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LIGHT AND ELECTRON MICROSCOPIC OBSERVATIONS ON THE DEVELOPMENT OF *BABESIA BIGEMINA* IN LARVAE, NYMPHAE AND NON-REPLETE FEMALES OF *BOOPHILUS DECOLORATUS*

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ABSTRACT

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In *Boophilus decoloratus* infected by transovarian passage with *B. bigemina*, primary schizogony occurred as a continuous repetitive process in all 3 stages of the tick's life cycle spent on the host. The primary schizonts and the large merozoites (= vermicules) produced by them were observed in the gut epithelium, haemocytes, muscles and peritracheal cells. Secondary schizogony which led to the formation of small merozoites (= infective forms) occurred mainly in the salivary glands, but was also observed in the cortex of the synganglion. Mature small merozoites were observed in nymphal and adult ticks only.

An infective stabilate was prepared from nymphae collected on Day 14 and Day 15 post larval infestation. The infections resulting from intravenous injection of the stabilate had a prepatent period of 8 days.

Résumé

OBSERVATIONS AUX MICROSCOPES OPTIQUE ET ÉLECTRONIQUE SUR LE DÉVELOPPEMENT DE BABESIA BIGEMINA *DANS LES LARVES, NYMPHES ET FEMELLES NON REPUES DE* BOOPHILUS DECOLORATUS

Chez Boophilus decoloratus, *après infestation à* B. bigemina *par passage transovarien, la schizogonie primaire apparaît sous forme d'un processus répétitif continu dans chacun des 3 stades du cycle vital de la tique sur son hôte. Les schizontes primaires et les grands mérozoïtes (=vermicules) qu'ils produisent ont été observés dans l'épithélium du tube digestif, les hémocytes, les muscles et les cellules péritrachéales. La schizogonie secondaire qui mène à la formation die petits mérozoïtes (=formes infectieuses) se déroule principalement dans les glandes salivaires, mais a aussi été observée dans le cortex du synganglion. Ce n'est que chez les nymphes et les tiques adultes qu'on a observé des petits mérozoïtes mûrs.*

Un stabilat infectieux a été préparé à partir de nymphes recueillies aux 14e et 15e jour après infestation larvaire. Les infections consécutives à l'inoculation intraveineuse de ce stabilat ont eu une durée prépatente de 8 jours.

INTRODUCTION

Bovine babesiosis, or redwater, is at present known to be caused by *Babesia bigemina* and *Babesia bovis* in the Republic of South Africa. Until recently, however, the only information on the natural transmission of these parasites in this country was based on observations made during the early part of this century and information on the developmental cycle of the parasites in their vectors was superficial or non-existent.

According to Hutcheon (1900), redwater was first observed by Europeans in Natal in 1870. However, Wiltshire (cited by Henning, 1949) reported the presence of redwater in Natal prior to European settlement in the 1830's. Detailed information on the history of redwater and the manner in which it spread through the country was recorded by Hutcheon (1900) and Theiler (1905), and later reviewed by Henning (1949).

The first local attempt to transmit the disease with ticks was apparently carried out by Theiler (1905). He infested a calf with *Boophilus decoloratus* (referred to as *Rhipicephalus decoloratus*) and subsequently inoculated the calf with *B. bigemina* (referred to as *Piroplasma bigeminum*)-infected blood. Eggs of female ticks collected from this animal during the ensuing redwater reaction were sent to McFadyean in London who fed the larval progeny on a calf and a steer, both of which contracted redwater. Blood smears of these animals were sent back to Theiler who identified the causative organisms as *B. bigemina*. Theiler (1907) infected 6 heifers with *B. bigemina* using the larval progeny of *B. decoloratus* and thus confirmed transovarian transmission of the infection. He extended his work by collecting engorged female ticks from horses and feeding their larval offspring on 3 heifers (Theiler, 1909a). All 3 animals contracted *B. bigemina* infections, thus demonstrating successful transovarian transmission as well as the fact that the ticks apparently retained the infection, even when fed on a non-bovine host.

Theiler (1908) noticed a *B. bigemina* infection in a bull which had been experimentally infested with adult *Rhipicephalus appendiculatus*. This observation was followed up by 3 successful attempts to transmit *B. bigemina* by adult *R. appendiculatus* (Theiler 1909b). The prepatent periods of these infections were given as 40, 24 and 23 days. Although larval transmission of the infection was also reported, no experimental details were given. At an international congress in The Hague, Theiler (1910), however, incriminated only ticks of the genus *Boophilus* as being responsible for the transmission of *B. bigemina* but did not elaborate any further on his statement.

No original research work was conducted on the transmission of bovine babesiosis in the years that followed. In 1938, Neitz & Du Toit published a review article on tick-borne diseases which indicated that *B. bigemina* was transmitted by *B. decoloratus*, *R. appendiculatus* and *Rhipicephalus evertsi* in South Africa. No specific references were given except the following: "According to Theiler the larvae bred from infected adults of *R. appendiculatus* are capable of transmitting the disease. Infected nymphae transmit the disease in the adult stage. In the case of *R. evertsi*, stage-to-stage transmission as well as passage through the egg to the larva takes place".

In a comprehensive review of the transmission of piroplasmas by ticks (Neitz, 1956), Theiler (1909b) is credited with having described the transstadial and transovarian transmission of *B. bigemina* by *R. appendiculatus* and *R. evertsi*. *R. evertsi* is, however, not mentioned as a possible vector in Theiler's paper

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and this particular reference is therefore apparently incorrect. However, in another paper, Theiler (1909c) did mention that both *R. appendiculatus* and *R. evertsi* could transmit *B. bigemina* but only in exceptional cases which he did not elaborate. No record of experiments in which specific attempts were made to transmit *B. bigemina* with *R. evertsi* could be traced during the course of this study.

Purnell, Branagan & Brown (1970), being unable to repeat the work done by Theiler (1909b) on the transmission of *B. bigemina* by adult *R. appendiculatus,* concluded that the somewhat lengthy and variable prepatent periods of the infections reported by Theiler indicate that infection may have been the result of unobserved *B. decoloratus* infestations.

Very little has been recorded on the role played by *Boophilus microplus* in the history of redwater in this country. Loundsbury (1905a) reported the widespread occurrence of *B. decoloratus* and *B. microplus* (referred to as *Boophilus australis*) in the Cape Colony. He also gave the following interesting information with regard to *B. microplus* in an unpublished report (1905b) by the Government Entomologist: "In only one instance has an unquestionable case of the disease been produced and this one followed a very heavy application of *B. australis* larvae, progeny of females, off a recently recovered animal. Fever appeared on the 10th day and the beast succumbed on the 17th". Based on the length of the incubation period of the disease and the identity of the vector, it is reasonable to assume that Lounsbury was dealing with a *B. bovis* infection. In this report he also concluded that the very common prevalent tick *B. decoloratus* was ordinarily responsible for the transmission of bovine piroplasmosis.

Howard (1908) produced a comprehensive list of the ticks of South Africa and mentioned that *B. microplus* (referred to as *Margaropus annulatus australis*) transmits redwater to cattle. Unfortunately no references were given.

It was not until 1941 that *B. bovis* was first identified with certainty in this country when it was incriminated as the causative organism of a redwater outbreak on a farm near Pretoria in the Transvaal (Neitz, 1941). Neitz observed that the cattle concerned were infested with *B. decoloratus,* but he believed that any of the 3 known vectors of *B. bigemina,* namely, *B. decoloratus, R. appendiculatus* and *R. evertsi,* might have transmitted the infection.

Neitz's (1962) initial attempts to transmit *B. bigemina* and *B. bovis* with *B. decoloratus* failed, but later (1969) he briefly reported 1 instance of successful transmission of *B. bigemina* with *B. decoloratus.* The vector of *B. bovis* remained unknown, however, until 1972 when Thomas (unpublished observations) established that a South African strain of *B. microplus* could transmit *B. bovis* as well as *B. bigemina.* This, and the transmission of *B. bigemina* by *B. decoloratus* have been confirmed, but attempts to transmit *B. bovis* with *B. decoloratus* have been unsuccessful (Potgieter, 1977), therefore this tick remains a conclusively proven vector of *B. bigemina* only.

Three phases can be recognized in the development of *B. bigemina* in the one-host tick *B. microplus* (Riek, 1964) and *B. decoloratus* (Potgieter, 1977), namely, the initial infection acquired by the engorging female, the transovarian passage of the infection, and the development in the next generation. The purpose of this investigation was to study the latter part of the life cycle, starting with development in unfed larvae infected transovarianly up to the formation of the infective form of the parasite.

A severe *B. bigemina* infection in a particular batch of *B. decoloratus* made it possible to study the development and to identify equivalent developmental stages with a reasonable degree of accuracy with both the light and electron microscope.

Materials and Methods

Infected ticks

Larvae of a laboratory-maintained strain of *B. decoloratus* transovarianly infected with a South African isolate of *B. bigemina* of known purity were used to infest a fully susceptible, splenectomized ox, as described by Potgieter (1977). The newly hatched larvae used were maintained in an acaridarium at 25 °C and 85% R.H. prior to infestation. This experiment was performed under strict supervision in tick-proof stables. The tick instars were manually removed from this ox at 24 h intervals after larval infestation and processed for light and electron microscopy as described below.

The following code was used to identify the different tick instars on a daily basis: L5, N14 and A16, for example, denote that larvae, nymphae and adult ticks were respectively removed for study 5, 14 and 16 days after larval infestation. In addition, preparations were made from unfed larvae of the same batch of ticks for light microscopic examination.

Light and electron microscopy

Smear preparations from individual intact larvae and nymphae as well as organs from nymphae and adult ticks, dissected as described previously (Potgieter, 1977), were made by placing the specimen on a glass slide, squashing it with another slide and smearing it out in a single movement. The smears were then air-dried, fixed in methanol for 3 min and stained in a 10% solution of Giemsa's stain for 30 min.

Intact ticks as well as dissected organs, for example, gut and salivary glands, were fixed in Carnoy's fixative for 1 h, dehydrated in two 1 h changes of absolute alcohol, a 1:1 mixture of absolute alcohol and chloroform and finally in pure chloroform before being embedded in Paraplast*. The blocks were shaped and sectioned on a Reichert Om U_2 ultramicrotome using glass knives. Sections approximately 1 μm thick were obtained and dried overnight at 37 °C. Subsequently the sections were immersed in xylol for 3–5 min to remove the Paraplast and washed in a graded series of ethanol concentrations for 2 min at a time and finally in distilled water for 1 min. The sections were stained in a 10% Giemsa solution for 10–20 min, differentiated in 0,1% acetic acid, dehydrated and cleared by means of standard acetone and xylol changes to pure xylol before the sections were mounted in Canada balsam in xylene.

A Wild binocular research microscope was used for the light microscopic examination of all stained smears and sections, and photomicrographs were taken with a Zeiss photomicroscope. The dimensions of the parasites were determined from colour negatives with the aid of a Nikon profile projector model 6C.

* Sherwood Medical Industries Inc.

N14 and N15 were selected for the ultrastructural study, primarily because of the large numbers of small merozoites seen in smears prepared from these ticks. The earlier developmental stages of *B. bigemina* seen in the smears of younger nymphae and larvae were also present in these nymphae, which made them ideally suited for fine structural studies.

The tick material was processed for electron microscopy as described by Potgieter, Els & Van Vuuren (1976). A Siemens 102 electron microscope was used.

Tick stabilate

Approximately 250–300 nymphal and adult instars were removed daily for 6 days from Day 14–19 after larval infestation. Stabilates were prepared from each collection as described by Potgieter & Van Vuuren (1974) for *B. bovis*-infected *B. microplus* larvae. Each 1 ml ampoule contained the equivalent of 20–25 triturated ticks.

The contents of 1 ampoule from the 6 batches of stabilate were pooled after thawing at 37 °C and the 6 ml thus obtained injected intravenously into a susceptible ox. The infectivity of each batch was determined by inoculating the contents of 1 ampoule (1 ml) intravenously into each of 6 oxen.

Thin and thick blood smears (Mahoney & Saal, 1961) were prepared and examined, and temperatures recorded daily from all inoculated animals. Reacting animals were treated with diminazene*, where necessary.

Results

Infectivity of ticks and stabilates

Larval ticks attached within 24 h of infestation. The first nymphae were collected from the animal on Day 7 and adult ticks on Day 16 after larval infestation. *B. bigemina* parasites were first seen in thick blood smears on Day 17.

The ox injected with 6 ml pooled stabilate of triturated tick material contracted a *B. bigemina* infection after a prepatent period of 8 days. Of the individual batches of stabilate prepared from N14–A19, only those of N14 and N15 proved to be infective. The prepatent period for each of these batches was also 8 days.

Microscopic observations

General description of developmental cycle in these ticks

Both light and electron microscopy indicated that multiplication of *B. bigemina* in larval, nymphal and adult ticks occurred by means of schizogony. Two morphological distinct types of merozoites were produced. One type, large merozoites (LM), known as vermicules, were formed by what will be referred to as primary schizogony in various organs and tissues; the other, so-called small merozoites (SM) or infective forms, were produced by what will be referred to as secondary schizogony, which occurred mainly in the salivary glands.

Larvae

Parasites were observed in considerably smaller numbers in unfed larvae than in feeding ones. The developmental stages in unfed larvae, which were only studied in smears with the aid of the light microscope, included small immature schizonts and LM. These parasites apparently only occurred in the epithelial cells of the gut, and the situation was one of low activity. However, in larvae that had fed for at least 24 h (L1), large actively growing schizonts were detected. Since no sections of infected larvae were obtained, it was not possible to determine which tissues were actually involved in primary schizogony. LM were plentiful in L4 and L5. Immature, mostly spherical, SM were seen in a very low number of L6 and L7, indicating that secondary schizogony had been initiated in their salivary glands.

Nymphae

Secondary schizogony continued in the salivary glands of the newly-moulted and feeding nymphae and was augmented by further infection of the glands with LM. The first mature piriform SM were observed in N9, and they reached peak numbers in N13–N15. Concurrently, primary schizogony continued in various nymphal tissues in which infections were initiated by LM.

Non-replete adults

Production of SM also proceeded in the newly-moulted and semi-engorged adult ticks but apparently at a lower level when compared with the situation in the nymphae. SM and secondary schizonts were detected in salivary glands of A16–A18. LM and primary schizonts were also seen in gut and in haemolymph smears prepared from these ticks, but they were more difficult to demonstrate than in the nymphae in which optimum parasitic activity appeared to have occurred.

LM were also found in the haemolymph of ovipositing females that had engorged on animals after the *B. bigemina* infections they had initiated had possibly been sterilized by therapy. Since the larval progeny of such ticks were capable of transmitting the infection to susceptible cattle (Potgieter, 1977), we concluded that the parasite was capable of uninterrupted development and even transovarian transmission in the absence of reinfection during the process of final engorgement of the tick.

