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Electron microscopic studies on the development of kinetes of *Theileria parva* Theiler, 1904 in the gut of the vector ticks *Rhipicephalus appendiculatus* Neumann, 1901*

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Summary

The development of motile stages, called kinetes, from a stationary stage (regarded as a zygote) has been followed in *Theileria parva* by means of electron microscopy. This process started after moult of the tick nymphs which had sucked on highly infected calves, i.e. about 20 days after repletion (a.r.) of the ticks. The transformation took place by formation of a growing protrusion (= anlage) into an inner, enlarging vacuole. During this process the limiting membrane of the enlarging vacuole serves as the outer membrane of the developing motile stage, whereas the two inner ones as well as the subpellicular microtubules are newly formed. This transformation proceeds rapidly, so that on the 25th day a.r. most of the kinetes have already left the gut cells and started penetration into the salivary gland cells. On the way to the salivary glands nuclear divisions occurred within the kinetes. The steps of the transformation described were compared to those in *T. annulata* and to ookinete formation in haemosporidia.

Key words: Theileria parva; piroplasms; kinete formation.

Introduction

For many decades the life cycles of *Theileria* species, which may often cause fatal fevers in cattle, were known only incompletely. Thus only asexual reproduction within the salivary glands of ticks and within the blood cells of cattle could be identified with any certainty (Wilde, 1967; Büttner, 1967; Bar-

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nett, 1968). Besides these multiplications, gamogony has been recently found to occur during the life cycle of *T. annulata* (Schein, 1975a, b; Schein et al., 1975; Mehlhorn et al., 1975) and *T. parva* (Schein et al., 1977a; Mehlhorn and Schein, 1976). This sexual process takes place within the gut of the vector ticks, beginning 12 to 24 h after detachment. Gamogony results in the formation of kinetes, which leave the intestinal cells of the tick, move actively within the haemolymph, and finally penetrate into the cells of the salivary glands (Schein, 1975a). The present study describes the fine structure of the differentiating motile stage in *T. parva* and compares it with the results in *T. annulata* (Mehlhorn and

Abbreviations used in the figures

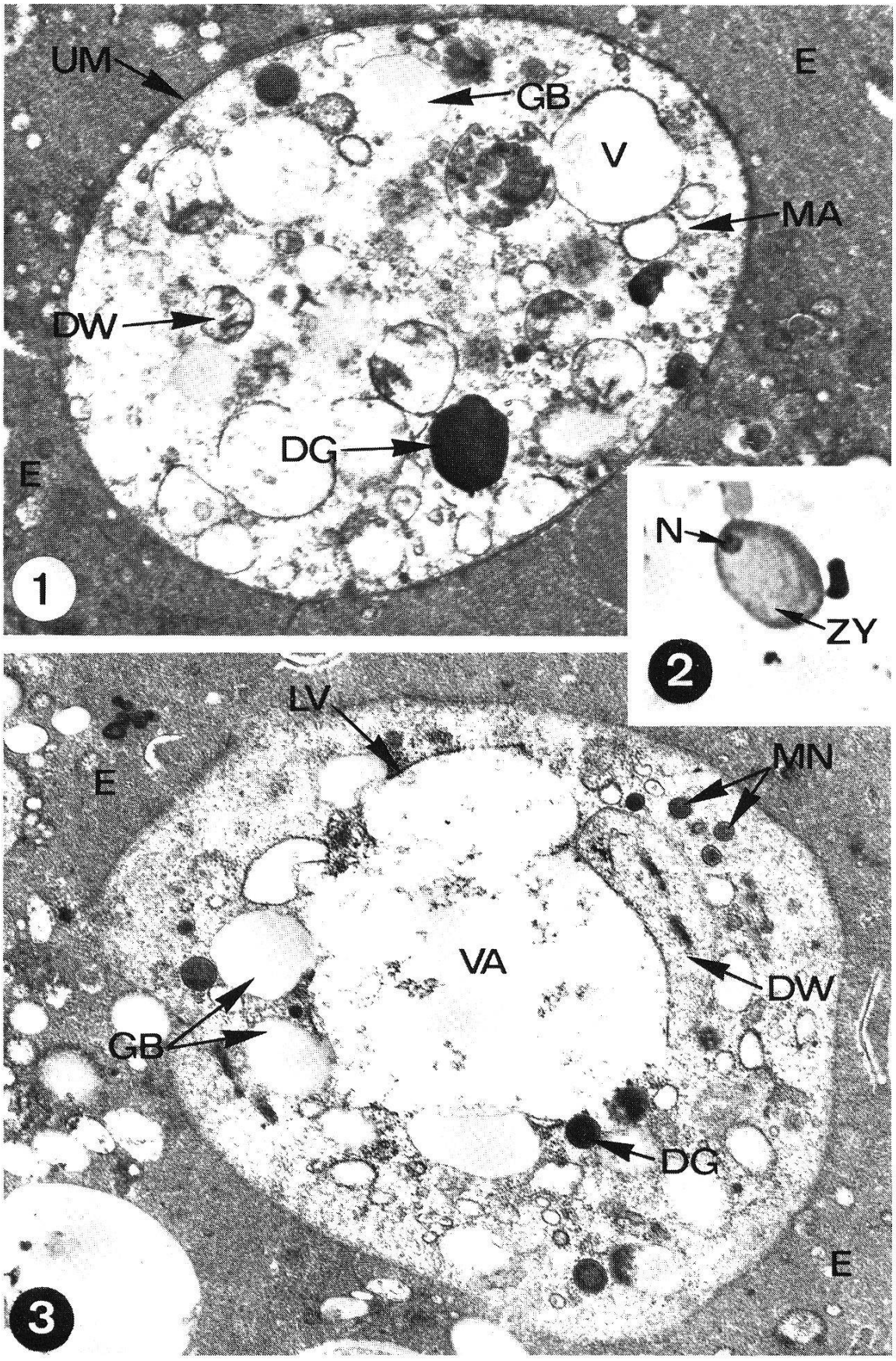
CR = Granules appearing crystalloidal
DG = Dense granules
DK = Developing kinete
DN = Dense intranuclear granules (nucleolus?)
DW = Double walled structures (mitochondria?)
E = Erythrocytic material being digested
ER = Endoplasmic reticulum
GB = Grey bodies
IM = Inner complex of the pellicle (two membranes)
K = Mature kinete
LV = Limiting membrane of the large vacuole (VA)
MA = Macrogamete
MN = Micronemes
MP = Micropore
MV = Multivesicular body
N = Nucleus
ND = Nucleus in division
NM = Nuclear membrane
OM = Outer membrane of the pellicle
P = Polar ring
PE = Pellicle
RB = Residual body
SP = Microtubules probably belonging to a spindle apparatus
ST = Subpellicular microtubules
UM = Unit membrane
V = Vacuole with electron lucid interior
VA = Large vacuole surrounding the differentiating motile stage
ZY = Zygote?

