Onderstepoort J. vet. Res., 46, 41–49 (1979)

AN ELECTRON MICROSCOPIC STUDY OF INTRA-ERYTHROCYTIC STAGES OF *BABESIA BOVIS* IN THE BRAIN CAPILLARIES OF INFECTED SPLENECTOMIZED CALVES

F. T. POTGIETER and H. J. ELS, Veterinary Research Institute, Onderstepoort 0110

ABSTRACT

POTGIETER, F. T. & ELS, H. J., 1979. An electron microscopic study of intra-erythrocytic stages of *Babesia bovis* in the brain capillaries of infected splenectomized calves. *Onderstepoort Journal of Veterinary Research*, 46, 41–49 (1979).

Splenectomized vaccine donor calves undergoing primary reactions to *Babesia bovis* infections may develop cerebral babesiosis which leads to death if not treated in time. A brain biopsy was performed on an artificially-infected animal showing nervous symptoms and the tissue was immediately processed for electron microscopic examination. Virtually every erythrocyte in the brain capillaries sectioned was infected with *B. bovis*. Intra-erythrocytic merozoites, trophozoites and dividing trophozoites were indentified. Important features of the piriform merozoites included a reduced apical complex consisting of the anterior polar ring, microtubules, rhoptries and micronemes. Unidentified membrane-bound bodies, mostly spherical in shape, were observed anterior to the nucleus. The trophozoites showed very little structural differentiation and no food vacuoles or micropores could be detected. Each trophozoite produced 2 identical merozoites and the parent cell became totally incorporated in the daughter merozoites in the multiplication process. Projections were seen radiating from the surface of infected erythrocytes which appeared to adhere to other surfaces on contact. This probably resulted in the sludging of infected erythrocytes in the capillaries. The latter observations coincide with those described for *Babesia argentina*.

Résumé

ÉTUDE AU MICROSCOPE ÉLECTRONIQUE DES STADES INTRA-ÉRYTHROCYTAIRES DE BABESIA BOVIS *DANS LES CAPILLAIRES CÉRÉBRAUX DE VEAUX SPLÉNECTOMISÉS ET INFECTÉS*

Des veaux splénectomisés pour fournir du vaccin peuvent, lorsqu'ils passent par les réactions primaires aux infections de Babesia bovis, *développer une babésiose cérébrale qui sera mortelle si l'on ne traite pas à temps. Une biopsie cérébrale a été effectuée sur un animal infecté artificiellement qui manifestait des symptômes nerveux et le tissu a été immédiatement préparé pour examen au microscope électronique. Pratiquement chaque érythrocyte dans les capillaires cérébraux sectionnés se trouvait parasité par* B. bovis. *On a identifié des mérozoïtes intra-érythrocytaires, des trophozoïtes et des trophozoïtes en division. D'importantes caractéristiques des mérozoïtes piriformes comprenant un complexe apical réduit composé de l'anneau polaire antérieur, de microtubules, de rhoptries et de micronèmes. On a observé en avant du noyau des corps non identifiés, de forme sphérique pour la plupart et liés à la membrane. Les trophozoïtes n'ont montré que très peu de différenciation structurelle et l'on n'a pu détecter ni vacuoles alimentaires ni micropores. Chaque trophozoïte produisait 2 mérozoïtes identiques et, lors du processus de multiplication, la cellule-mère s'incorporait totalement aux mérozoïtes-files. On a pu voir, irradiant à partir de la surface de l'érythrocyte infecté, des projections qui semblaient adhérer au contact à d'autres surfaces, une conséquence probable de ce phénomène étant l'agglutination, dans les capillaires, des érythrocytes infectés. Ces dernières observations concordent avec celles que l'on a faites à propos de* Babesia argentina.

INTRODUCTION

Both *Babesia bovis* and *Babesia bigemina* occur in South Africa. Redwater vaccine, which consists of blood obtained from splenectomized donor cattle infected with these parasites, is produced at this Institute. In order to obtain satisfactory levels of infection in the vaccine it is desirable that the highest possible parasitaemia should develop in these animals before they are bled. However, extreme precaution must always be taken when donor animals undergoing primary *B. bovis* reactions are used for vaccine purposes, since cerebral babesiosis may develop, and this usually leads to the death of the animal if babesiacidal treatment is delayed for too long. The phenomenon of cerebral babesiosis is not restricted to splenectomized experimental animals, however, as natural cases are often reported (Anon, 1976).