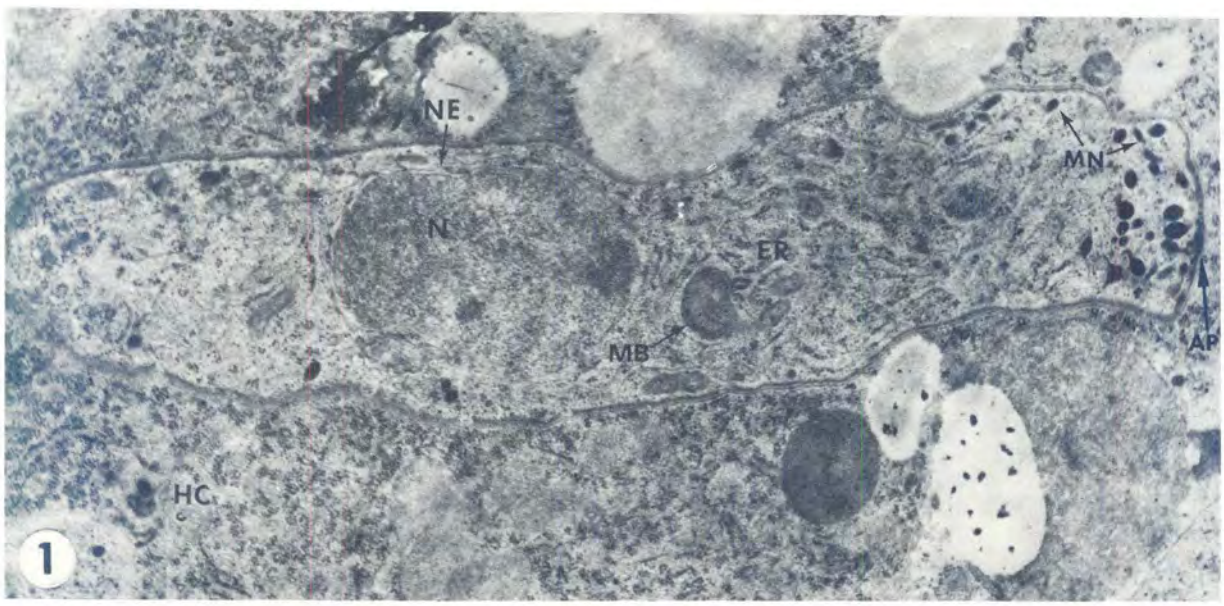
Production of large merozoites (primary schizogony)

During this study it became evident that LM of *B. bigemina* were formed in the tick through successive cycles of schizogony. This occurred in the larval, nymphal and adult stages and continued even after the formation of the infective forms in the salivary glands.

The LM (Fig. 1, 24–26) commonly observed throughout the life cycle of the parasite, as well as those undergoing transformation to become schizonts (Fig. 27 & 28), were studied under the electron microscope. Upon entry into a host cell the thick inner membrane of the pellicle of the LM disappeared. This process apparently started at the posterior end of the parasite and resulted in the loss of the typical merozoite shape as the parasite began to round off (Fig. 2) to form an initial spherical schizont (Fig. 12). This formation was readily identifiable under the light microscope (Fig. 29). At this stage, traces of the pellicular complex, namely, the thick inner membrane, anterior polar ring (see transforming LM in Fig. 20) and associated micronemes, could still be identified, but no microtubules were observed (Fig. 12). The first indications of nuclear division were also evident at this and at slightly earlier stages (Fig. 2 & 12). The schizonts increased in size, micronemes became scattered in the cytoplasm and many membrane-bound, diversely-folded, cylindrically-shaped structures, which were possibly mitochondria, appeared (Fig. 3).

* Berenil, Hoechst

FIG. 1–23 Electronmicrographs of developmental stages of *B. bigemina* in the nymphae (N 14 & N 15) of *B. decoloratus*. Fig. 1–10 illustrate the stages involved in the formation of LM (primary schizogony)



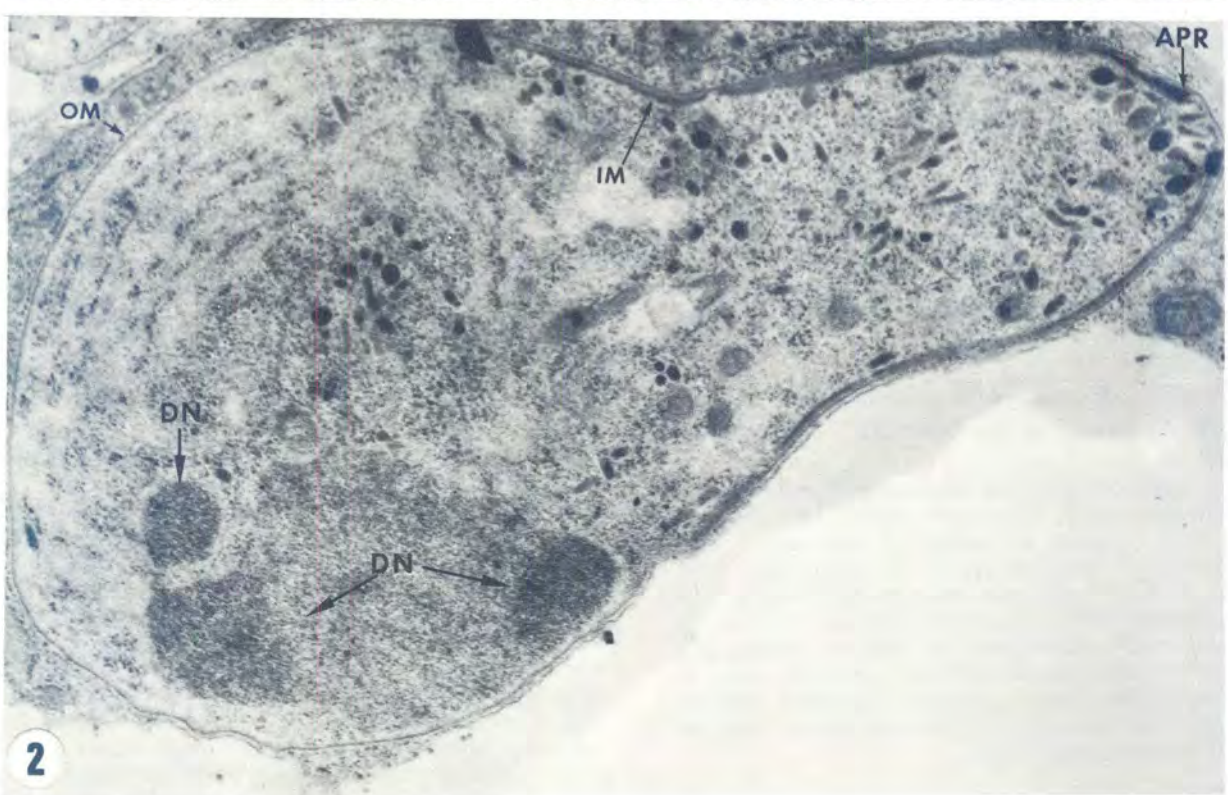
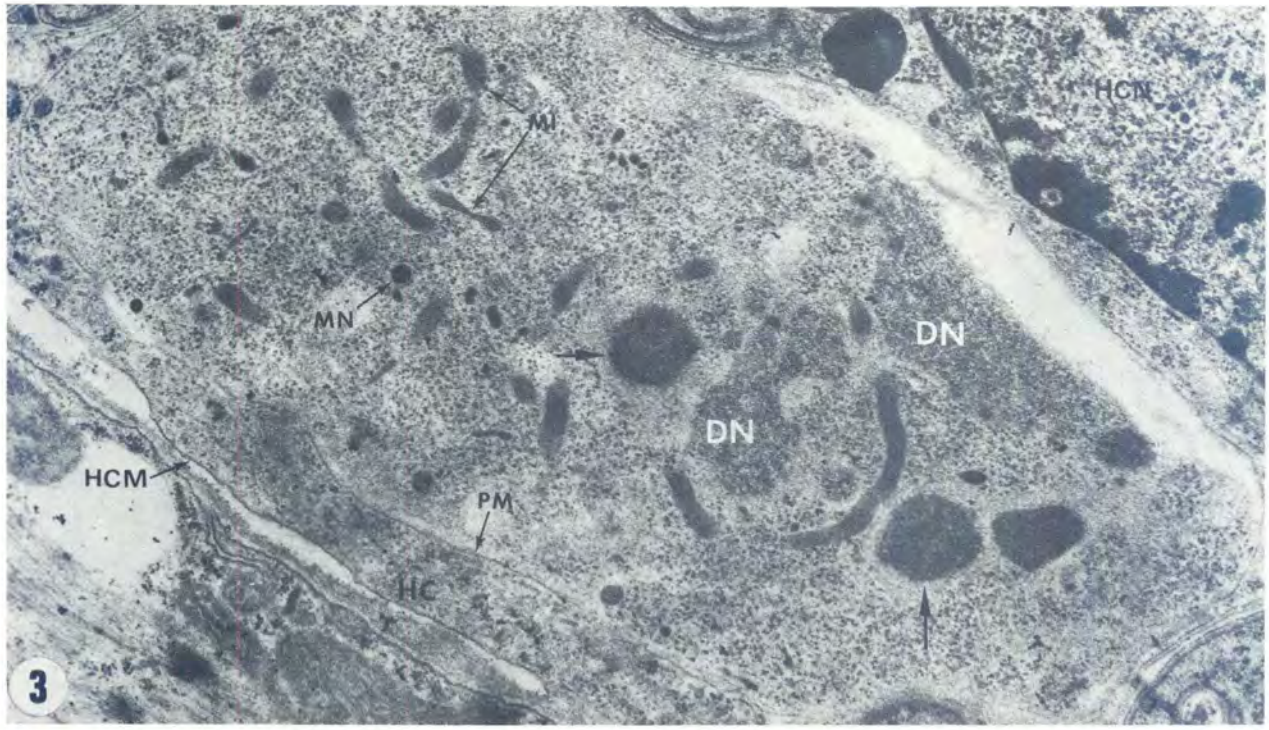


FIG. 1 Longitudinal section of intracellular LM in a gut epithelial cell. × 20 000
FIG. 2 Longitudinal section of transforming LM in a gut epithelial cell in the process of rounding off before onset of schizogony. Note dividing nucleus. × 28 000



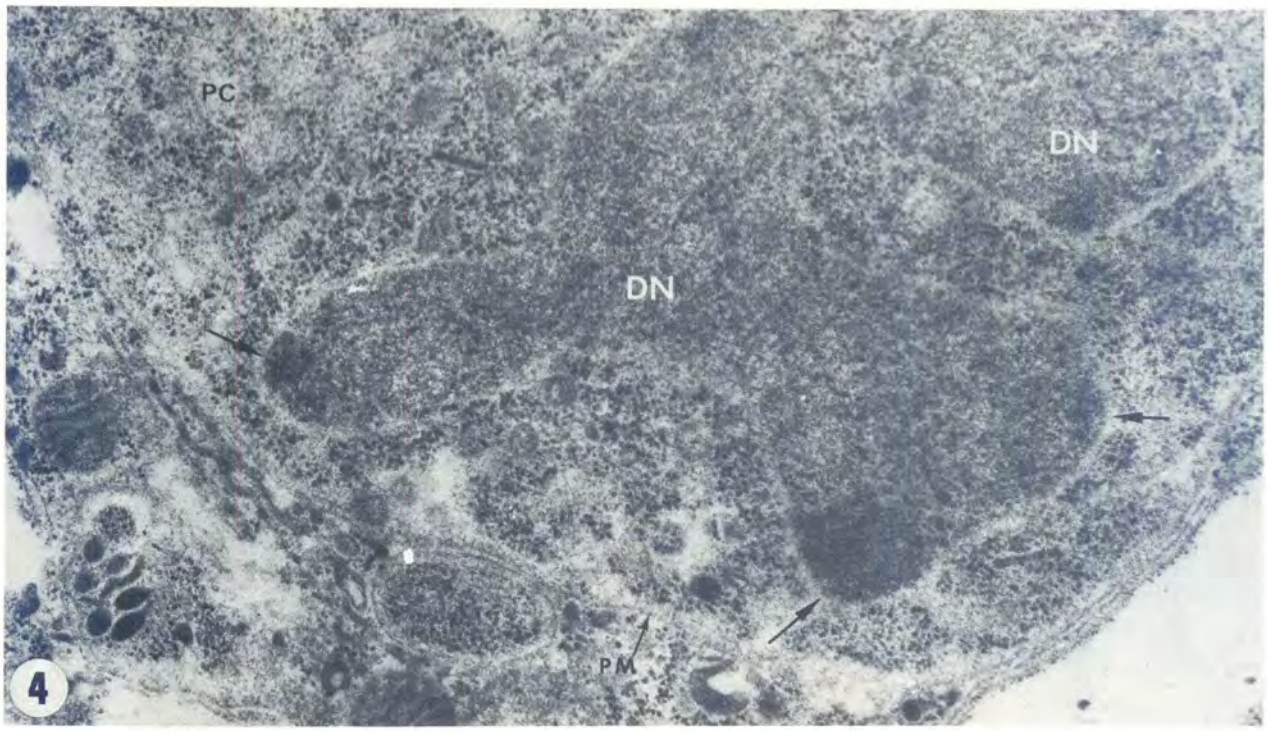
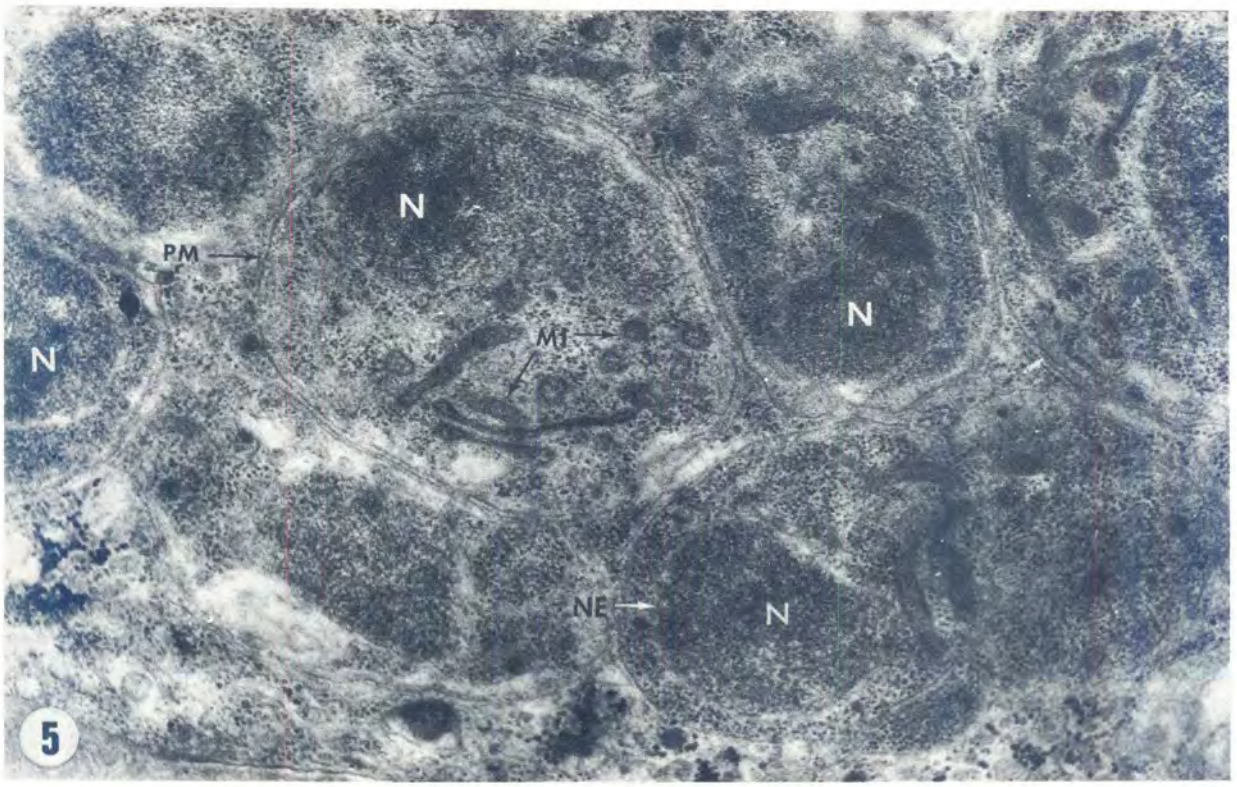
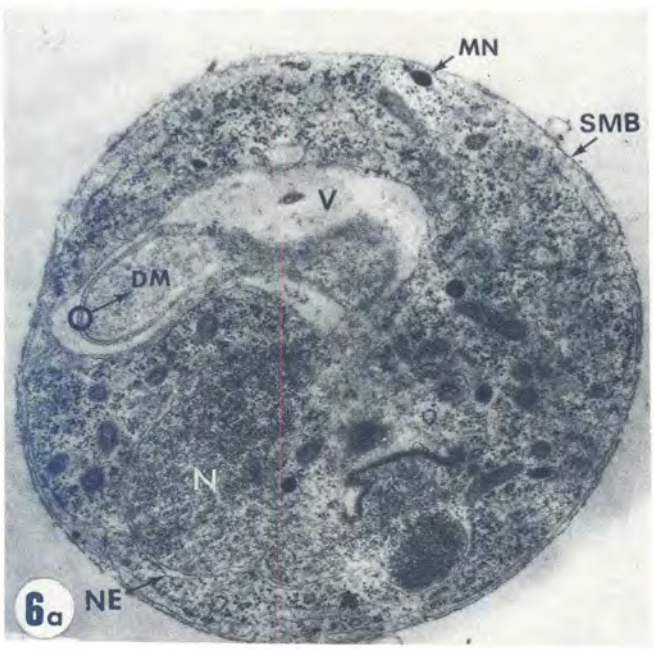