Figs. 1–14. *Theileria parva* within gut cells of the vector tick. Fig. 1, 3, 4–7, 12–13 electron micrographs; 2, 8–11 light micrographs.

Fig. 1. Section through a stage on the 4th day a.r. probably representing a macrogamete not yet fertilized. × 18,000.

Fig. 2. Giemsa-stained preparation of a parasite on the 16th day a.r. probably representing a fertilized macrogamete (= zygote). × 2,000.

Fig. 3. Section through a supposed zygote on the 20th day p.r.; note the presence of a large vacuole (VA) and that the size of the dense granules (DG) is reduced. × 14,000.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Schein, 1977), in haemosporidia (Davies, 1974; Gallucci, 1974; Trefiak and Desser, 1973; Garnham et al., 1969) and in *Babesia* (Friedhoff and Scholtyseck, 1968, 1969).

Materials and methods

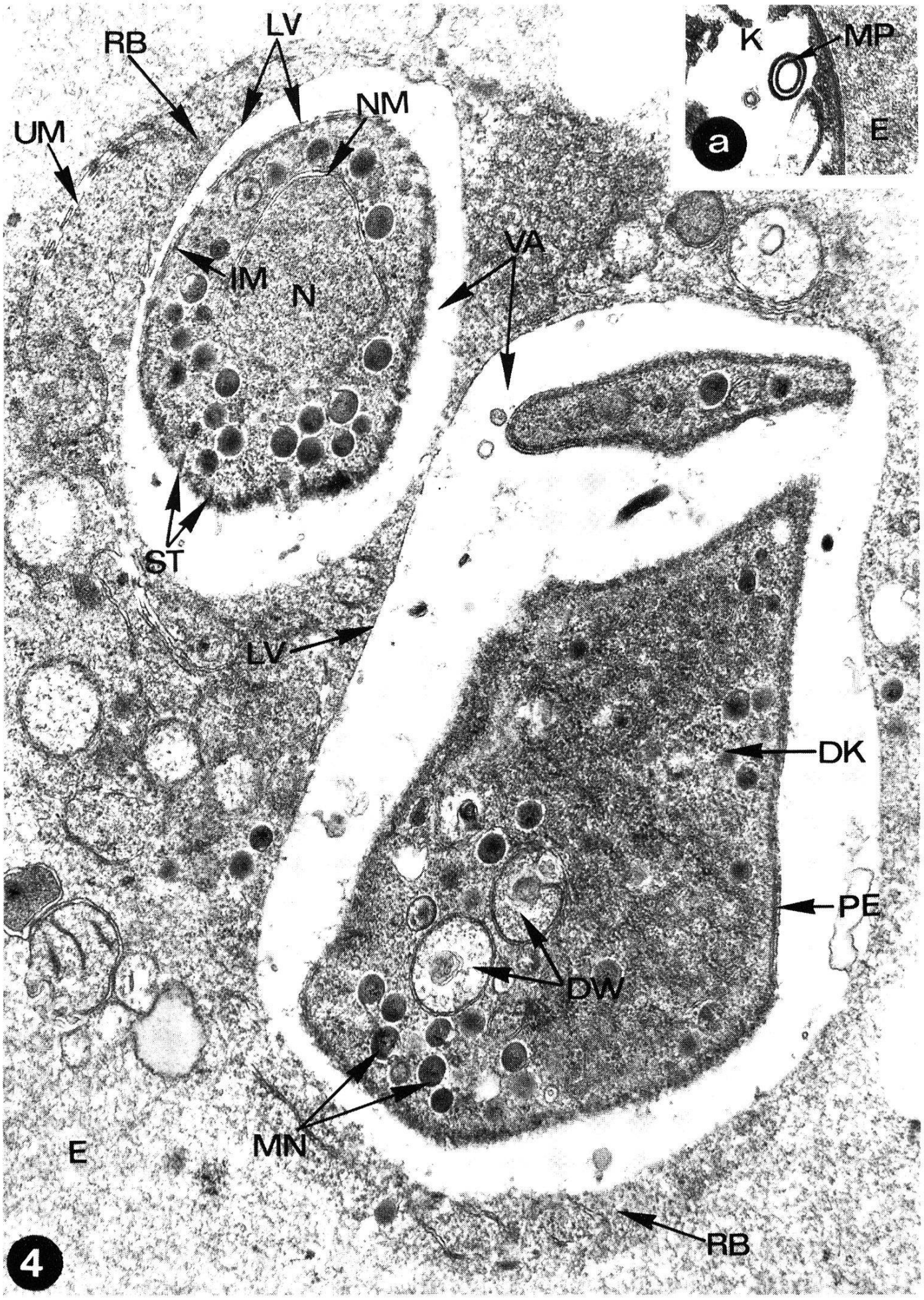
The strain of *Theileria parva* Theiler, 1904, originated from Muguga, Kenya and has been bred in the Berlin laboratory since 1971, after its isolation from a naturally infected calf. In the laboratory the parasites were transmitted to 4–6-month-old calves by adults of the tick *Rhipicephalus appendiculatus* Neumann, 1901. Further details of the maintenance of the parasites and hosts are published elsewhere (Schein et al., 1977a). For the present study nymphs which had engorged on calves with a parasitaemia of at least 40% were used. From the 2nd to the 25th day after detachment of the ticks (*after repletion* = a.r.), the whole gut was removed and placed in a container of fresh 2.5% (v/v) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3), and was fixed for 4–14 h at 4° C. The preparations were then rinsed in 0.1 M cacodylate buffer for 2–12 h, treated for 2 h in 2% (w/v) OsO_4 and dehydrated in an ethanol series before being embedded in Araldite (Ciba-Geigy). Ultrathin sections of embedded guts were cut on a