likely to hinder its secretory function, since the other cells of the alveolus and its excretory duct showed a normal morphology.

Discussion

Since there is some evidence for the occurrence of gamogony during the *Babesia* life cycle (Friedhoff and Büscher, 1976; Weber and Friedhoff, 1977; Rudzinska et al., 1979; Mehlhorn et al., 1980b, 1981) the infectious stages can be considered as sporozoites and their formation as sporogony. The development of *Babesia ovis* sporozoites inside salivary gland cells of the tick *Rhipicephalus bursa* proceeds as an agamogonic reproduction in the course of which a large fission body divides into cytomeres. Later on these form numerous sporozoites as protrusions at the cytomere surface. The first parasitic stages seen on the second day after infestation (a.i.) of the tick on its host in the salivary glands are kinetes. During the next three days all parasites are found to be more or less in the same developmental stage, until on the fifth day a.i. the infected host cells are filled with differentiated sporozoites. In the species *Babesia canis* and *Babesia bigemina* the comparable development does not show this synchronization (Schein et al., 1979; Weber and Friedhoff, 1979).

The process of sporozoite differentiation in the genus *Babesia* has been object of several light microscopic observations (Dennis, 1932; Li, 1958; Riek, 1964) which revealed a fundamental process which is confirmed here for *B. ovis*. An exception is *B. canis*, where reproduction of the parasite in the salivary glands occurs as repeating binary fission, until the stages gradually acquire the morphology of sporozoites (Regendanz and Reichenow, 1933; Schein et al., 1979).

The development of sporozoites in the genus *Theileria* was also described as binary fission (Reichenow, 1940). Recently, however, Mehlhorn et al. (1979) observed that the transformation of *Theileria ovis* kinetes in the salivary glands occurs as a fragmentation, being rather similar to the development of *B. ovis* described in this paper. In both species the whole process is completed within 4–5 days.

The developmental stages of *Babesia* in the salivary glands of ticks were formerly regarded as schizonts and merozoites (Weber and Friedhoff, 1971; Friedhoff et al., 1972; Potgieter and Els, 1977). Since a 3-phase life cycle, as seen in the Coccidia, can be assumed for *Babesia* species, the parasite differentiation in the salivary glands should be considered as the last step of sporogony. This led to some considerations on phylogenetic relationships between the Piroplas-

mia, Haemosporina and Adeleina (Mehlhorn et al., 1980; Friedhoff, 1981). The kinetes of the genus *Babesia* may be compared with the sporokinetes which occur within the invertebrate host of *Karyolysus* (Reichenow, 1921). An ultrastructural description of the sporogony in the mite as vector of this adeleidian parasite is still lacking. On the other hand, there are some detailed ultrastructural studies on sporogony in the genus *Plasmodium* (Vanderberg et al., 1967; Terzakis, 1971; Schrével et. al., 1977; Sinden and Strong, 1978). Here the sporozoites arise in a more «peripheral» or more «internal» budding process (Sinden and Strong, 1978) from a fissured parasite cytoplasm which lies enclosed in an oocyst. The wall of this oocyst derives from the host cells and can therefore not be compared to the eimerian oocysts (Mehlhorn et al., 1980a). The sporozoites of the Piroplasmia are formed directly in the cytoplasm of the host cell, but the general development is similar to the sporogony of *Plasmodium*.

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