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Autor(en): **Schein, E. / Mehlhorn, H. / Voigt, W.P.**

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[1] Institut für Parasitologie und Tropenveterinärmedizin der Freien Universität Berlin
[2] Institut für Zoologie II der Universität Düsseldorf, BRD

Electron microscopical studies on the development of *Babesia canis* (Sporozoa) in the salivary glands of the vector tick *Dermacentor reticulatus**

E. Schein[1], H. Mehlhorn[2], W. P. Voigt[1]

Summary

The formation of sporozoites of *Babesia canis* was studied by light- and electron microscopy in the salivary gland cells of adult female ticks from the 2nd day after attachment until 1 day after detachment. It was found that this process was initiated by the binary division of kinetes that had already entered or entered during the period examined. During division the kinetes (15×2.5 μm) lost their typical organelles, reduced their three-layered pellicle to a single membrane and became spherical. After nuclear division and a further time-lag cell division occurred, giving rise to two cells in which this process was repeated. After numerous binary divisions the parasites acquired more and more closely the shape of the later infectious, pyriform sporozoite. These sporozoites measured about 2.5×1.5 μm and also had a three-layered pellicle, with rhoptries and a few micronemes, but never contained "spherical bodies". The formation process needed about 2–3 days so that the transmission to the dog could be carried out while the tick engorges and this is probably the stimulation for the development. Finally the cytological features of this sporozoite formation were compared to those in the *Theileria* species studied by our group.

Key words: Babesia canis; vector tick *Dermacentor reticulatus;* ultrastructure.

Introduction

In *Babesia* and *Theileria* species asexual reproduction has been observed in the cells of the salivary gland of appropriate ticks, leading to the formation of

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Correspondence: Prof. Dr. H. Mehlhorn, Institut für Zoologie II, Universitätsstrasse 1, D-4000 Düsseldorf

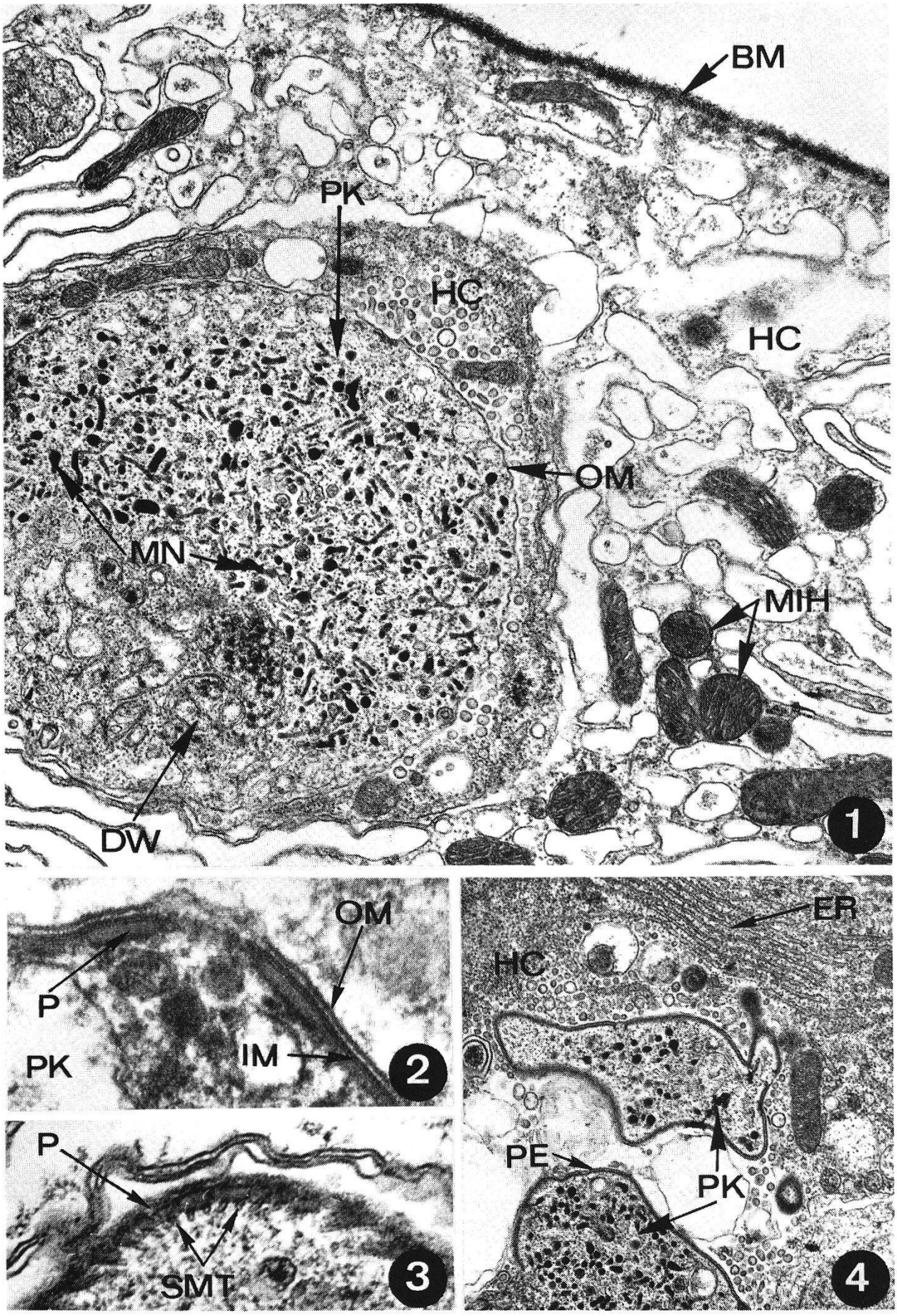
very small infectious stages (Lit. cf. Regendanz and Reichenow, 1933; Regendanz, 1936; Riek, 1964, 1966; Purnell and Joyner, 1968; Friedhoff, 1969; Weber and Friedhoff, 1971; Schein and Friedhoff, 1978). When the ticks suck blood these stages are transmitted to the vertebrate host. Recent work on the development of *Babesia* and *Theileria* has shown that these stages can be considered as sporozoites and their formation as sporogony (Schein, 1975; Scheint et al., 1975, 1977; Mehlhorn et al., 1975, 1978; Mehlhorn and Schein, 1976, 1977; Weber and Friedhoff, 1977; Friedhoff and Büscher, 1976). Few details of the process can be observed in the light microscope as the nuclei and mature sporozoites are very small. Thus electron microscopical studies may throw more light on this important developmental phase in the salivary gland of the vector ticks. The present study is concerned with the ultrastructural features in *Babesia canis* and a comparison is made with the process in *B. bovis*, *B. bigemina* (Potgieter and Els, 1976, 1977) and in several species of *Theileria* (Mehlhorn et al., 1979).