Reichert ultratome OMU 3, mounted on copper grids without a film, stained with an alcoholic solution of uranyl acetate for ½ h, and finally were laid in lead citrate for 10 min. Specimens were then examined with a Zeiss electron microscope EM 9 S 2. The methods of handling the parasites within the haemolymph were the same as in previous studies (Mehlhorn et al., 1975; Mehlhorn and Schein, 1977). Light microscopical micrographs were taken from Giemsa-stained smears. The parasitic stages described were only found in ticks which had been fed on calves infected with *T. parva*, and were not found in ticks which had sucked on uninfected calves.

Results

On the 2nd to the 6th day after repletion the gut of the tick nymphs contained numerous microgamonts and microgametes with characteristic features (Mehlhorn and Schein, 1976). Besides these stages a very few ovoid parasites occurred, measuring about 4–5 μm as maximum (Fig. 1), which were taken to be macrogametes. These parasites being limited by a single membrane were characterized by a relatively pale cytoplasm, which contained small vacuoles, double walled structures and several electron dense granules with a maximum diameter of about 0.8 μm. Besides the electron lucid vacuoles some others occurred, the interior of which was slightly stained (Fig. 1). Around the 20th day a.r. other spherical or ovoid parasites were found within the gut cells of the now adult ticks. These stages measured about 6–7 μm in diameter and were considered to be zygotes, although fertilization was not observed. The nucleus of these stages was often found at the margin of the cells (Fig. 2) and a large vacuole with a diameter of 2–3 μm occurred close to the nucleus (Fig. 3). The cytoplasm of these cells, which were also limited by a single membrane, appeared more dense and contained smaller dense inclusions of about 0.3 μm in diameter (Fig.