Wright (1972) conducted an electron microscopic study on "intravascular agglutination" which was caused by *Babesia argentina* infection in the cerebral cortex of splenectomized calves. In the present investigation, a comparable study was made of the fine structure of *B. bovis* and of infected erythrocytes in the brain capillaries.

MATERIALS AND METHODS

A brain biopsy was performed, as described by Johnston & Callow (1963), on a splenectomized ox, undergoing a primary, blood-induced *B. bovis* reaction. At the time the parasitaemia was 5,2% and the animal was showing nervous symptoms.

The brain specimens of mainly the cerebral cortex so obtained, were immediately fixed in a 2,5% solution of glutaraldehyde, buffered with 0,05 M sodium-cacodylate (pH 7,2), at 4 °C for 1 h. The specimens were processed for electron microscopic examination in the standard way, namely, post-fixed in a 1% solution of osmium tetroxide, dehydrated in ethanol and embedded in Epon embedding medium. The blocks were shaped and sectioned on a Reichert Om U2 ultramicrotome. The sections were stained with a 5% aqueous solution of uranyl acetate, counter-stained in lead citrate and viewed in a Siemens 102 electron microscope.

RESULTS

Development of B. bovis *in erythrocytes*

Apart from a few extracellular merozoites, 2 morphologically distinct, intracellular, developmental stages, namely, merozoites and trophozoites, were encountered. The merozoites were easily identified as typical paired forms. The trophozoite stage was more difficult to identify as such, since the merozoites, upon entering the host cell, gave rise to the generally spherical trophozoites through a dedifferentiating process of the ultrastructural features, the onset and completion of which are not clearly defined.

Received 7 Otctober 1978—Editor

Multiplication took place as the trophozoite stage produced 2 piriform merozoites, and typical of many *Babesia* spp., they remained joined for some time at their posterior poles.

Merozoites

The intracellular merozoites encountered in the sections were paired forms (Fig. 1, 8 & 9). The pellicle of the merozoite consisted of a delicate outer membrane (difficult to demonstrate) and a relatively thick osmiophilic inner layer (Fig. 1 & 12) which formed the anterior and posterior polar rings. This inner layer appeared to comprise a double membrane (Fig. 3) and will be referred to as the inner membrane of the pellicle for the purpose of this study.

Microtubules (not demonstrated here) were occasionally obeserved in cross-section below the inner membrane at the anterior polar ring. Numerous micronemes and usually 1 or 2 larger rhoptries were seen at the anterior pole (Fig. 1, 9 & 12).

Membrane-bound bodies, mostly spherical in shape, were seen anterior to the nucleus (Fig. 1, 3, 4, 8 & 12) and contained a dense agregation of granular material which stained with varying intensity (compare Fig. 1 & 4). One pair of merozoites was sectioned in which these spherical bodies were absent (Fig. 9).

The comparatively large nucleus was surrounded by a nuclear envelope which often formed large perinuclear spaces (Fig. 1 & 8).

The presence of a membranous type of endoplasmic reticulum is clearly demonstrated in the merozoite found extracellularly (Fig. 12).

An organelle surounded by a double membrane was often found in a position posterior to the nucleus. In most sections it appeared dumb-bell-shaped (Fig. 1), but diverse forms which appeared as folded cylindrical structures were often seen (Fig. 3 & 9). It is believed that these organelles are in fact mitochondria, although cristae could not be demonstrated.

Parasites were also encountered in lysed erythrocytes (Fig. 3) as well as extracellularly (Fig. 10 & 12). Most of these forms were apparently undergoing degeneration. One particular merozoite encountered extracellularly appeared almost intact and showed many of the fine structural characteristics of a typical *B. bovis* merozoite (Fig. 12).

Trophozoites

The trophozoites were mostly spherical in shape, surrounded by a single membrane and the osmiophilic inner layer of the pellicle, polar rings, rhoptries and most of the micronemes were absent (Fig. 2 & 5). Relatively large, membrane-bound, irregularly-shaped organelles, possibly mitochondria, were frequently seen in the cytoplasm (Fig. 6 & 7). Groups of mostly 4–5 microtubules observed in the cytoplasm of trophozoites (Fig. 2 & 5) were frequently associated with a specific area in the cytoplasm which was completely devoid of ribosomes. These were not membrane-bound and showed the presence of a very fine homogeneous substance (Fig. 2, 5, 6 & 7).

The absence of amoeboid forms or indications of phagocytosis of haemoglobin, coincided with the absence of food vacuoles, cytostomes and micropores which could possibly have been involved in food uptake.