Abbreviations used in the figures

BM = Basal membrane of the acinus
CH = Chromatin material
DI = Stage reproducing by binary fission
DS = Developmental stage
DW = Double walled organelle
ER = Endoplasmic reticulum
HC = Host cell
IM = Inner membranes of the pellicle deriving from ER
IN = Invagination
LH = Limiting membrane of the host cell
MIH = Mitochondria of the host cell
MN = Micronemes
N = Nucleus
NH = Nucleus of host cells
NM = Nuclear membrane
OM = Outer membrane of the pellicle
P = Anterior polar ring
PE = Pellicle
PK = Kinete that has penetrated
PP = Posterior polar ring
R = Rhoptries
RI = Ribosomal structures
S = Sporozoite
SMT = Subpellicular microtubules
V = Vacuole

Figs. 1–12. *Babesia canis* within salivary glands of the vector tick *Dermacentor reticulatus*. Figs. 1–6, 8, 10–11 electron micrographs; 7, 9 light micrographs.

Figs. 1–4. Sections through kinetes (PK) that had entered the salivary gland cells on the second day after attachment of the tick. Note that in Fig. 1 the original pellicle (PE) is already reduced to a single cell membrane (OM). 1. ×25,000; 2. ×40,000; 3. ×42,000; 4. ×16,000.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Materials and methods

1. Ticks: Dermacentor reticulatus Fabricius 1794, Ixodidae. – Adult ticks of the strain Bordeaux 1976 were exclusively used in these experiments. The ticks were collected from a dog with natural tick infestation and maintained in the laboratory for four generations. Larvae and nymphs were fed on gerbils *(Meriones unguiculatus);* adult ticks were fed on dogs and rabbits.

2. Piroplasms: Babesia canis Piana and Galli-Valerio 1895. – The strain of *Babesia* was isolated from a dog infested with *D. reticulatus* in the vicinity of Bordeaux in 1976.

3. Dogs: Beagle. – Five dogs of 6–9 months of age were employed for the pathogenicity- and transmission experiments. The test animals were accommodated in climatized boxes and received pelleted dog food and water ad libitum. Previous to the experiments these dogs were found to be free of piroplasm infection.

4. Experimental procedure. – For the investigation of the salivary gland of *D. reticulatus* for the developing stages of *B. canis* only the progeny of those ticks which had engorged themselves as adults on erythrocytes with a parasitaemia of 9–14% were used.