Fig. 4. Section through a developing kinete (DK) on the 23rd day a.r. within the large vacuole (VA), which is kidney-shaped thus giving the impression of two vacuoles in this section. The residual body (RB) is in part already very thin. ×28,000.

Fig. 4a. Cross section through the periphery of a mature kinete. ×20,000.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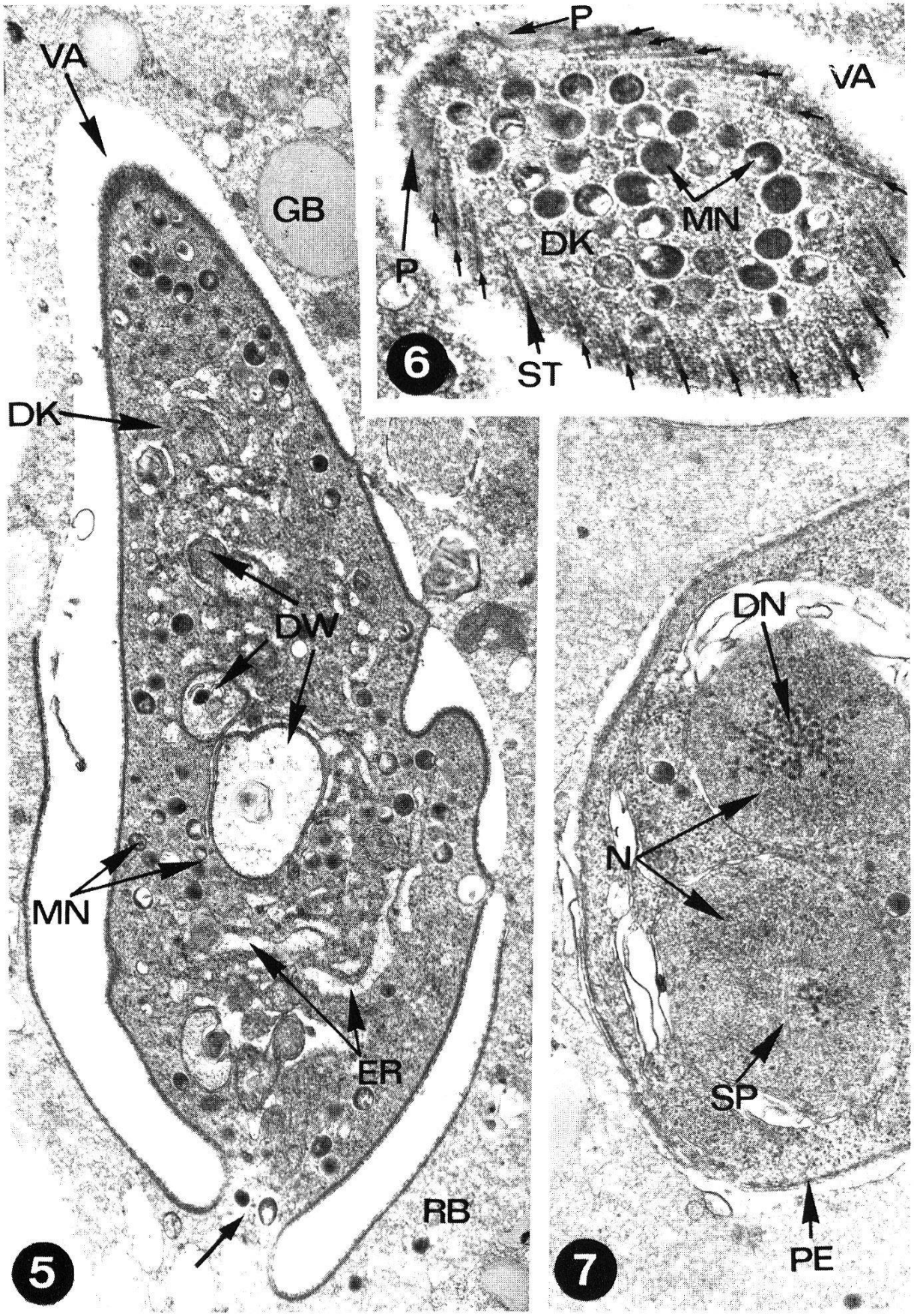
3) and already some microneme-like structures (Fig. 3). This parasitic stage grew somewhat in volume measuring about 9 μm on the 21st to the 23rd day a.r. A typical micropore, however, was not observed at this developmental phase, nor an endoplasmic reticulum, golgi apparatus, polysaccharide granules or typical mitochondria. Few days after moult of the tick, differentiation of a motile stage began within this spherical or ovoid stage. The cytoplasm and the nucleus formed a protrusion into the large vacuole adjacent to the nucleus (Fig. 14b). This protrusion was limited by the vacuolar membrane (Fig. 14b), under which 2 newly formed membranes, adjacent to each other, appeared (Figs. 4, 14b, c). Thus a typical coccidian pellicle surrounded this protrusion. The inner complex of this pellicle was interrupted at the apical pole of the protrusion, forming there a thick electron opaque polar ring, which was canopy-like and seemed to extend into ribs (Fig. 6). No conoid nor any similar structure ever occurred within the interior of this polar ring, at which about 40 subpellicular microtubules were anchored. These were present even in the very short protrusions (Figs. 4, 6). In this phase of development the subpellicular microtubules ran beneath the inner complex of the pellicle to the base of the protrusion. At this stage the number of micronemes had increased within the parasite and were almost exclusively present within the developing kinete (Figs. 4, 6). As development proceeded, the large vacuole extended, while the protrusion was considerably enlarged by the intake of more and more cytoplasm of the originally spherical cell (Fig. 14c, d). Finally only a small zone of cytoplasm surrounded the large vacuole containing the differentiating stage (Fig. 14d), which, however, remained connected with the residual body by a small cytoplasmic bridge (Fig. 5). In every case only a single kinete was formed from a spherical stage, although in some micrographs two seemed to be present. Such micrographs (Fig. 4) are due to the folding of the kinete during development. It thus filled the large, often kidney-shaped vacuole; this fact can easily be demonstrated by serial sections. Finally the kinete attained a club-shaped appearance (Figs. 8, 14d) within the vacuole, prior to the residual body being ruptured and thus setting free the motile parasite. This "free" kinete then became more elongated measuring 19 μm $\times$ 5.5 μm (Figs. 9, 12) and started moving within the intestinal cells of the tick, which were closely filled with blood being digested**. On the 3rd to the 5th day after moult of the tick numerous kinetes were observed, indicating that their development in *T. parva* proceeds at the same rapid pace in all specimens, probably starting immediately after moult. These motile stages, the nucleus of which was situated centrally or at the apical pole (Figs. 9,