Multiplication

The appearance of rhoptries, micronemes and a segment of the inner membrane of the pellicle somewhere along the periphery of the parasite were the first signs of division of the trophozoites (Fig. 6). Simultaneously, the nucleus, normally spherical in shape, became either elongated or irregular in shape and appeared to extend towards the areas where the rhoptries and micronemes were concentrated (Fig. 6). Subsequently, the cytoplasm bulged out from the mother cell (Fig. 7). Nuclear material moved into the developing daughter cell which, in contrast to the mother cell, had a thick osmiophilic inner membrane (Fig. 7).

Two daughter cells were formed simultaneously, and the size of the mother cell progressively decreased until it finally disappeared. At the completion of this process of division a pair of piriform merozoites were produced which remained attached at their posterior poles for some time before they separated (Fig. 1, 8 & 9). The mode of nuclear division remains somewhat obscure but it was evident that division was completed only during the final stages of cytoplasmic differentiation in the developing merozoites.

Host cells

Infected cells appeared to undergo certain ultrastructural changes. The membrane of the host cell formed projections which radiated from the cell surface and gave rise to scalloped peripheries (Fig. 10 & 11). Where these projections came into contact with other cell surfaces, there were indications of adhesions (Fig. 11 & 4).

Small vesicles (Fig. 5, 8 & 10) were present in the cytoplasm of most of the infected erythrocytes, but what relationship, if any, exists between these structures and the parasites is not known.

The pathological changes detected in the brain tissue fall outside the scope of this study and are not dealt with.

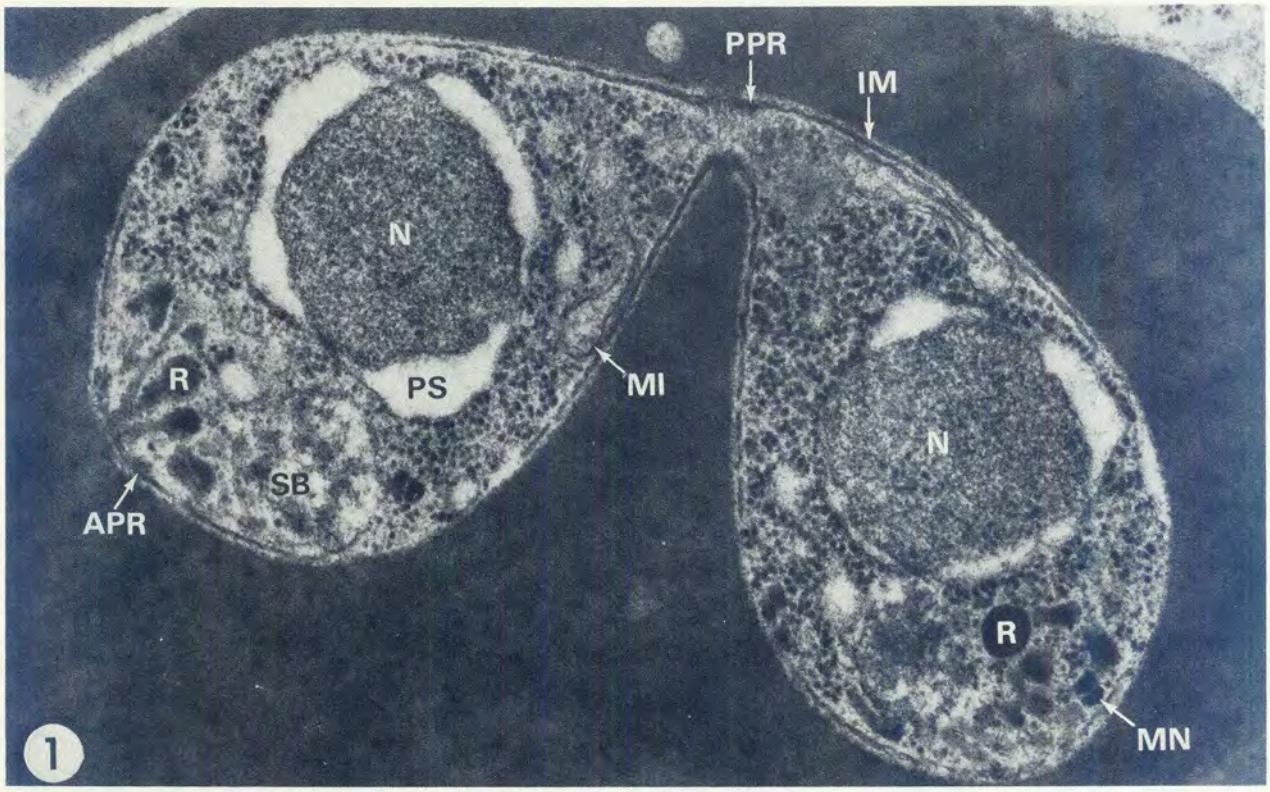
Discussion

The parasitaemia, as determined in a thin blood smear prepared from peripheral blood, rarely exceeded 5% by the time the first nervous symptoms were observed and babesiacidal treatment was given. Blood, collected concurrently from the jugular vein for fine structural studies, invariably showed a lower parasitaemia whereas virtually all the red blood cells encountered in the brain capillaries were parasitized. The quality of sections obtained from the brain tissue was superior to that of whole blood specimens.