FIG. 3* Section through immature schizont. Note nuclear material spread through cytoplasm. × 25 000
FIG. 4* Section through schizont illustrating nuclear material at high magnification. × 35 000

* Bold arrows indicate intensely staining terminal ends of chromatin strands





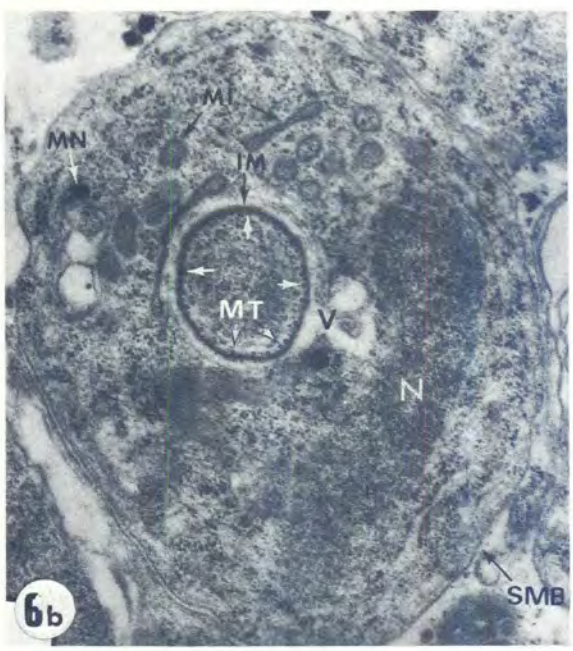


FIG. 5 First phase of cytoplasmic differentiation in schizont. Note membranes that surround individual daughter nuclei and cytoplasm. × 25 000

FIG. 6a & b Sections of immature LM in which more advanced cytoplasmic differentiation can be seen. × 23 500

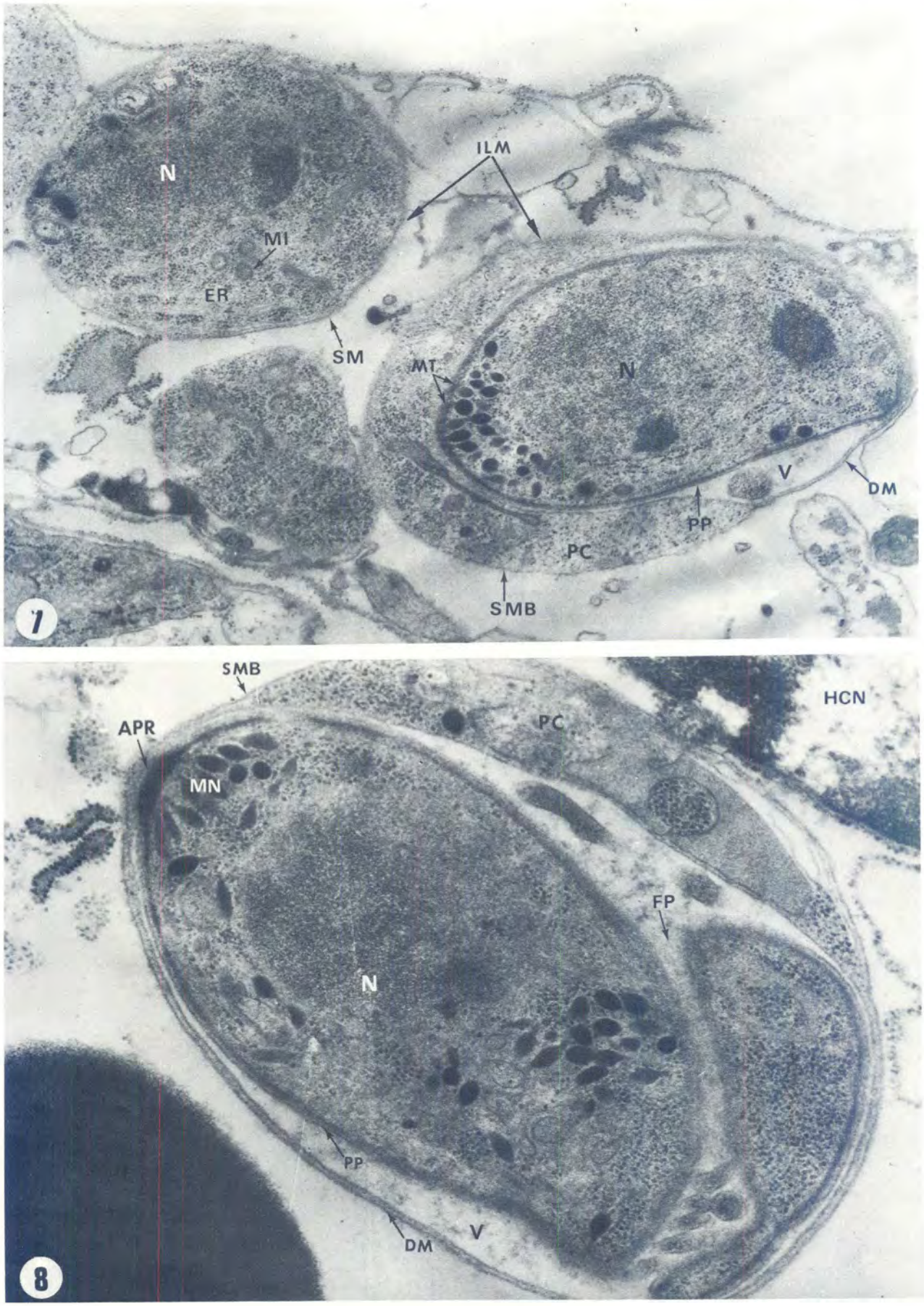
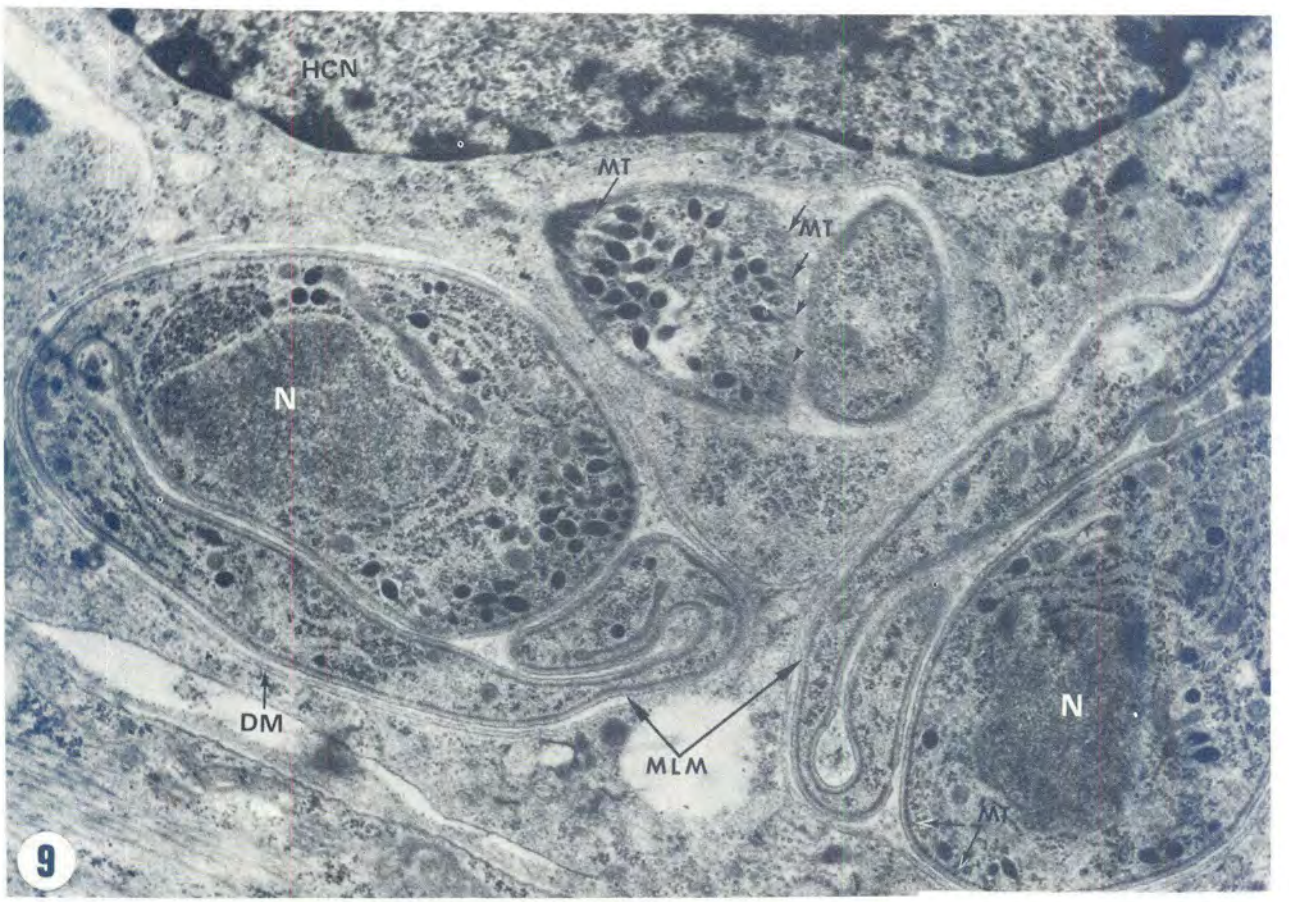


FIG. 7 Developing LM in nearly mature schizont. Note that LM on right has reached a more advanced stage of differentiation than the one on the left. × 25 000

FIG. 8 High magnification of nearly mature LM. × 46 000



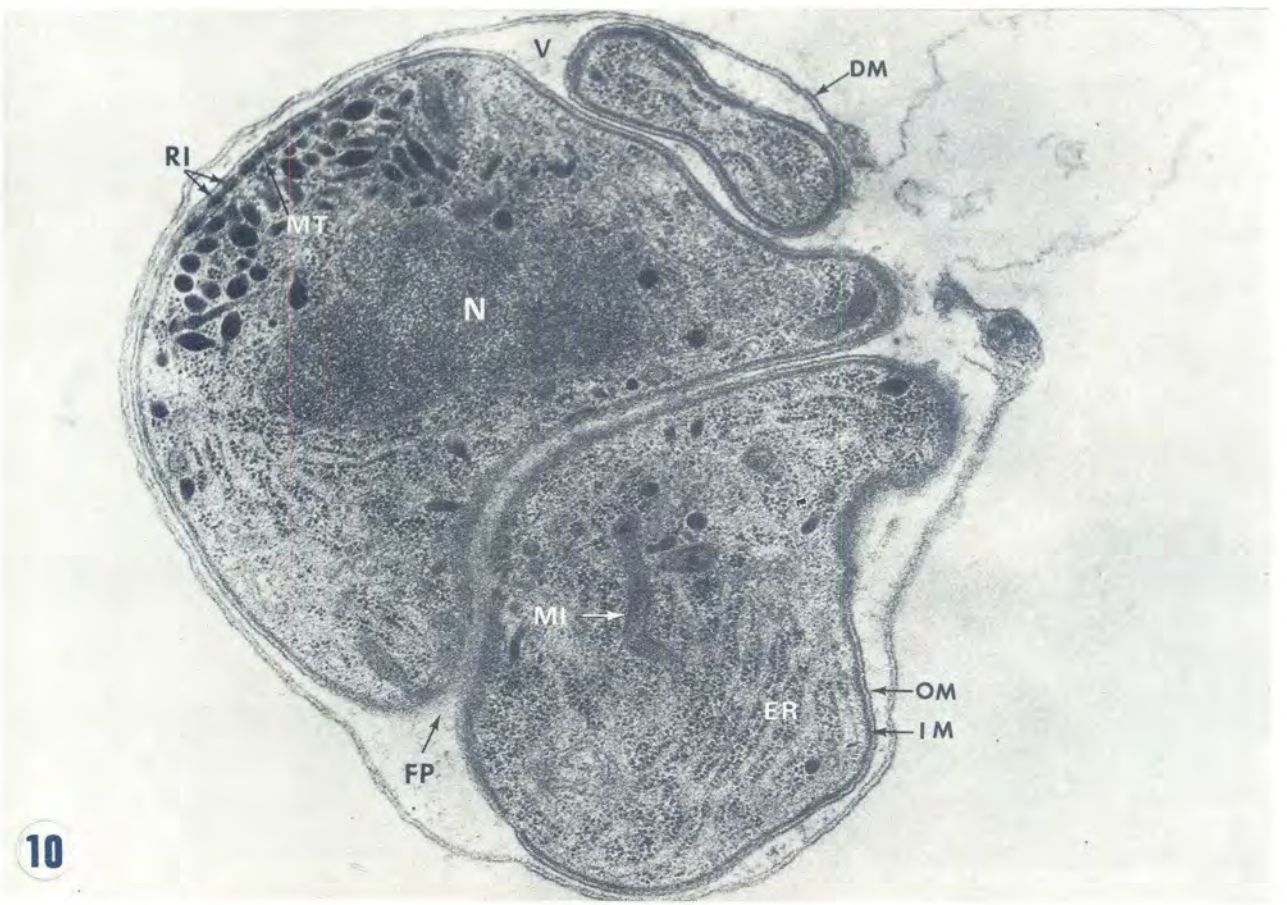


FIG. 9 Section through part of schizont illustrating mature folded LM in gut epithelial cell. × 32 000
FIG. 10 Mature extracellular LM prior to unfolding. × 30 000