** The moving of the kinetes was proved in native preparations being filmed (TV-camera!).

Fig. 5. Longitudinal section through a developing kinete. Note that the differentiating stage is still connected with the residual body (arrow). $\times$20,000.

Fig. 6. Tangential section through the apical pole of the developing kinete. $\times$20,000.

Fig. 7. Cross section through the middle region of a mature kinete. $\times$27,000.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12), contained large numbers of micronemes measuring 70–90 nm in diameter, which were scattered throughout the whole cytoplasm (Fig. 12). Their typical pellicle was provided in some cases with micropore-like structures (Fig. 4a), measuring 0.15 μm at the inner diameter. The number of the double walled structures had increased and during development, the inner membrane of these organelles formed a few invaginations, giving a mitochondria-like appearance (Figs. 5, 12). The late developing stages as well as the elongated kinetes contained lacunes which were very similar to the endoplasmic reticulum of other cells (Figs. 5, 12), whereas typical dictyosomes as well as rhoptries and polysaccharide granules were lacking. In several kinetes nuclear divisions started, so that stages with up to 4 nuclei arranged in a line were found (Figs. 10, 11, 13, 14f). Within the nuclei, the karyoplasm of which appeared relatively homogeneous, dense granules of 20 nm were spherically arranged (Figs. 7, 13). These were also visible in light microscopy when seen together (Figs. 8, 10). During nuclear division microtubules occurred which apparently belonged to the spindle apparatus (Fig. 7). Close to such nuclei granules of 15–16 nm in diameter were found, which together appear as a slightly crystalloidal pattern (Fig. 13). In mature kinetes the subpellicular microtubules extended for only a third of the cell. Beginning with the 3rd day after moult of the ticks, these stages left the intestinal cells of the tick, were found in the haemolymph and finally penetrated into the alveoles of the salivary glands of the tick. After they had become spherical and carrying out further nuclear divisions, a new developmental phase starts here which can be considered as sporogony. Summarizing, it can be stated that the transformation described of a stationary developmental stage (probably a zygote) into a motile one occurs via an inner protrusion – depicted in Figs. 14a–d. The following developmental phase (sporogony) is initiated by nuclear divisions which often begin within the kinete (Fig. 14e, f). It is noteworthy that the transformation described does not begin before the moult of the tick is completed, but is then carried out in a few days in most of the parasites observed.

Discussion

As reported previously, *T. annulata* and *T. parva* develop sexual stages on the 1st to the 6th day a.r. within the gut cells of their vector ticks (Schein, 1975a, b; Schein et al., 1975; 1977a; Mehlhorn et al., 1975; Mehlhorn and Schein,

Figs. 8–11. Giemsa-stained kinetes 4–5 days after moult.

Fig. 8. Kinete almost completely differentiated. ×2,500.