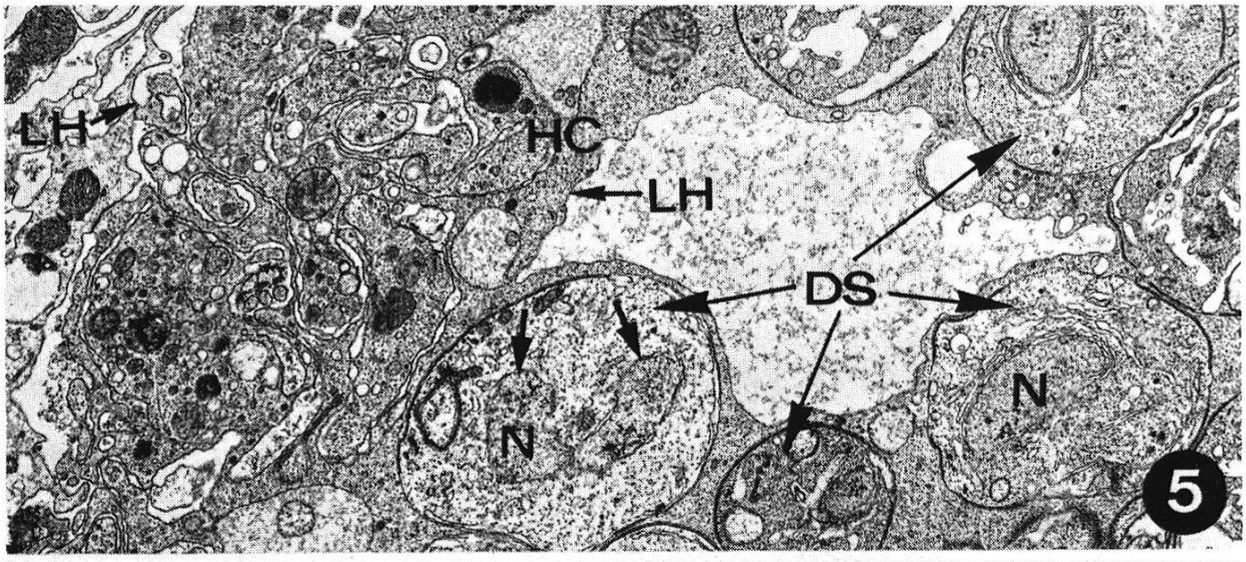
The infected adult ticks of the next generation (50 male and 50 female ticks) were attached to rabbits until the adults started to fall off. Five male and five female ticks were removed daily from the host and examined by light- and electron microscopy for developing stages of *B. canis.* Ticks were prepared in 2.5% glutaraldehyde under the dissecting microscope. The saliva of the ticks was examined for developing stages of *B. canis.* Infected ticks which had been engorged for 4–6 days were warmed under the dissecting microscope or injected into the coelom with a 10% Pilocarpin solution according to the method of Tatchell (1967). The recovered saliva was then caught in a capillary tube and fixed in 2.5% glutaraldehyde.

5. Cytological investigation. – For cytological investigations the salivary glands of the ticks or their saliva were fixed in 2.5% (v/v) glutaraldehyde in 0.1 M cacodylate-buffer (pH 7.3) for at least 24 h at 4° C. The preparations were then rinsed in this buffer, treated for 2 h in 2% OsO_4 (w/v) and dehydrated in ethanol before being embedded in Araldite (Ciba-Geigy). Ultrathin sections of embedded salivary alveoles were cut on a Reichert OMU 3 ultramicrotome, mounted on copper grids without a film, stained with an alcoholic solution of uranyl acetate for ½ h, and were finally laid in lead citrate for 10 min. Specimens were then examined with a Zeiss electron microscope EM 9 S 2. Light microscopical micrographs were taken form Giemsa-stained smears.

Results

In the salivary glands of unengorged adult ticks no kinetes were observed. Only 24 h after attachment could kinetes be demonstrated in the haemolymph. Single kinetes of *Babesia canis* were found in the cytoplasm of the salivary gland cells of engorging adult ticks from the 2nd until the 6th day after attachment and even 1 day after detachment. These kinetes measured about 11.5–18.5 × 2.5 μm and were surrounded by a typical three-layered coccidian pellicle under which about 40 subpellicular microtubules were observed in the anterior third (Figs. 1–4). At the apical pole a thick polar ring was prominent, and this was retained even in those kinetes in which the inner pellicular layer had disappeared. The kinetes were closely filled with micronemes, mitochondria-like organelles, accumulations of the endoplasmic reticulum, electron lucid vacuoles

Figs. 5–6. Sections through developmental stages on the 4th day after attachment. Note that these stages are ovoid and have an inner, incomplete layer of pellicle formed by cisternae of ER. The nucleus becomes u-shaped before division (Fig. 5, arrows) and cell division proceeds by invagination of the cell boundary (IN). 5. × 8500; 6. × 40,000.