FIG. 11–15 Illustrate various developmental stages of the parasite in different tissues

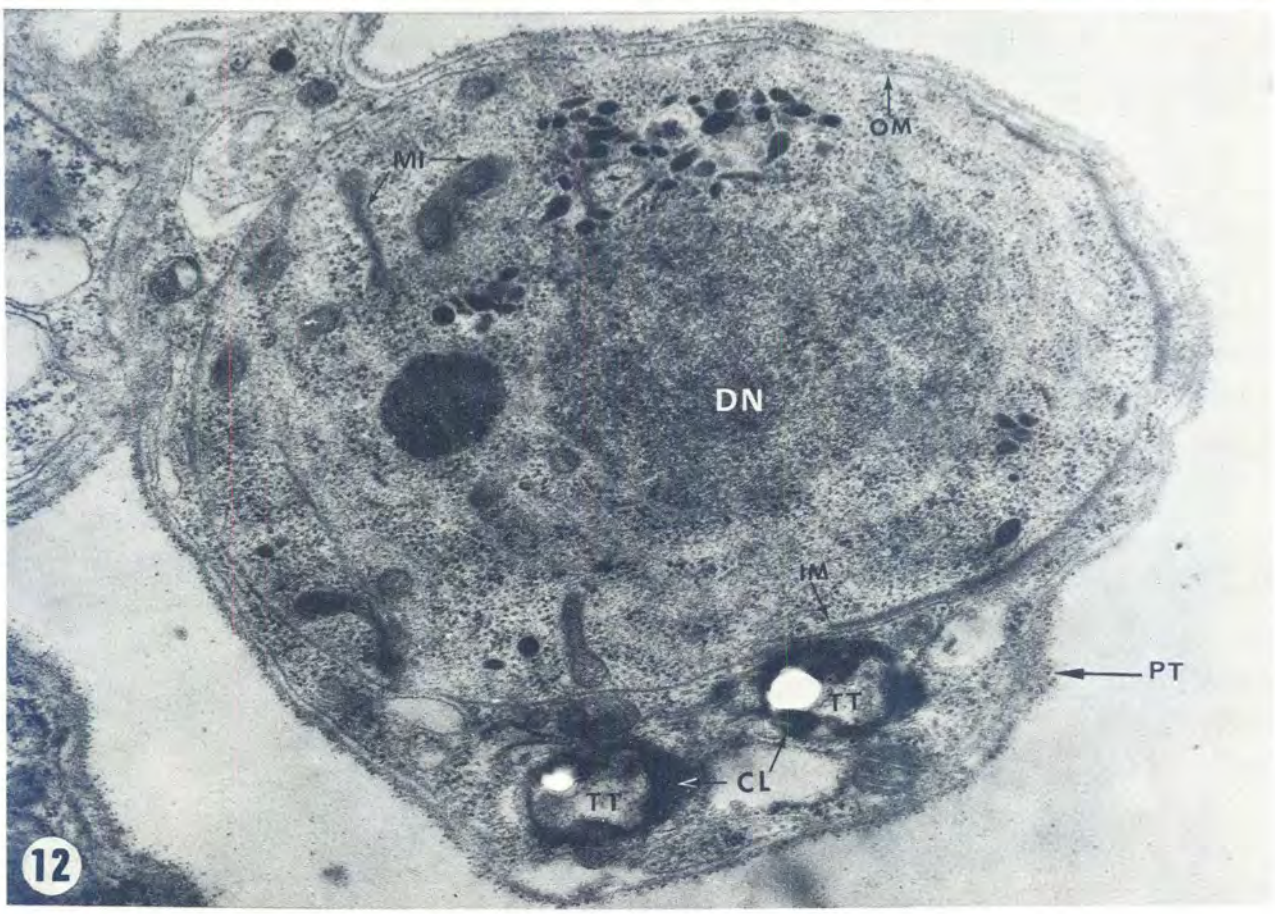
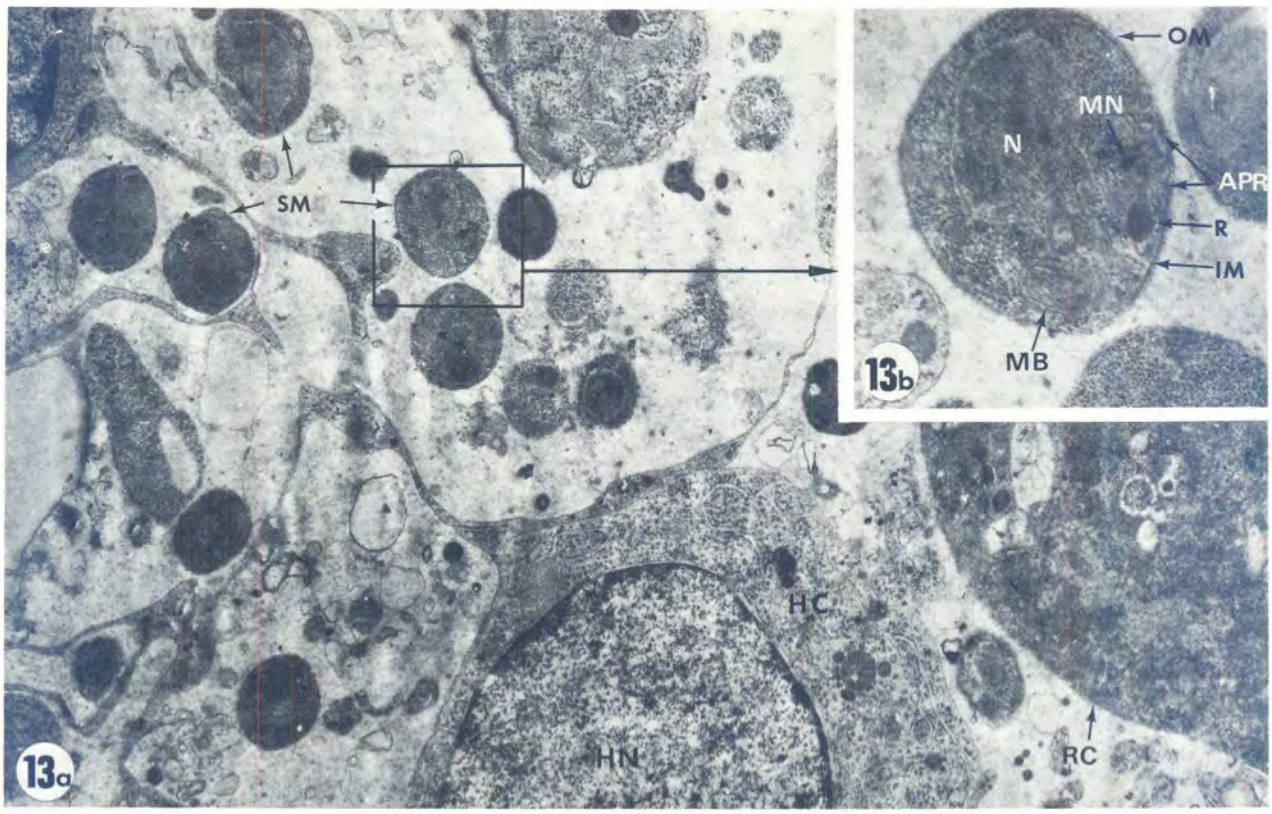


FIG. 11 Longitudinal section through LM in muscle tissue. × 27 000
FIG. 12 Spherical form (transformed LM) in a peritracheal cell. Note cuticular-lined tracheal tubes. × 30 000



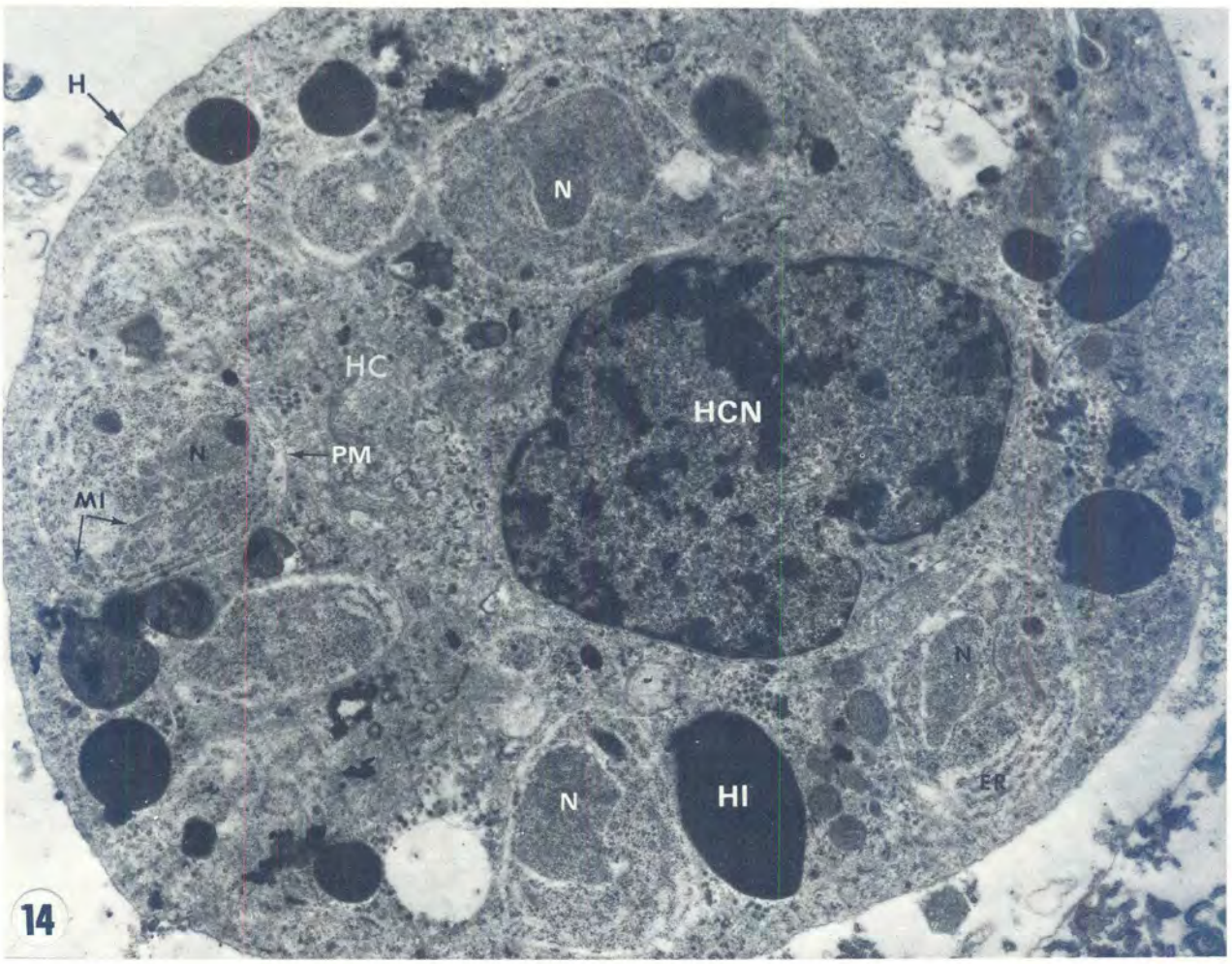
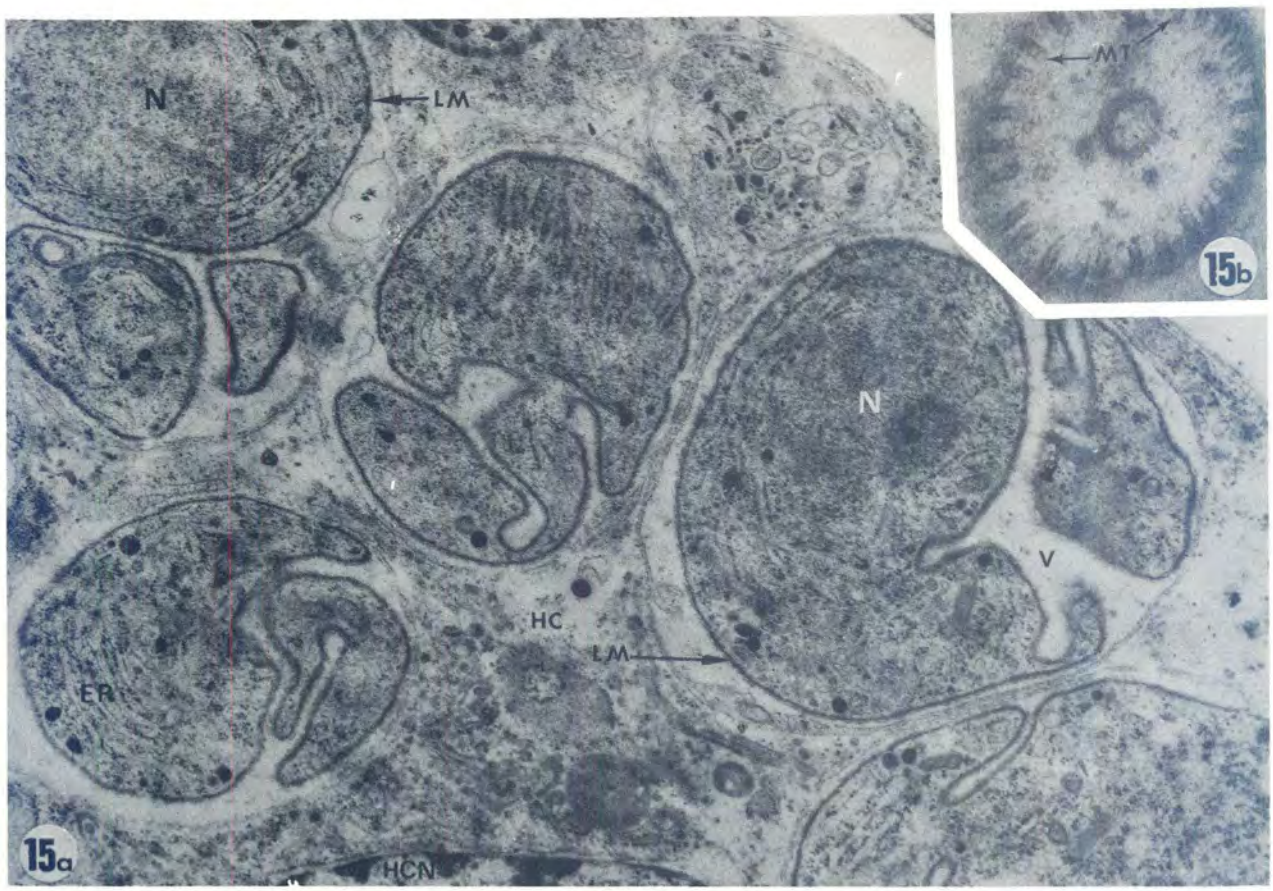
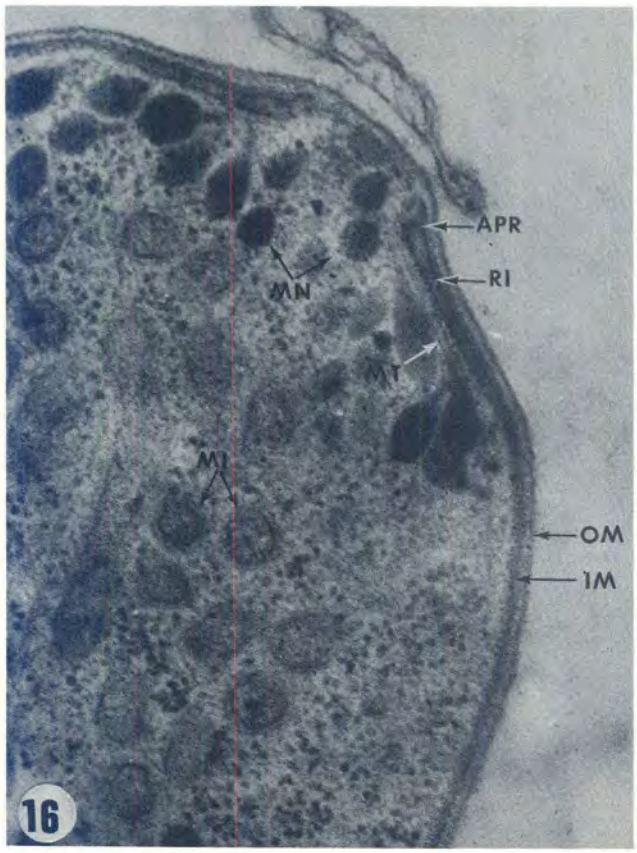