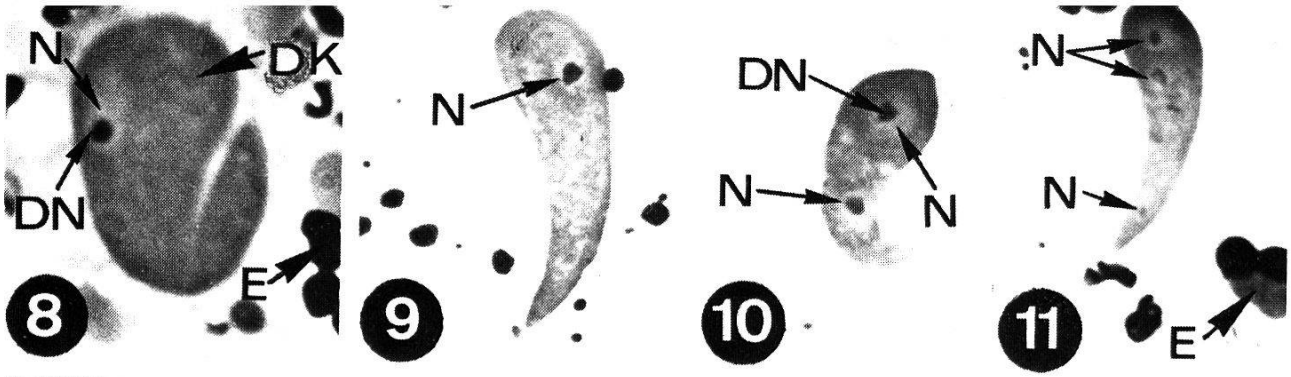
Fig. 9. Mature kinete with 1 nucleus. ×2,000.

Fig. 10. Mature kinete with 2 nuclei. ×2,000.

Fig. 11. Mature kinete with 3 visible nuclei. ×2,000.

Fig. 12. Longitudinal section through a mature kinete with 1 nucleus. × 10,500.