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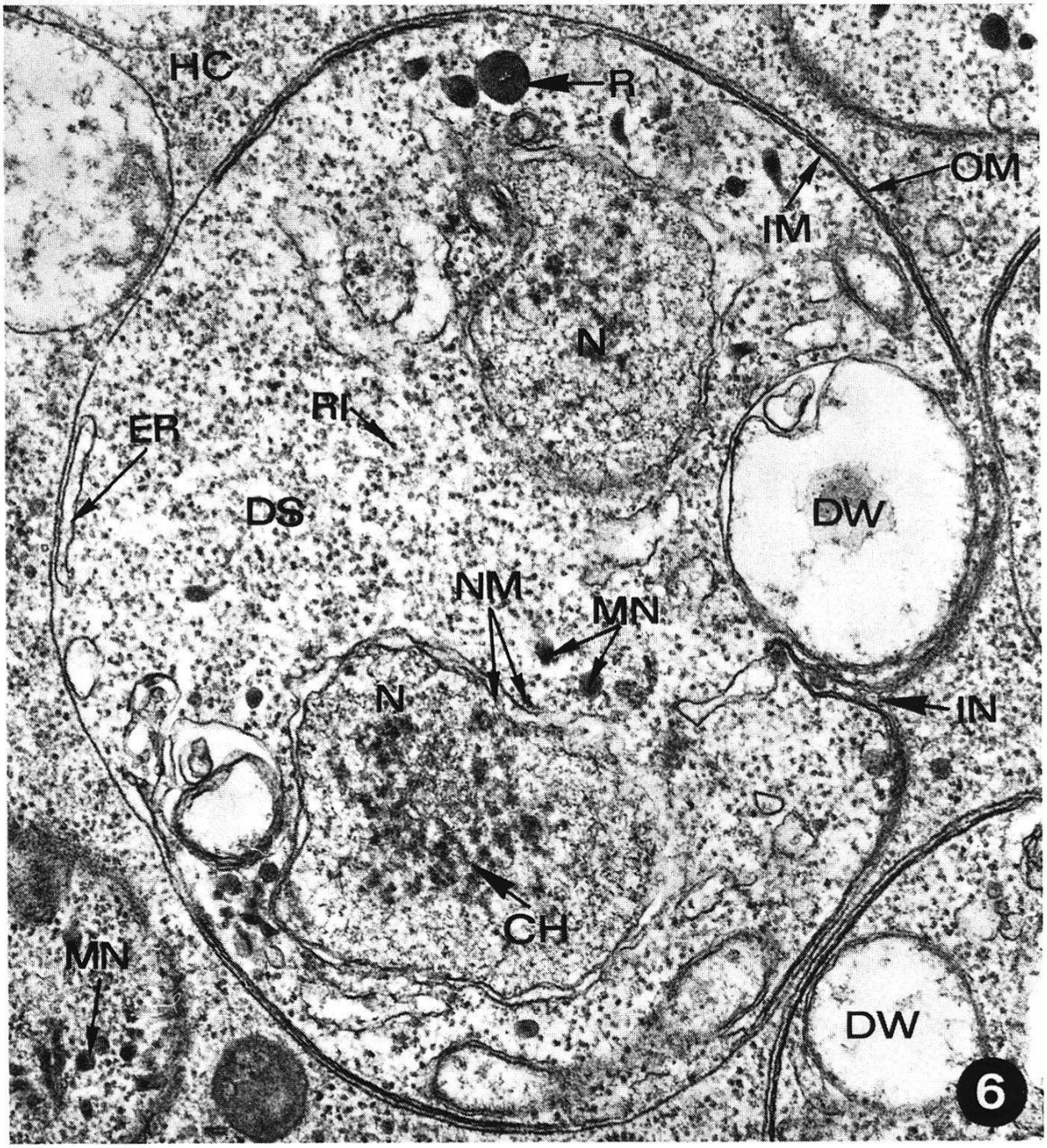