FIG. 13a SM in cortex of the synganglion. × 10 000
FIG. 13b Higher magnification of section through single SM seen in Fig. 34a. × 28 000
FIG. 14 Section through haemocyte showing immature LM. × 12 500





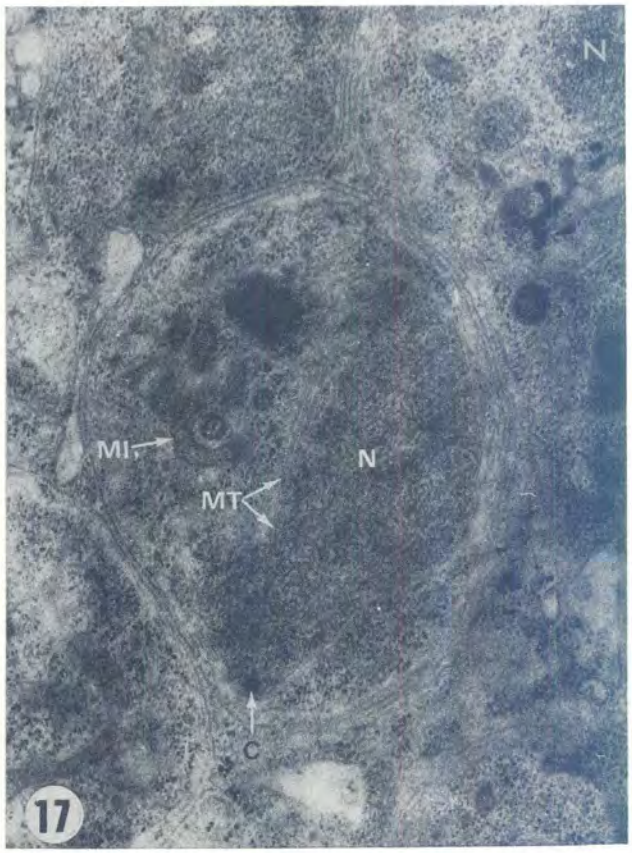


FIG. 15a Mature schizont in gut epithelial cell. × 20 000
FIG. 15b High magnification of section through anterior polar region of mature LM. Note arrangement and number of microtubules. × 80 000
FIG. 16 High magnification of longitudinal section through apical region of mature LM. × 60 000
FIG. 17 Section through schizont during phase of initial cytoplasmic differentiation. Note microtubules associated with the nuclear material and the presence of an organelle which might be a centriole. × 25 000

FIG. 18–23 Illustrate the terminal development of the parasite in the salivary glands of the tick (secondary schizogony)

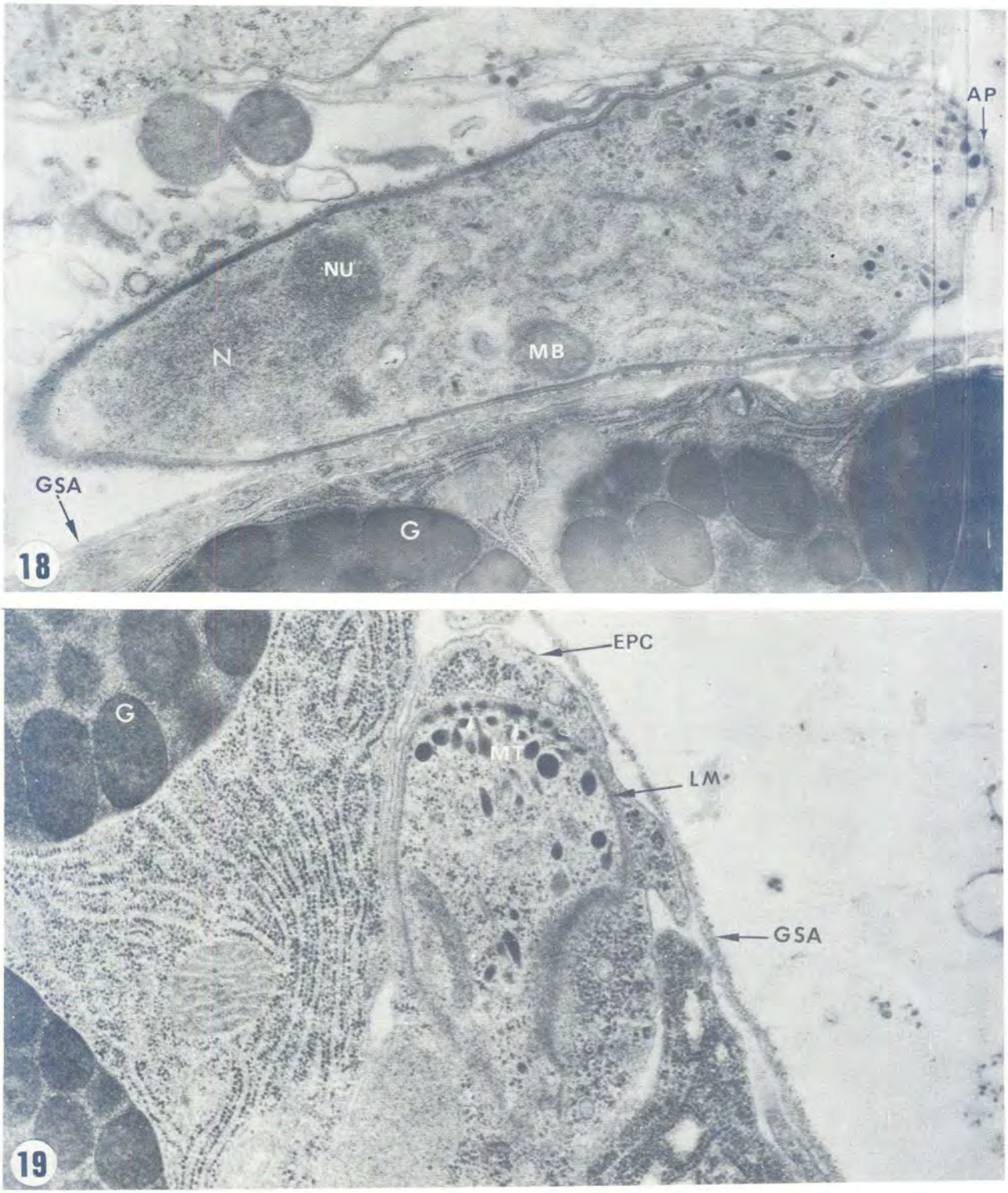
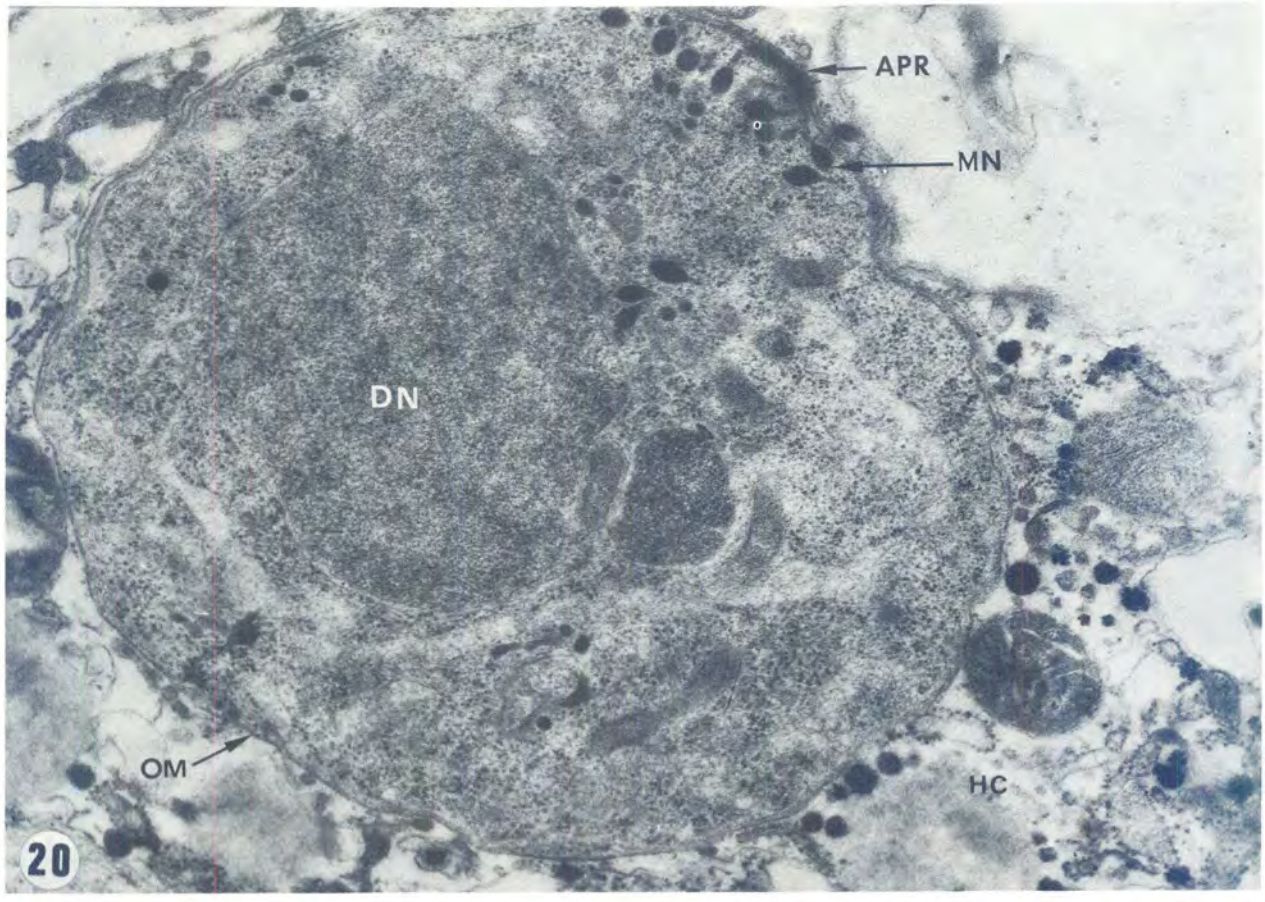


FIG. 18 LM in immediate vicinity of granular alveolus. × 21 500
FIG. 19 Oblique section through LM in epithelial cell of granular alveolus. × 30 000