Fig. 13. Longitudinal section through a kinete, the nuclei of which are in division. ×25,000.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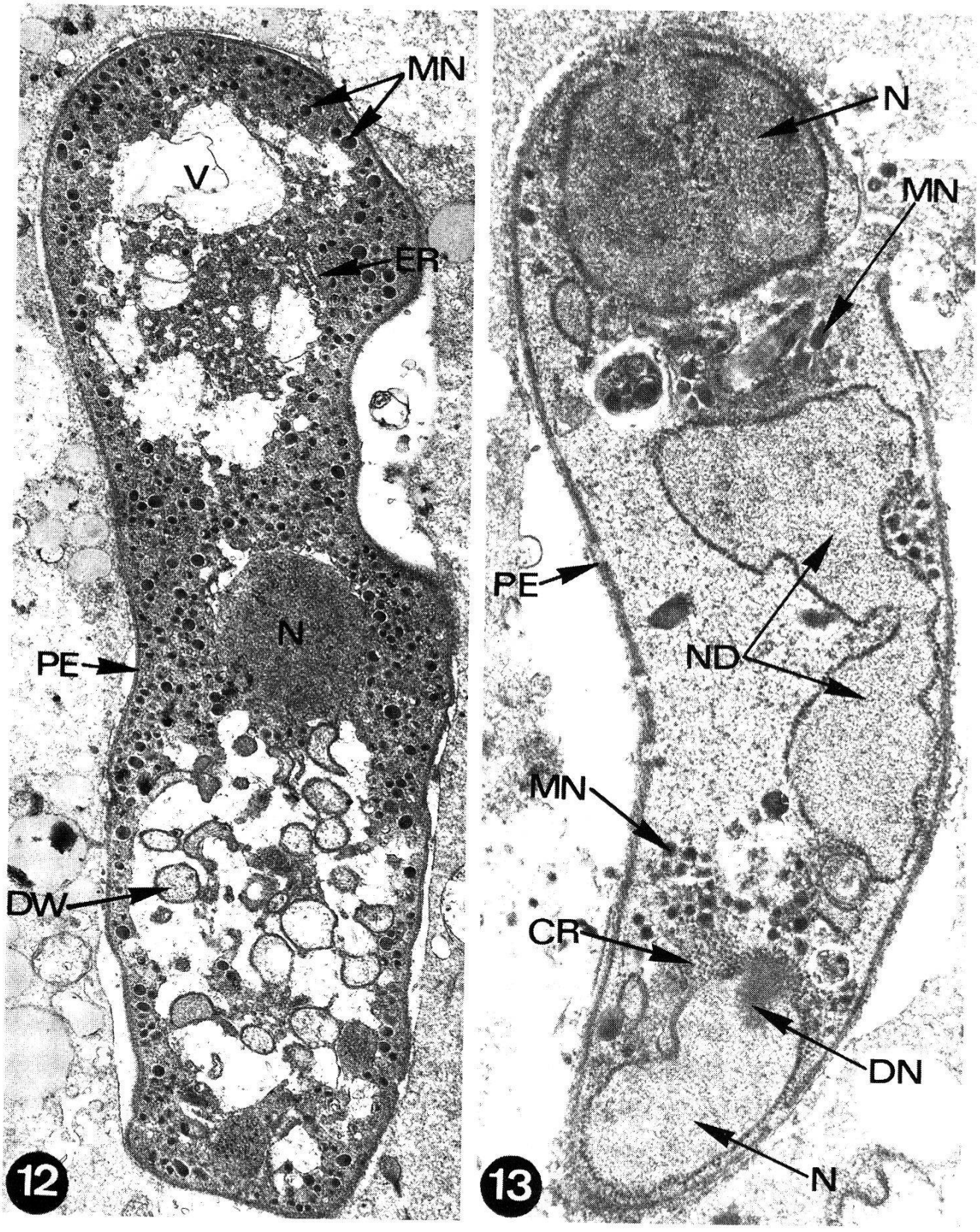
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1976; 1977). Such stages had already been observed by Koch (1906), Gonder (1911) and Cowdry and Ham (1932), but were neglected by Nuttall and Hindle (1913), Reichenow (1935, 1937, 1940), Martin et al. (1964) and other authors, thus hindering the discovery of the complete life cycle of these piroplasms. During gamogony in both species numerous microgamonts and microgametes are formed, but apparently only a few stages occur which are to be considered as macrogametes. Although fertilization has not yet been observed, it seems very probable that the stages from which the transformation described here proceeds are fertilized macrogametes (= zygotes). Considering that this zygote increases in size and that only one motile stage is formed, these stages of *T. annulata* and *T. parva* can be considered as ookinetes analogous to those of the haemosporidia. We describe these motile stages as "kinetes", which in our opinion is a more neutral term. In *T. annulata* the transformation starts relatively soon after the supposed fertilization, i.e. on the 9–10th day a.r. numerous developing stages occur within the intestinal cells of the nymph. In *T. parva*, however, the transformation does not begin before the moult of the nymph is completed (i.e. about 20 days a.r.), but this transformation is then carried out very quickly – in about 3–5 days at the maximum. In haemosporidia the transformation of the stationary zygote into the ookinete differs from that in *Theileria*. In haemosporidia a protrusion becomes visible on the zygote's surface (Schumacher, 1973; Gallucci, 1974; Rosales-Ronquillo and Silverman, 1974) and by enlargement of this protrusion the final elongate shape of the ookinete is attained. However, in the *Theileria* species studied the transformation takes place by formation of a growing protrusion into an inner, enlarging vacuole. During this process, which in light microscopy seems to be an invagination, the limiting membrane of the enlarging vacuole becomes the outer membrane of the later motile stage. On the other hand, the two inner membranes of its pellicle as well as the subpellicular microtubules are newly formed. Typical crystalloidal bodies as observed in haemosporidia (Trefiak and Desser, 1973) are not present in the *Theileria* kinetes, but a few granules occur in *T. parva*, giving a slightly similar impression. Typical rhoptries are found in the ookinetes of haemosporidia (Canning and Sinden, 1973; Davies, 1974; Desser, 1970; Garnham et al., 1969), wheras in *Theileria* only micronemes of varying diameter occur. A conoid was never present in *Theileria* kinetes as in the genus *Plasmodium*, but is suggested in the ookinetes of *Haemoproteus columbae* (Gallucci, 1974). Micropores or larger cytostomes were not found in the spherical, probably fertilized macrogametes of the *Theileria* species, although this organelle is present in the erythrocytic stages in cattle (Schein et al., 1977b) and in the mature kinetes of *T. parva*. It is therefore somewhat difficult to imagine how the zygotes ingest the food that is necessary for their considerable increase in size.

The fine structure of the kinetes in *Theileria* is furthermore very similar to that of the so-called "vermicules" of *Babesia* species from the ovaries of the ticks (Friedhoff and Scholtyseck, 1969). The final development of these "vermicules"