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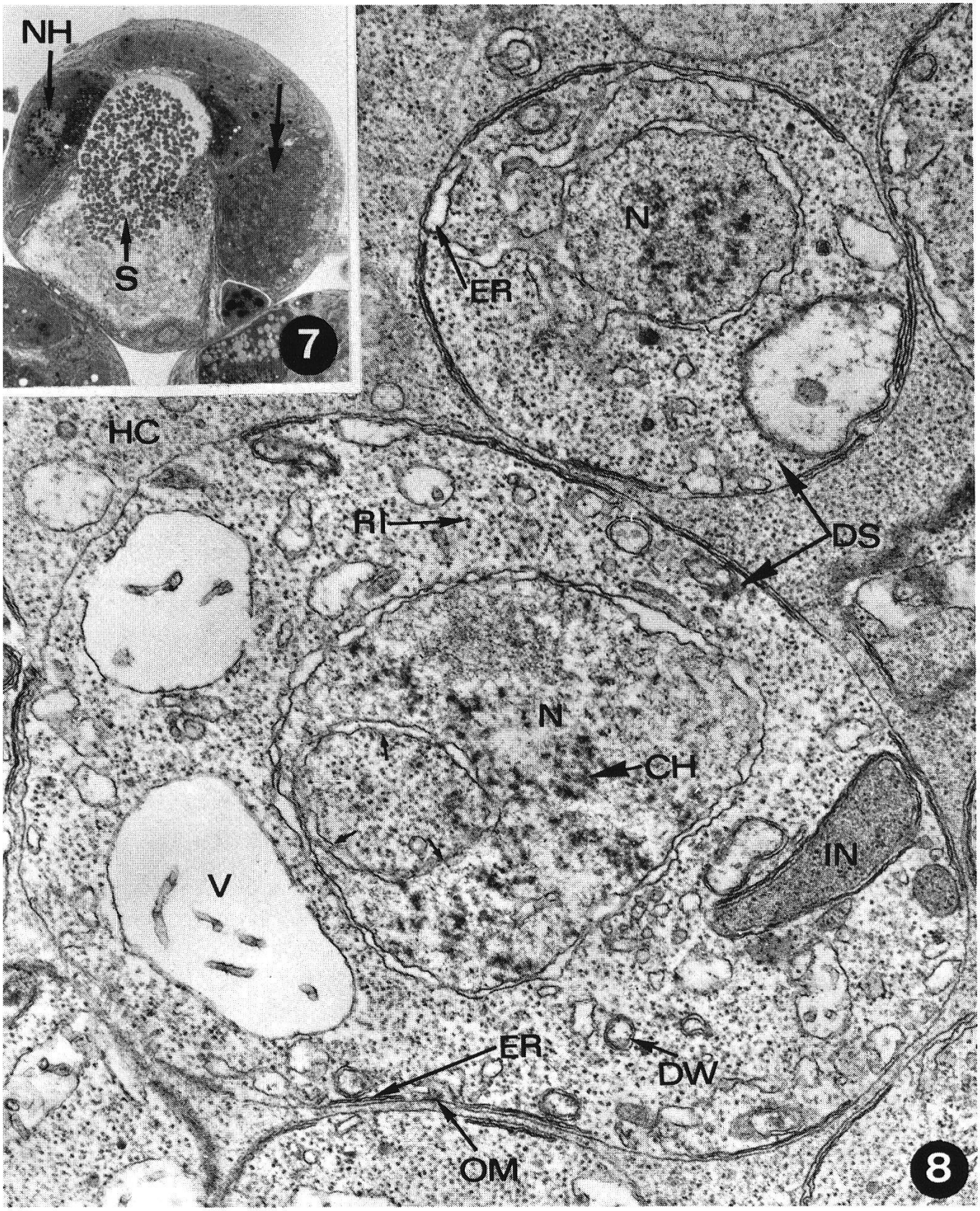
and a 2–3 μm sized nucleus containing osmiophilic chromatin concentrations. These kinetes were situated directly in the cytoplasm of the host cell and were not surrounded by a parasitophorous vacuole (Figs. 1, 4). Apparently these cigar-shaped kinetes became ovoid or spherical in shape during development, reduced their inner layer of the pellicle and were thus only bounded by the outer membrane (Fig. 1). Binary fission of this parasitic cell soon began, the nucleus dividing first and then the cell. In a few light micrographs, however, it seemed that more than two nuclei were formed before cell division. The binary fission was initiated by concentric invagination of the cell membrane and lead to two globular cells measuring about 4–6 $\times$ 5 μm, which apparently continued to divide, so that the parasitized host cell became continuously more and more filled. The division rate was very high, so that most of the parasitized host cells on the 2nd–3rd day after attachment of the ticks were crowded with these dividing parasites (Fig. 5), which under the light microscope had the appearance of a compact body (Fig. 7). During divisions the globular parasites became constantly smaller until they reached about 3–4 μm in diameter and had completely removed those organelles mentioned above as typical of the kinete (Figs. 5, 6, 8). Their cytoplasm appeared relatively electron-pale and was provided with 1.2 μm sized vacuoles and numerous ribosome-like granules (Fig. 6). At several places beneath the limiting cell membrane the cisternae of the endoplasmic reticulum began to form a new inner layer for a future pellicle (Figs. 6, 8). However, typical micropores were not found along the surface of such developmental stages. The dividing stages constantly became more and more pyriform and some rhoptries were visible at their apical poles (Figs. 10, 11). Furthermore a polar ring now occurred at the apical and also at the posterior pole, where division was carried out by constriction (Fig. 11). Finally on the 4–5th day after attachment of the ticks most of the infected salivary gland cells were filled with the pyriform stages, which were also present in the saliva of ticks after detachment (Figs. 9, 11). These pyriform parasites were considered as sporozoites infective for the vertebrate host, the dog. These sporozoites measured about 2.5 μm in length and had a diameter of about 1.5 μm at the maximum. They were limited by a coccidian three-layered pellicle (Fig. 11), the inner membranes of which had a few breaks – relatively scarce in parasites taken from the saliva. At the apical pole 4–6 rhoptries were present close to the polar ring (Figs. 10, 11), where some microneme-like structures were also observed. The nucleus was spherical with a diameter of about 0.8 μm and was situated central-