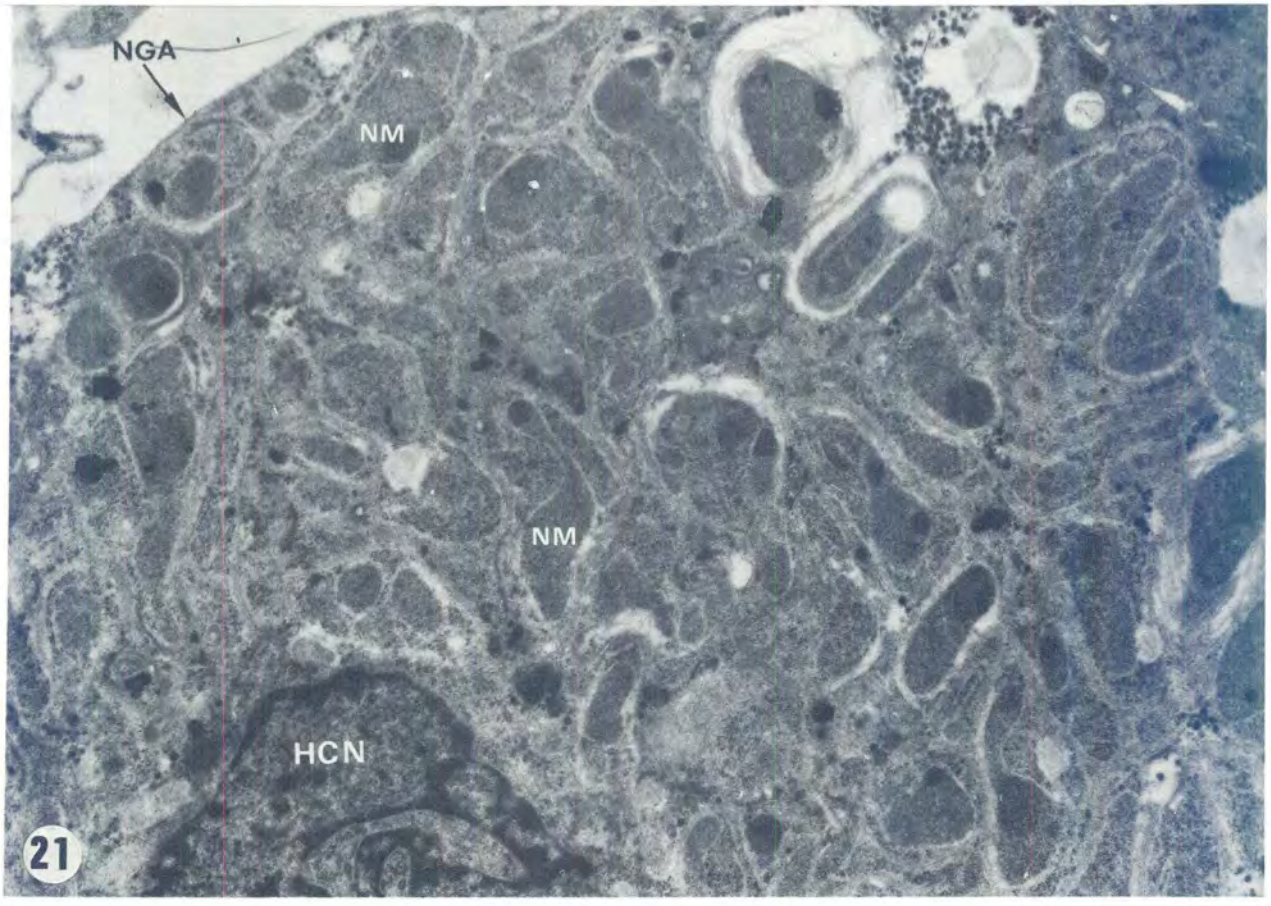


FIG. 20 Intracellular spherical form (transforming LM) with dividing nucleus in cell of non-granular alveolus. Note that anterior polar ring is still visible. × 30 000
FIG. 21 Large secondary schizont in cell of non-granular alveolus. × 12 000

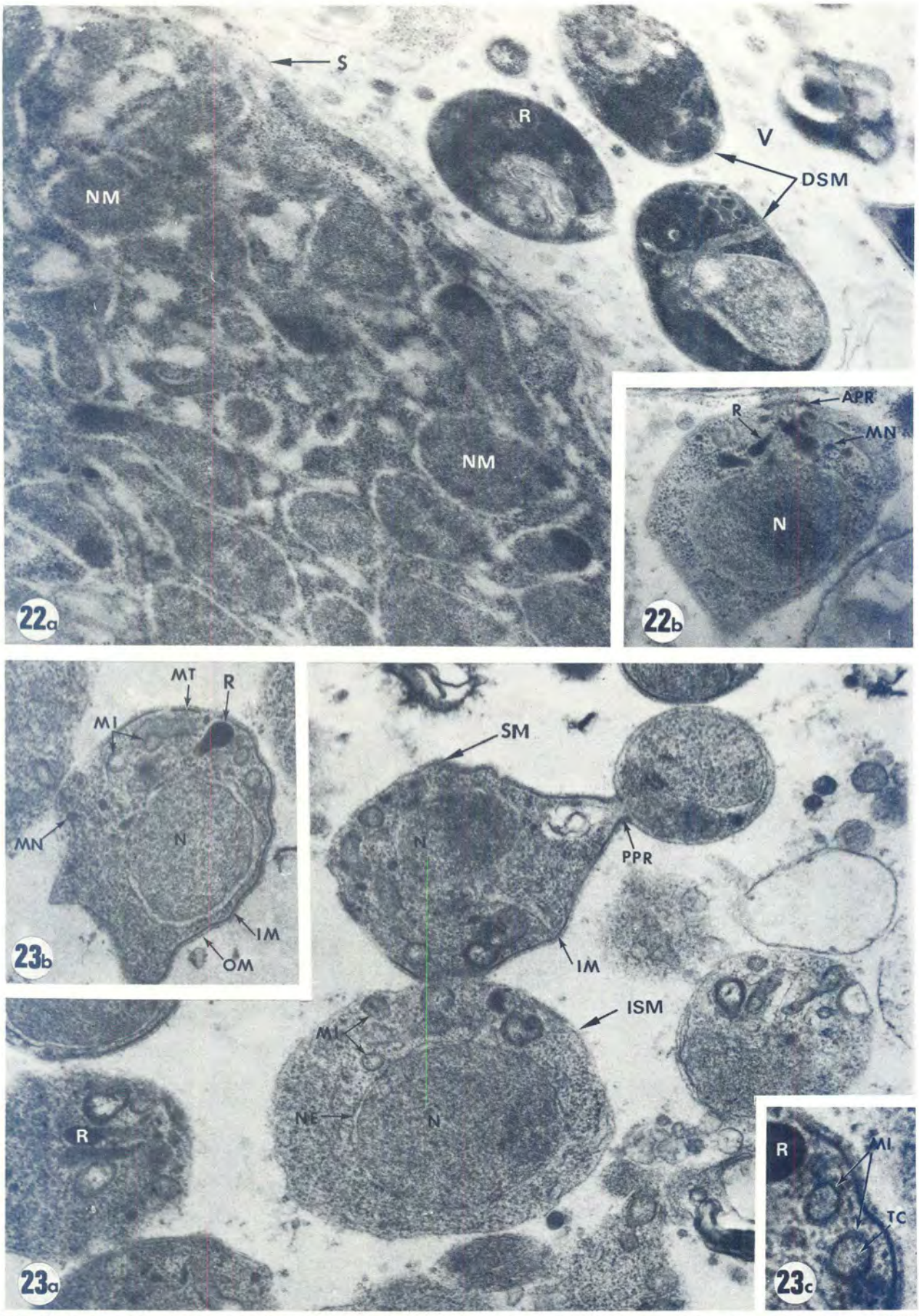


FIG. 22a Section through part of similar schizont seen in Fig. 21. Note degenerated SM from neighbouring spent schizont. × 25 000
FIG. 22b Longitudinal section through SM. × 25 000
FIG. 23a Differentiating SM as seen in section through secondary schizont. Note undifferentiated spherical form (immature SM). × 25 000
FIG. 23b Single SM showing appearance of thick inner membrane of pellicle and a rhoptry. × 25 000
FIG. 23c High magnification of cross section through mitochondria of SM seen in Fig. 44b, showing tubular cristae. × 60 000

FIG. 24–34 Photomicrographs of primary schizogony of *B. bigemina* in smears of *B. decoloratus* larvae and nymphae. × 1 250

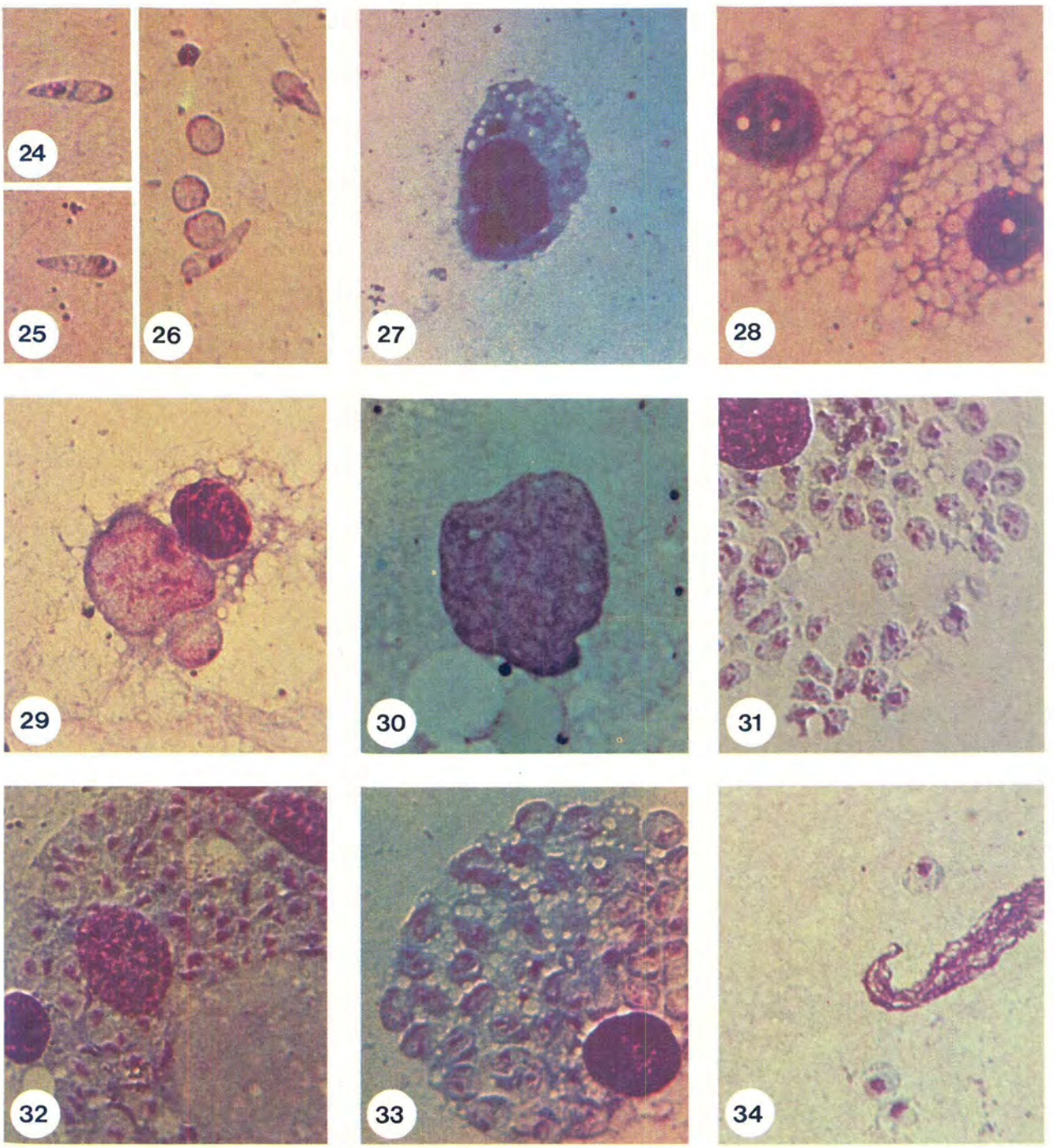


FIG. 24 LM with nucleus situated in the posterior half of body (L3)
FIG. 25 LM with anteriorly placed nucleus (L4)
FIG. 26 Two LM of unequal shape and size and 3 immature spherical forms, the latter released from a ruptured schizont (N7)
FIG. 27 Two LM which have entered a haemocyte (N9)
FIG. 28 Transformed LM developing into a schizont (L5)
FIG. 29 Two immature schizonts which differ considerably in size. Note uneven strands of chromatin material extending through the cytoplasm (N10)
FIG. 30 Large schizonts showing diffuse distribution of aggregations of chromatin material (N8)
FIG. 31 Ruptured schizont showing cytoplasmic aggregation around chromatin particles (N12)
FIG. 32 Schizont showing large individual daughter nuclei each surrounded by cytoplasm (N12)
FIG. 33 Schizont with nearly mature, spherically-shaped LM (N12)
FIG. 34 Folded LM outside schizont (N13)

FIG. 35–43 Photomicrographs illustrating secondary schizogony of *B. bigemina* in smears and sections of the salivary glands of *B. decoloratus* larvae and nymphae. × 1 250

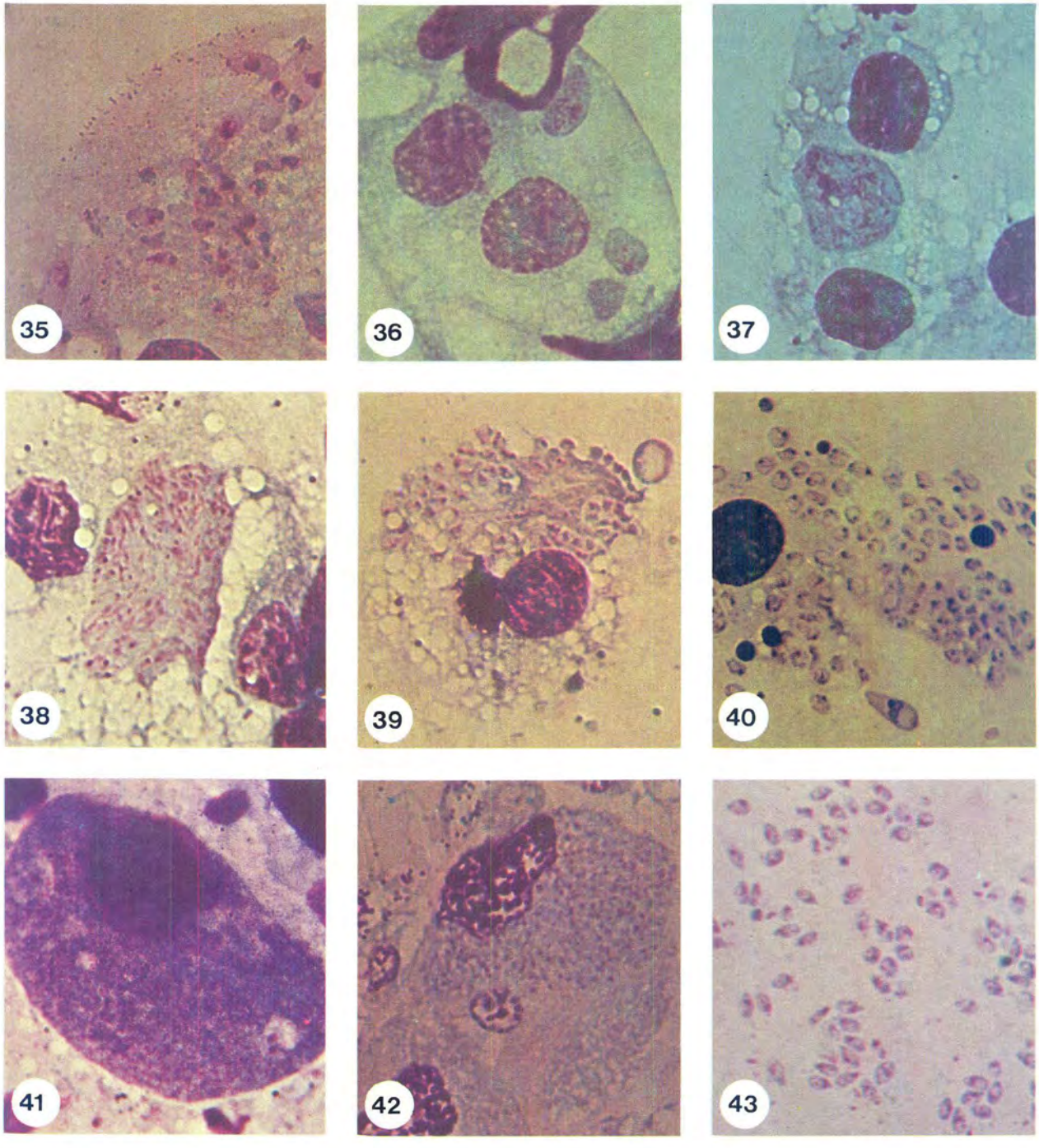


FIG. 35 Multiple infection of a non-granular alveolus with LM (N12)
FIG. 36 Three transforming LM developing into secondary schizonts in a non-granular alveolus (N9)
FIG. 37 Similar schizont as seen in Fig. 36, but increased in size (N13)
FIG. 38 Large schizont prior to onset of cytoplasmic differentiation (N14)
FIG. 39 Secondary schizont showing SM being formed (N13)
FIG. 40 Ruptured immature secondary schizont. Note spherical shape of majority of immature small merozoites. A LM can be seen in the same field (N12)
FIG. 41 Intact cell of a non-granular alveolus packed with SM contained in a secondary schizont (N14)
FIG. 42 Section through a granular alveolus showing secondary schizont in one cell (N14)
FIG. 43 Mature piriform SM released from secondary schizont. Note small red-staining dot in cytoplasm of parasite (N13)

Not much information could be obtained about the actual process of karyokinesis. In preparations examined with the light microscope a network of nuclear strands or projections were seen extending through the cytoplasm of young developing schizonts (Fig. 29 & 30). These projections of nuclear material were also studied with the aid of an electron microscope and found to stain very intensely at their terminal ends (Fig. 4). In cross-section these osmiophilic structures appeared as roughly spherical bodies (Fig. 3). A single observation was made of nuclear division in an immature schizont involving microtubules and the presence of organelles which might represent a centriole (Fig. 17).