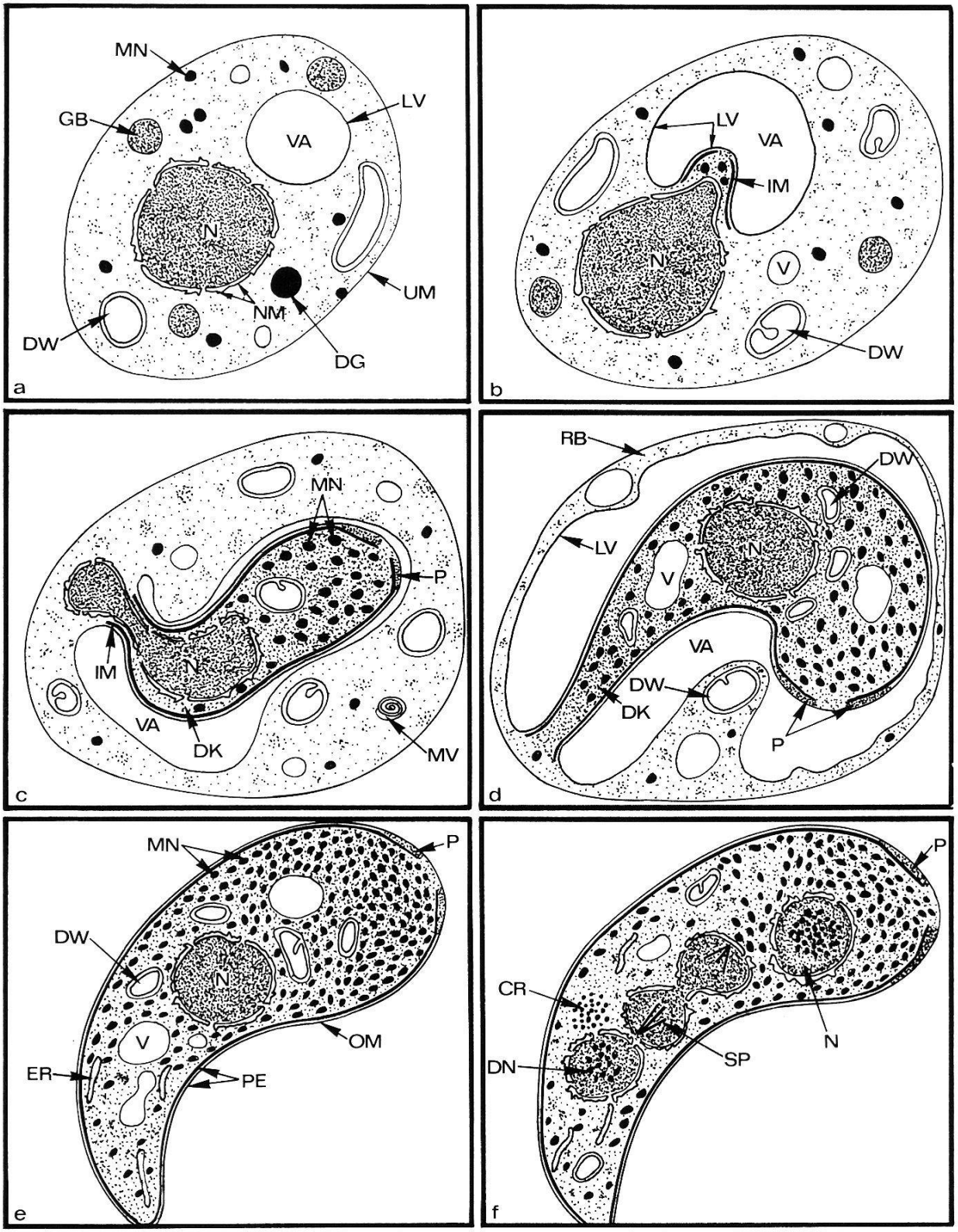

Fig. 14. Diagrammatic representation of the transformation of a spherical stationary stage (fertilized macrogamete) into a motile one (kinete) as seen in four steps (a–d). The nucleus of the mature kinete often starts already divisions (e–f) before penetration into the salivary glands.

seems, however, somewhat different from the transformation described here in *Theileria*. Furthermore, there may be preceding nuclear divisions in *Babesia*, leading to the suggestion that the "vermicules" might be "sporokinetes" of the *Babesia* cycle. Such "sporokinetes" are known from light microscopical studies of adeleidean coccidia of the genus *Karyolysus* (Bergle, 1974; Reichenow, 1921), where up to 64 "sporokinetes" are formed more or less simultaneously along the surface of a single enlarged zygote. These observations, however, still need confirmation by intensive electron microscopical investigation.

In *T. parva* the nucleus of the kinete may start with divisions, even when the motile stage is still stretching to reach its final shape. However, cytomere-like structures were never observed to develop from such kinetes, which were provided with up to 4 nuclei. Therefore these divisions do not give rise to "sporokinetes", but initiate the following developmental phase (sporogony), which normally has its nuclear divisions in the cells of the salivary glands of the tick. In *T. annulata* such an early initiation (of sporogony) was not observed (Mehlhorn and Schein, 1977) nor in haemosporidia (Davies, 1974). However, a similar cytological phenomenon of early initiation of the following developmental phase was observed in *Eimeria callospermophili* (Roberts et al., 1970), where sporozoites (motile) still contained numerous nuclei, the division of which already belonged to the following schizogony.

Concluding, it can be stated that the timing of kinete formation is different in *T. parva* from that in *T. annulata*. This might be the reason why sporogony of *T. parva* may be already initiated within the motile, mature kinetes. However, considering the whole development of the *Theileria* species within the gut of the vector ticks, a closer relationship to the haemosporidia (Sinden et al., 1976) than to the adeleidea can be assumed.

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