Fig. 7. Semi-thin section through an acinus of *Dermacentor reticulatus* on the 4th day after attachment. One cell is filled with sporozoites (S), another host cell (double arrow) is closely packed with developing stages thus giving the aspect of a compact body. $\times$400.

Fig. 8. Section through developing stages (DS) on the 4th day after attachment. Note that the nucleus (N) may have invaginations (small arrows) as well as the surface of the parasite (IN). The parasites are situated in the granular cytoplasm (without numerous distinct organelles) of the host cell (HC). $\times$41,000.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ly (Fig. 11). The karyoplasm contained extended chromatin material, which was probably condensed in light microscopical preparations, thus giving the impression of a nucleolus (Fig. 9), which was, however, not observed in electron microscopy. Close to the nucleus a spherical, 0.7 μm sized, organelle was observed (Figs. 10, 11). This organelle was bounded by two membranes running adjacent to each other (Figs. 10, 11), which formed a few invaginations into the electron pale interior. The cytoplasm contained numerous ribosome-like granules, that together caused a relatively dense appearance (Fig. 11). Cisternae of endoplasmic reticulum were extremely scarce and polysaccharide granules did not occur.

The formation of these infectious stages, which took 2–3 days at the maximum, is diagrammtically represented in Fig. 12. This development was exclusively observed in non-secretory host cells of which the cytoplasm was completely dissimilated while the parasites were being formed inside. The organelles gradually disappeared and finally the enormously stretched host cell was filled with granular masses surrounding the sporozoites. However, the nucleus of the host cell, which at first grew to a considerable degree, was present even in cells filled with masses of sporozoites, but was in most cases pushed to the periphery and later contained apparently empty spaces.