These schizonts continued to increase in size and eventually differentiation started in the cytoplasm with the appearance of membranes around portions of cytoplasm containing chromatin units (Fig. 5). Fig. 31 depicts a schizont, which is probably in the same or a slightly more advanced, stage of development than that illustrated in Fig. 5, as it appears under the light microscope. Subsequently, relatively large daughter nuclei could be identified (Fig. 32 & 33), which were characteristic of LM-formation (primary schizogony). In smear preparations the immature, mostly spherically-shaped, immature LM were commonly encountered extracellularly, probably on account of schizonts rupturing during the preparation of the smears (Fig. 26).

Nuclei, mitochondria, endoplasmic reticulum and a few isolated electron dense bodies, presumably micronemes, appeared in the immature spherical LM (Fig. 6a & 6b). At this stage these merozoites were still bound by a single limiting membrane. The development of the complete parasite pellicle took place during the final phase of differentiation.

The transformation from the immature, spherically-shaped merozoite to a mature vermicule-shaped merozoite (=LM) is an elaborate process and as yet not fully understood. It started with the formation of the pellicular complex, which consisted of the thick osmiophilic inner membrane with the associated microtubules within the cytoplasm of the spherical form (Fig. 6b). This process took place in an intracytoplasmic vacuole. The osmiophilic membrane and vacuole continued to increase in size as the cytoplasm of the original merozoite was progressively incorporated into the maturing LM. At this stage the latter was partly surrounded by the pellicle consisting of an outer membrane and a thick inner membrane (Fig. 7 & 8). The rest of its cytoplasm was still located outside the body of the merozoite and was bound by a single membrane (Fig. 7 & 8). Eventually this cytoplasm became totally incorporated into the developing merozoite and the mature folded merozoite was left in a vacuole surrounded by a double membrane (Fig. 9 & 10). The latter membrane was therefore of parasitic origin.

The limiting membrane of the schizont apparently disappeared and played no part in the development of the LM. The final development of the LM therefore appeared to take place in direct association with the host cell cytoplasm (Fig. 9 & 15a).

Damage to host cells was extensive in most cases (Fig. 7 & 8). In many instances the LM were observed to develop close together, occupying the whole cell in the process and leaving very little recognizable host cell cytoplasm (Fig. 9 & 15a). Some of the folded LM were also encountered extracellularly in both light (Fig. 34) and electron microscope preparations (Fig. 10). It therefore seems possible for mature LM to be released from the host cell in the folded form and to unfold thereafter to appear as the typical vermicule-shaped LM (Fig. 18, 24 & 25).

The production of LM by schizogony, as described above, appeared to have a cyclic nature which was continuous throughout the life cycle of the tick. After the production of the first LM in the replete female tick (Potgieter, 1977), primary schizogony is carried on basically as a multiplication process of LM. It is not known, however, how many cycles occur before the onset of secondary schizogony (see below).

Morphological characteristics of large merozoites

Light microscope studies: The LM encountered in smears varied in size, shape, position of nucleus, the presence of a red staining "cap" and vacuoles in the cytoplasm. A fair degree of pleomorphism existed (Fig. 24–26) and it was not possible to distinguish between LM from ticks in different stages of development.

In smear preparations the size of the LM ranged from 8,2 μm–11,9 μm in length and 2,9 μm–3,9 μm in width with a mean value of 10,7 μm$\times$3,2 μm. They had a typical club shape with the shorter ones tapering less posteriorly and therefore appearing more thick-set than the longer ones (Fig. 26). The nuclei were seen in various positions but mostly in the middle third of the body. The nuclei stained dark-red and were normally spherical in shape. In some cases the nuclei appeared as irregular bands of red-staining material extending across the width of the parasite (Fig. 26). Extra-nuclear material which also stained red with Giemsa's stain was observed as a red staining "cap" in the anterior polar area of some LM (Fig. 24). This was not seen in all LM and it may be related to the age of the LM. The same applied to vacuoles which were present in the cytoplasm of some presumably older, or perhaps degenerating, LM (Fig. 24).

Electron microscope studies: Mature LM were surrounded by a pellicle composed of an outer plasma membrane and an underlying, thick, osmiophilic inner membrane (Fig. 2 & 16). In the vicinity of the anterior pole of the merozoite the latter appeared to form osmiophilic "ribs" which converged to form the anterior polar ring (Fig. 10 & 16). Microtubules were found directly underneath the "ribs", as seen in cross-section (Fig. 10 & 19). These microtubules converged towards the anterior polar ring and were apparently involved in its formation (Fig. 15b). In Fig. 15b, 28 of these microtubules could be identified. The "opening" of the anterior polar ring was covered by the outer membrane of the pellicle (Fig. 16). The slight protuberance at the tip of the anterior pole was probably formed by the robust polar ring lying underneath the outer membrane (Fig. 16). The majority of the micronemes were concentrated at the anterior pole and in some sections micronemes were observed to be directed towards interruptions (micropores) in the inner membrane of the pellicle (Fig. 11). No rhoptries were observed in LM. A single nucleus and a rough type of endoplasmic reticulum were present (Fig. 1 & 10). Other organelles in the cytoplasm include membrane-bound, cylindrical, dumbbell and spherical structures, all of which were considered to be mitochondria sectioned in different planes (Fig. 10 & 16). Unidentified organelles consisting of folded membranes lying parallel to each other were occasionally seen (Fig. 1).

No indication could be obtained from the fine structural features of the parasites that different types of LM existed in the nymphal ticks.

Site of development of primary schizonts

The exact identity of the tick tissues involved in primary schizogony could only be established with a reasonable degree of certainty from the examination of sections. Electron microscope preparations were superior to ordinary histological sections in this respect and were therefore preferred. Owing to the extensive damage done by the parasite, accurate identification of the cells involved was, however, impossible in some cases.

The highest incidence of primary schizonts in nymphae was observed in the epithelial cells of the gut (Fig. 9 & 15a) and in haemocytes (Fig. 14). Infection of muscle cells (Fig. 11) and peritracheal cells (Fig. 12) by LM was also common. Whether the LM underwent successful schizogony in muscle tissue remains unknown. Multiple infections of cells by LM were commonly observed (Fig. 27 & 35), especially in heavily infected ticks.

Production of small merozoites (secondary schizogony)

The development of the SM of *B. bigemina* took place mainly in the salivary glands. Not only were unfolded LM seen in the vicinity of the salivary gland alveoli (Fig. 18), but they were also found in alveoli (Fig. 19 & 35) where they transformed to spherical schizonts (Fig. 20 & 36) similar to those seen in other tissues (Fig. 2 & 12). In the initial stages of development the secondary schizonts seen in the salivary gland cells were indistinguishable from the young primary schizonts described above. (Compare Fig. 4, 21, 22, 27–30 & 36–38.) The early stages of both primary and secondary schizonts could therefore only be identified on the basis of their location, a useful if not very sound criterion. Only at the stage of karyogenesis, when smaller and more numerous daughter nuclei were produced in the secondary schizonts (Fig. 39–42) than in the primary schizonts (Fig. 31–33), could they be differentiated from each other.

Eventually, extremely large schizonts developed which occupied the entire host cell (Fig. 21 & 41). In sections prepared for examination with the light microscope these schizonts measured up to 45 μm in diameter (Fig. 42).

No fine structural differences were observed between the immature spherical forms seen in the secondary schizonts (Fig. 23a) and those in the primary schizonts (Fig. 5 & 7).

At the completion of karyokinesis, membranes developed in the cytoplasm which enclosed individual daughter nuclei together with some cytoplasm, resulting in the formation of spherical immature SM (Fig. 39 & 40). These immature forms showed little evidence of differentiation of their cytoplasm, only membrane-bound organelles resembling mitochondria being discernible (Fig. 23a).

Subsequently, the typical thick inner membrane of the merozoite pellicle appeared (Fig. 23a & 23b). Rhoptries and micronemes were observed concurrently and the parasite developed into a pear-shaped SM (Fig. 22b & 43).

These SM were assumed to be the terminal stage in the development of *B. bigemina* in the tick since they apparently did not undergo further development. Numerous degenerated, unreleased SM were also observed (Fig. 22a).

Morphological characteristics of small merozoites

Mature SM have a typical pear shape. In smear preparations the shape and size of these organisms could, however, only be determined upon their release from ruptured schizonts (Fig. 40 & 43) when it was found that they measured 2,9 by 1,8 μm. The dark red-staining nucleus was mostly situated near the tapered end of the body. A 2nd unidentified small body, which stained less intensely than the nucleus, occurred in various positions in relation to the nucleus (Fig. 43).

In the fine structural study, the development of the pellicle, characterized especially by the appearance of the thick osmiophilic inner membrane (Fig. 23a & 23b), indicated a final phase of maturation. Anterior and posterior polar rings were identified as well as rhoptries (Fig. 22b, 23a & 23b), microtubules and mitochondria with tubular cristae (Fig. 23c). As in the case of the LM, unidentified multimembraned structures were observed (Fig. 13b).

Spent schizonts with a few scattered remaining SM were found in the salivary glands (Fig. 22a). The parasites were rounded off, stained very intensely and were probably degenerating, as was also the case with the SM observed in the cortex of the synganglion (brain) of the tick (Fig. 13a & 13b).

Site of development of secondary schizonts

This stage was most commonly found in the salivary glands. The precursor LM were encountered in the epithelial cells of the granular alveoli (Fig. 19), as well as in the non-granular alveoli of the salivary gland (Fig. 35). Secondary schizonts developed in these cells and eventually occupied large portions of the alveoli (Fig. 21, 41 & 42). The development of SM was not entirely limited to the salivary glands but SM were also observed in the cortex of the synganglion. In Fig. 13a, SM, and what appears to be residual cytoplasm of a schizont, can be seen between the neurons in areas showing extensive damage to host tissue.

Discussion

Transmission aspects

As mentioned above, *B. bigemina* is transmitted by both *B. decoloratus* and *B. microplus* in the R.S.A. and these and other studies (Potgieter, 1977) indicate that the life cycle of this parasite is identical in both tick species. Although extensive transmission experiments carried out in this laboratory have revealed that both the nymphal and adult stages of these ticks can transmit the parasite to cattle (Potgieter, 1977), [and this agrees with the observations recorded by Callow & Hoyte (1961) for *B. microplus* in Australia], this investigation has shown that the nymphal stages of *B. decoloratus* are mainly responsible.

The position with regard to transmission by means of larval ticks was also studied. Neitz (1956) indicated in a review article that larvae of *Boophilus* spp. were able to transmit *B. bigemina*. However, Callow & Hoyte (1961) reported that attempts to transmit *B. bigemina* with *B. microplus* larvae in Australia were unsuccessful. Local attempts to transmit *B. bigemina* with infected *B. decoloratus* larvae also failed (Potgieter, 1977). Not only were the small spherically and irregularly shaped parasites observed in smears of L6 and L7 in this investigation apparently immature, but it is quite possible that these particular smears were made from pharate nymphae, which were not actively feeding at the time, rather than from larvae.

This can be inferred from the observation made by Arthur & Londt (1973) that the feeding period of *B. decoloratus* larvae is limited to the first 5 days after attachment, and would offer an acceptable explanation for the failure of the instar concerned to transmit the infection even if the SM were infective at that stage.

In this experiment, the prepatent period of the *B. bigemina* infection was 17 days after larval infestation and 8 days after intravenous inoculation of the tick stabilates prepared from N14 & N15. If it is assumed that the magnitude of the infective inocula received by these animals was very similar, it can be calculated that emission of the infective forms probably starts at the N9 stage when they are still relatively difficult to demonstrate in smears.

Developmental aspects

The only developmental stages of *B. bigemina* observed by Riek (1964) in larvae of *B. microplus* were fission bodies (= primary schizonts) that occurred in the gut epithelial cells, and vermicules (= LM). The smears of infected larvae examined in this investigation revealed marked activity. In addition to primary schizonts, immature and mature LM, which occurred mainly in the gut, secondary schizonts and immature SM in the salivary glands were also seen.

Riek (1964) believed that development in the salivary glands took place only after the moult from larva to nymph occurred, but he gave no indication whether the vermicules which are carried over from the larvae initiated any other form of parasitic development in the nymphae. Particular attention was paid to this aspect in this study. It was found that cycles of primary schizogony continued to occur in various tissues throughout the nymphal stage, concurrent with, and after the production of the first SM in the salivary glands, a process which was repeated in the adult stage. This would furnish an alternative interpretation to that submitted by Riek (1964) who believed that the "infection" seen in the salivary glands of engorged and semi-engorged females had been carried over as such from the nymphae or resulted from an "infection" of an earlier instar. The fact that LM, SM, as well as schizonts were found in newly-moulted and engorging females, indicates that infection is carried over from the nymphae. This explains why the transmission of *B. bigemina* infections by adult ticks could be demonstrated with relative ease by Callow & Hoyte (1961) and Potgieter (1977). Whether the immature secondary schizonts resulted from a new cycle in the adult tick could not be determined.

Transstadial transfer of a developmental stage like the LM, which is capable of initiating further schizogony, offers an explanation for the transovarian transmission of *B. bigemina* by *B. decoloratus* (Theiler, 1909a) and *B. microplus* (Callow, 1965) which have fed on non-bovine hosts. As early as 1937 Brumpt (cited by Callow, 1965) proposed that piroplasms can be transmitted hereditarily from one generation to the next without passing through the vertebrate host. Callow (1965) accepted this postulation but in addition suggested that ticks could possibly become reinfected from such a host.

The fact that advanced developmental stages of the parasite can occur in unfed and semi-engorged female ticks explains why it is essential to obtain a non-infected strain in order to study the development of *B. bigemina* in this one-host tick.

At the ultrastructural level, the development of mature vermicule-shaped LM from precursor spherical forms emerged as an elaborate process, which resembles the transformation of the so-called spherical or stationary forms of *B. ovis* into motile ones (= vermicules) (Friedhoff & Scholtyseck, 1968a; Scholtyseck & Mehlhorn, 1970). Although more information on the transformation process was obtained in this study, the actual way in which the pellicle was formed and the cytoplasm gradually incorporated to leave the mature LM in a vacuole surrounded by a double membrane could not be clearly established.

Friedhoff & Scholtyseck (1969) found that the folded merozoites (= LM) of *B. bigemina* in the tick ovary were surrounded by more than 1 membrane which seemed to be continuous with the endoplasmic reticulum of the host cell. In the case of *B. ovis*, Friedhoff & Scholtyseck (1968b) observed that the outer membrane of the double membrane surrounding the folded merozoite was derived from the host cell cytoplasm, whereas the origin of the second membrane was uncertain. Mature folded LM of *B. bovis* were found in parasitophorous vacuoles lined by membranes thought to have originated from the host cell (Potgieter, *et al.*, 1976). In this study it was demonstrated, however, that the double membrane which surrounded the mature folded merozoites of *B. bigemina* was of parasitic origin.

Riek (1964) described the release of club-shaped merozoites (= LM) from ruptured fission bodies. In this investigation, however, LM were evidently also released in the folded form to unfold outside the schizont. It is clear that mature LM that reached the haemocoele from the gut had the potential to infect a wide variety of tissues to which free access was afforded via the haemolymph, including haemocytes, Malpighian tubes, muscles, peritracheal cells, the synganglion, salivary glands and the ovaries in adult female ticks.

The only difference between these observations on the morphology of LM and those recorded by Riek (1964) is that binucleate forms were not encountered in this study. The fine structure of the LM of *B. bigemina* closely resembles that reported by Friedhoff & Scholtyseck (1969) for the merozoites of this parasite in the ovaries of *B. microplus* and *B. decoloratus*, except that they did not observe the micropores in the inner membrane of the pellicle. Micropores were also observed in merozoites of *B. bovis* (Potgieter *et al.*, 1976). Whereas Friedhoff & Scholtyseck (1969) recorded the presence of 32 microtubules at the anterior pole of *B. bigemina*, only 28 could be identified with certainty in this study.

It is interesting to note that the ultrastructure of merozoites of *Babesia major* in the gut and ovaries of *Haemaphysalis punctata* (Morzaria, Bland & Brocklesby, 1976) was very similar to that of the LM of *B. bigemina*. Novel distinguishing features were: the occasional presence of a "primitive conoid", marked striations across the outer and inner membranes of the pellicle and the presence of rhoptries. The maximum number of microtubules counted was 27.

Riek (1964) was able to distinguish between the development of *B. bigemina* in the salivary glands and in the other cells of the tick. He recorded that in the salivary glands the chromatin material remained in a more central position after the vermicules had rounded off and enlarged. In this investigation both peripherally and centrally placed chromatin were seen in this form of the parasite and this feature could therefore not be

used to distinguish between primary and secondary schizonts. The first noticeable difference between them was the greater number and smaller size of the daughter nuclei in secondary schizonts.

According to Riek (1964), the development in the salivary glands only proceeds after the moult from larva to nymph has occurred, whereas immature SM were detected in L6 and L7 in this investigation. Riek (1964) concluded that the infection was transmitted in the nymphal stage 8–10 days after larval infestation. His description of the infective form of the parasite resembles the SM seen in this study. Although developmental stages other than SM were present in the ticks at the time the infective stabilate was prepared, it is believed that the piriform SM are the infective forms of the parasite. The fact that they are formed in the cells of the salivary glands, which provide them with a passage-way out of the tick, suggests that they are the terminal developmental stage in the tick. In addition to this, their morphology closely resembles that of the intra-erythrocytic merozoites, an observation which also applies to *B. argentina* (Riek, 1966; Mahoney & Mirre, 1971). *B. ovis* (Weber & Friedhoff, 1971; Friedhoff, Scholtyseck & Weber, 1972) and *B. bovis* (Potgieter & Els, 1976, 1977).

In the electron microscope examination it was seen that the formation of the pellicle and the appearance of rhoptries and micronemes heralded the final development phase of the SM. Apparently the pellicle supports the typical piriform shape of the mature SM. Such piriform SM were first identified in smears prepared from N9, which coincided with the time of transmission recorded by Riek (1964). It is believed, however, that SM may reach maturity in even earlier nymphal stages.

The fine structure of the SM resembled that of *B. ovis* in *R. bursa* (Friedhoff *et al.*, 1972) and of *B. bovis* in *B. microplus* (Potgieter & Els, 1976). The spherical bodies observed in SM of *B. ovis* and *B. bovis* could not be demonstrated in the SM of *B. bigemina* in this study, but it is possible that the red-staining dot seen in mature SM with the light microscope represents this organelle. Since spherical bodies have been seen in the intra-erythrocytic merozoites of *B. bigemina* with the electron microscope by Friedhoff (1970; 1974) and Potgieter & Els (1977), failure to demonstrate their presence in SM is probably because very few SM could be found in the sections studied.

List of Abbreviations Used in all Micrographs

AP	Anterior pole
APR	Anterior polar ring
C	Centriole (?)
CL	Cuticular lining
DM	Double membrane
DN	Dividing nucleus
DSM	Degenerated small merozoites
EPC	Epithelial cell
ER	Endoplasmic reticulum
FP	Fold in pellicle
G	Secretory granule
GSA	Granular-secreting alveolus (granular alveolus)
H	Haemocyte
HC	Host cell cytoplasm
HCM	Host cell membrane
HCN	Host cell nucleus
HI	Haemocyte inclusion
ILM	Immature large merozoite
IM	Inner membrane
ISM	Immature small merozoite
LM	Large merozoite
M	Muscle cell
MB	Multimembraned body
MI	Mitochondrion
MLM	Mature large merozoite
MN	Micronemes
MP	Micropore (in inner membrane)
MT	Microtubule
N	Nucleus
NE	Nuclear envelope
NGA	Non-granular alveolus
NM	Nuclear material
NU	Nucleolus
OM	Outer membrane
PC	Parasite cytoplasm
PM	Parasite membrane
PP	Parasite pellicle
PPR	Posterior polar ring
PT	Peritracheal cell
R	Rhoptries
RC	Residual cytoplasm of parasite origin
RI	Ribs
S	Schizont
SM	Small merozoite
SMB	Single membrane
TC	Tubular cristae
TT	Tracheal tube
V	Vacuole

References

ARTHUR, D. R. & LONDT, J. G. H., 1973. The parasitic cycle of *Boophilus decoloratus* (Koch, 1844) (Acarina: Ixodidae). *Journal of the Entomological Society of Southern Africa*, 36, 87–116.

CALLOW, L. L., 1965. *Babesia bigemina* in ticks grown on non-bovine hosts and its transmission to these hosts. *Parasitology*, 55, 375–381.

CALLOW, L. L. & HOYTE, H. M. D., 1961. Transmission experiments using *Babesia bigemina, Theileria mutans, Borrelia* sp., and the cattle tick *Boophilus microplus*. *Australian Veterinary Journal*, 37, 381–390.

FRIEDHOFF, K., 1970. Studies on the fine structure of *Babesia bigemina, B. divergens* and *B. ovis*. Proceedings, 2nd International Congress of Parasitology. *Journal of Parasitology*, 56 (4). Section 2, Part 1, 110–111.

FRIEDHOFF, K. T., 1974. The ultrastructure of the Piroplasmea. Proceedings, 3rd International Congress of Parasitology, München, 25–31 August, 1974. 1, 135–136.

FRIEDHOFF, K. & SCHOLTYSECK, E., 1968a. Feinstrukturen von *Babesia ovis* (Piroplasmidea) in *Rhipicephalus bursa* (Ixodoidea): Transformation sphäroider Formen zu Vermiculaformen. *Zeitschrift für Parasitenkunde*, 30, 347–359.

FRIEDHOF, K. & SCHOLTYSECK, E., 1968b. Fine structure of *Babesia ovis* trophozoites in *Rhipicephalus bursa* ticks. *Journal of Parasitology*, 54, 1 246–1 250.

FRIEDHOFF, K. & SCHOLTYSECK, E., 1969. Feinstrukturen der Merozoiten von *Babesia bigemina* im Ovar von *Boophilus microplus* und *Boophilus decoloratus*. *Zeitschrift für Parasitenkunde*, 32, 266–283.

FRIEDHOFF, K., SCHOLTYSECK, E. & WEBER, G., 1972. Die Feinstruktur der differenzierten Merozoiten von *Babesia ovis* in den Speicheldrüsen weiblicher Zecken. *Zeitschrift für Parasitenkunde*, 38, 132–140.

HENNING, M. W., 1949. Animal diseases in South Africa. 2nd ed. South Africa: Central News Agency Ltd.

HOWARD, C. W., 1908. A list of the ticks of South Africa with descriptions and keys. *Annals of the Transvaal Museum* 1, 73–169.

HUTCHEON, D., 1900. Redwater and its history. *Agricultural Journal of the Cape of Good Hope*, 17, 331–339; 395–409.

LOUNSBURY, C. P., 1905a. Report of the government entomologist for the half year ended June 30th, 1904. *Report of the Department of Agriculture. Cape of Good Hope* (1904), 35 pp.

LOUNSBURY, C. P., 1905b. Transmission of certain diseases by ticks. Bovine piroplasmosis (Unpublished manuscript).

MAHONEY, D. F. & MIRRE, G. B., 1971. Bovine babesiasis: estimation of infection rates in the tick vector *Boophilus microplus* (Canestrini). *Annals of Tropical Medicine and Parasitology*, 65, 309–317.

MAHONEY, D. F. & SAAL, J. R., 1961. Bovine babesiosis: Thick blood films for the detection of parasitaemia. *Australian Veterinary Journal*, 37, 44–47.

MORZARIA, S. P., BLAND, P. & BROCKLESBY, D. W., 1976. Ultrastructure of *Babesia major* in the tick *Haemaphysalis punctata*. *Research in Veterinary Science*, 21, 1–11.

NEITZ, W. O., 1941. The occurrence of *Babesia bovis* in South Africa. *Journal of the South African Veterinary Medical Association*, 12, 62–66.

NEITZ, W. O., 1956. Some protozoan diseases of man and animals: anaplasmosis, babesiosis and toxoplasmosis. *Annals of the New York Academy of Sciences,* 64, 25–277.

NEITZ, W. O., 1962. Review of recent developments in the protozoology of tick-borne diseases. Working paper, 2nd Meeting of FAO/OIE. Expert panel on tick-borne disease of livestock (Cairo, December 3–10, 1962), 1–17.

NEITZ, W. O., 1969. Observations on the duration of premunity following administration of the bivalent redwater vaccine. *Journal of the South African Veterinary Medical Association,* 40, 419–420.

NEITZ, W. O. & DU TOIT, P. J., 1938. Tick-borne diseases. *Journal of the South African Veterinary Medical Association,* 9, 85–124.

POTGIETER, F. T., 1977. The life-cycle of *Babesia bovis* and *Babesia bigemina* in ticks and in cattle in South Africa. Ph.D.Thesis, Rand Afrikaans University.

POTGIETER, F. T. & ELS, H. J., 1976. Light and electron microscopic observations on the development of small merozoites of *Babesia bovis* in *Boophilus microplus* larvae. *Onderstepoort Journal of Veterinary Research,* 43, 123–128.

POTGIETER, F. T. & ELS, H. J., 1977. Fine structure of intra-erythrocytic stages of *Babesia bigemina. Onderstepoort Journal of Veterinary Research.* In press.

POTGIETER, F. T. & VAN VUUREN, A. S., 1974. The transmission of *Babesia bovis* using frozen infective material obtained from *Boophilus microplus* larvae. *Onderstepoort Journal of Veterinary Research,* 41, 79–80.

POTGIETER, F. T., ELS, H. J. & VAN VUUREN, A. S., 1976. The fine structure of merozoites of *Babesia bovis* in the gut epithelium of *Boophilus microplus. Onderstepoort Journal of Veterinary Research,* 43, 1–10.

PURNELL, R. E., BRANAGAN, D. & BROWN, C. G. D., 1970. Attempted transmission of some piroplasms by Rhipicephalid ticks. *Tropical Animal Health and Production,* 2, 146–150.

RIEK, R. F., 1964. The life cycle of *Babesia bigemina* (Smith & Kilborne, 1893) in the tick vector *Boophilus microplus* (Canestrini). *Australian Journal of Agricultural Research,* 15, 802–821.

RIEK, R. F., 1966. The life cycle of *Babesia argentina* (Ligniéres, 1903) (Sporozoa: Piroplasmidea) in the tick vector *Boophilus microplus* (Canestrini). *Australian Journal of Agricultural Research,* 17, 247–254.

SCHOLTYSECK, E. & MEHLHORN, N., 1970. Ultrastructural study of characteristic organelles (paired organelles, micronemes, micropores) of Sporozoa and related organisms. *Zeitschrift für Parasitenkunde,* 34, 97–127.

THEILER, A., 1905. Redwater. *Transvaal Agricultural Journal,* 3, 476–496.

THEILER, A., 1907. Further notes on *Piroplasma mutans*—a new species of *Piroplasma* in South African cattle. *Journal of Comparative Pathology and Therapeutics,* 20, 1–18.

THEILER, A., 1908. Further transmission experiments with East Coast fever. *Transvaal Agricultural Journal* 6, 413–425.

THEILER, A., 1909a. Quelques observations concernant la transmission du *Piroplasma bigeminum* par des tiques. *Bulletin de la Société de pathologie exotique,* 2, 384–385.

THEILER, A., 1909b. Transmission des spirilles et des piroplasmes par differentes éspéces de tiques. *Bulletin de la Société de pathologie exotique,* 2, 293–294.

THEILER, A., 1909c. Diseases, ticks and their eradication. *Transvaal Agricultural Journal,* 7, 685–699.

THEILER, A., 1910. The prophylaxis of tropical and subtropical diseases of domesticated stock. *Transactions of the 9th International Veterinary Congress, The Hague,* September 13–19, 1909. I, S.G. 7, 8, 1–32.

WEBER, G. & FRIEDHOFF, K., 1971. Lichtmikroskopische Untersuchungen über die Entwicklung von *Babesia ovis* (Piroplasmidea) in *Rhipicephalus bursa* (Ixodoidea), II. Cytochemische Untersuchungen an differenzierten Merozoiten in den Speicheldrüsen weiblicher Zecken. *Zeitschrift für Parasitenkunde,* 35, 218–233.