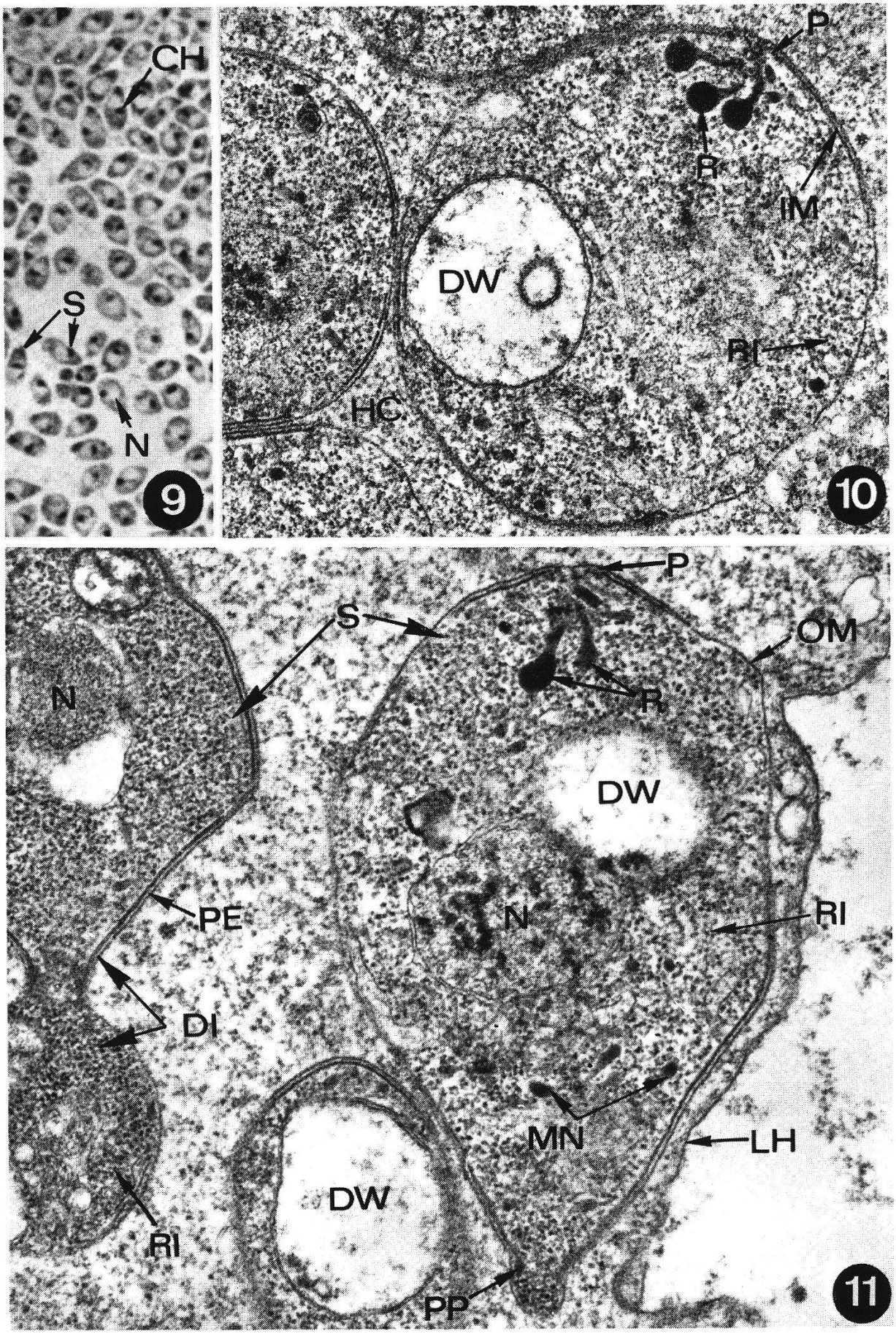
Discussion

The development of sporozoites of *B. canis* inside the cells of the salivary glands of *D. reticulatus* is an agamogonic process, proceeding as constant binary fission, during which the stages produced possess more and more features of the final infectious sporozoites. In all cases the development starts from kinetes (called vermicules in older publications), which have entered the salivary gland cells probably during the attachment of the ticks. However, it was noted that such kinetes may enter even later, because they were found in salivary gland cells which were close to others already filled with infectious sporozoites. With respect to the transmission of the sporozoites to the canine host, however, only those kinetes that penetrated initially seem to be successful, as sporozoite formation needs about 2–3 days. Probably development initiated by those kinetes that penetrate later does not lead to infectious sporozoites before the detachment of the ticks.

The cytological procedure of sporozoite formation described here was also observed by Regendanz und Reichenow (1933) in a light microscopical study of the same species. However, Riek (1964, 1966), Friedhoff et al. (1972), and Weber and Friedhoff (1971) suggested formation of sporozoites by multiple

Fig. 9. Giemsa-stained smear of mature sporozoites (S). ×2000.

Figs. 10–11. Sections through nearly mature sporozoites within host cells (HC) on the 5th and 6th day after attachment of the tick. 10. ×30,000; 11. ×31,000.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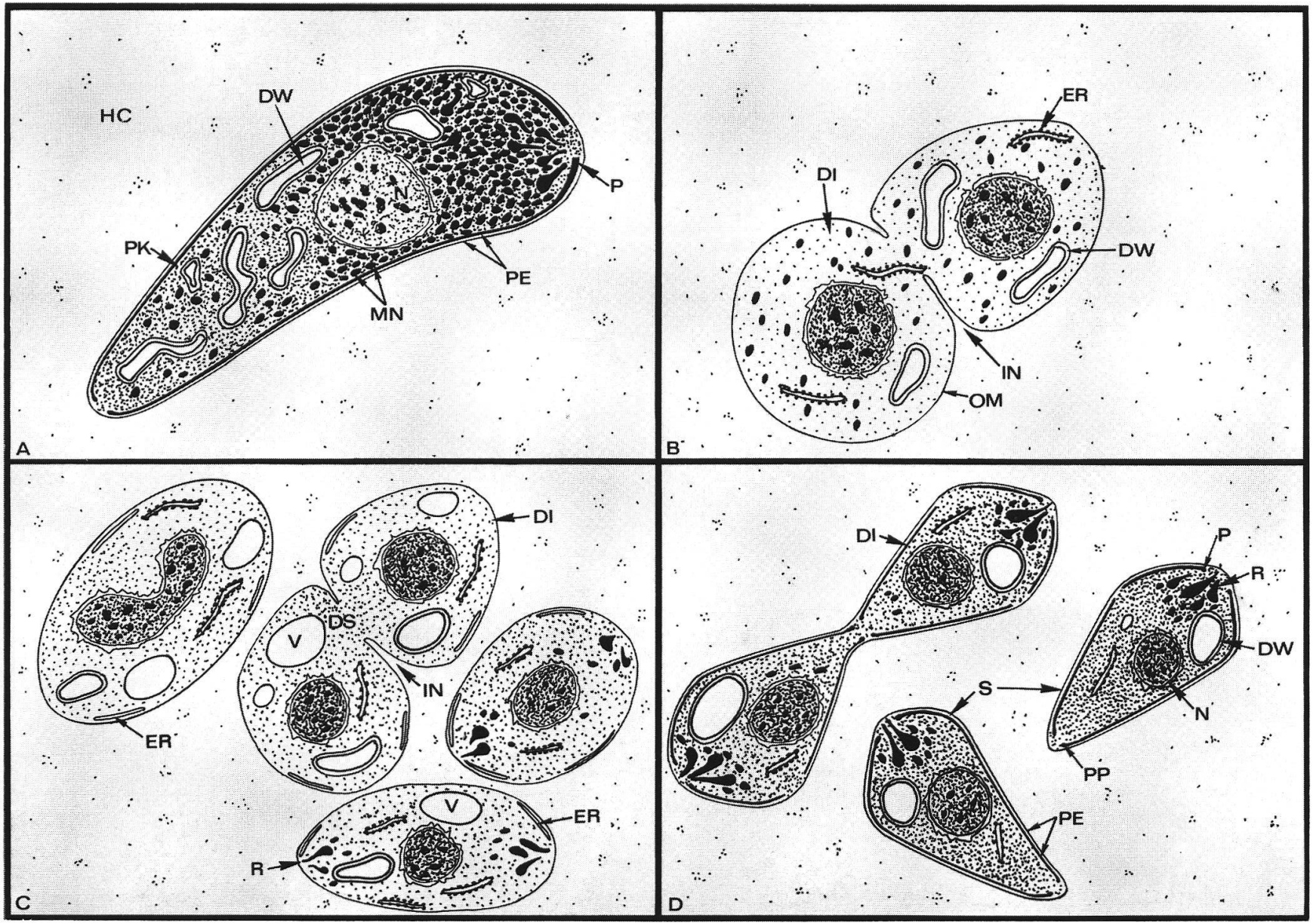

Fig. 12. Diagrammatic representation of the formation of sporozoites of *Babesia canis* within the salivary gland cells of *Dermacentor reticulatus*. This formation proceeds as binary fission. Note that first the inner layers of the pellicle are reduced and that later this layer becomes more and more complete thus indicating its importance for the infectious sporozoites.

division within the salivary gland cells of the vector ticks. This interpretation probably resulted from the small size of the dividing stages and their close packing in light microscopical preparations, as could also be demonstrated in our material before ultrathin sectioning. Potgieter and Els (1976, 1977) also described binary fissions in *B. bovis* and *B. bigemina*, but suggested that initially "schizonts" occur. The electron micrographs given of "immature schizonts", however, indicate that these authors considered the granular cytoplasm of the altered host cell as cytoplasm of a "schizont", within which the "merozoites" were already present. Thus according to electron microscopy, features of a typical schizont as in intestinal coccidia (Lit. cf. Scholtyseck, 1973) or a typical sporont as in haemosporidia (Schrével et al., 1977) do not occur in *Babesia* during the formation of sporozoites in the salivary glands of the ticks.

In *Theileria* species the formation of sporozoites is different from that in *Babesia*, as has been described in recent light and electron microscopical investigations (Schein and Friedhoff, 1978; Mehlhorn et al., 1979). In *Theileria* multiple fissions of the large cytomeres and their stretched nuclei simultaneously give rise to several small sporozoites, although in light microscopical studies a binary fission process has been suggested by some authors (Regendanz, 1936; Reichenow, 1940). Considering the timing and the start of sporozoite formation, *Babesia* and *Theileria* are similar. The start of this process is probably stimulated by the sucking of the vector tick, and needs about 2–3 days until formation of the infectious sporozoites, which may then, during the same sucking process, be transmitted to the vertebrate host.

With respect to the morphological features of the infectious sporozoites it can be demonstrated that there is no basic difference between those in *Babesia* and those in *Theileria*, where, however, they reach only half the size. They are both provided with a more or less complete coccidian pellicle, with a few rhoptries and neither has a conoid. In our preparations of *Babesia* and *Theileria* species the sporozoites never contained structures like a "sphäroider Körper" described by Friedhoff et al. (1972) in *B. ovis*. The space that appears pale in light microscopical preparations represents the nucleus, the chromatin of which was condensed to a dense dot, which was believed to be a nucleolus. Potgieter and Els (1976, 1977) did not observe such a peculiar body either. Thus this "sphäroider Körper" cannot be considered as a general criterion of the genus *Babesia*.

Summarizing it can be stated that the formation of sporozoites in *Babesia* differs from that in *Theileria* by proceeding as binary fission instead of as a multiple one and by the bigger size of the sporozoites in *Babesia*, but not in their basic structure.

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