The different developmental forms of *B. bovis* in the bovine host and the fine structure of these parasites closely resemble those of *Babesia bigemina* (Potgieter, 1977). The small piriform merozoites of *B. bovis* inoculated by the tick (Potgieter & Els, 1976) probably enter the red blood cells directly and transform into trophozoites. Eventually each trophozoite produces 2 merozoites (typical paired forms) which may separate and infect other erythrocytes where the process is repeated.

The identity and function of the "spherical bodies" (see Potgieter & Els, 1977, for a more comprehensive discussion), which featured prominently in the intra-erythrocytic forms of *B. bovis* remain unknown.

FIG. 1–12 Electron micropgraphs of intra-erythrocytic forms of *B. bovis* as seen in the brain capillaries of a splenectomized calf.



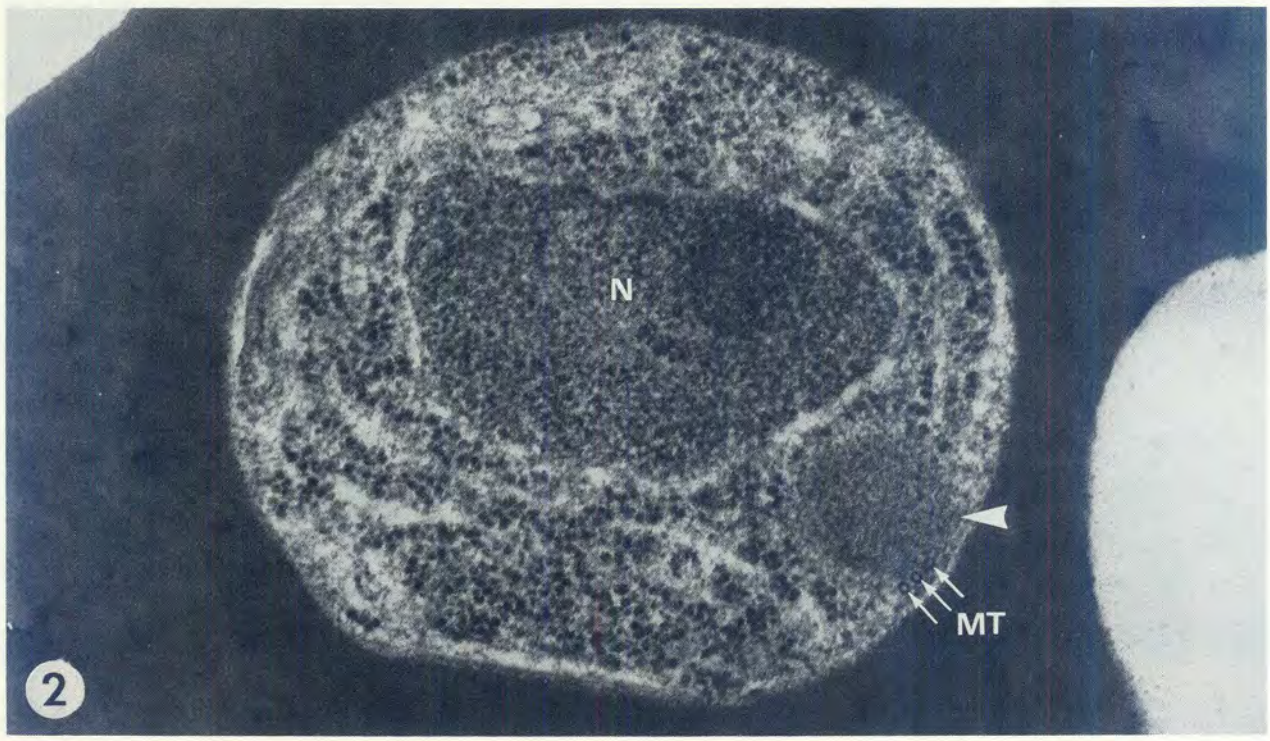
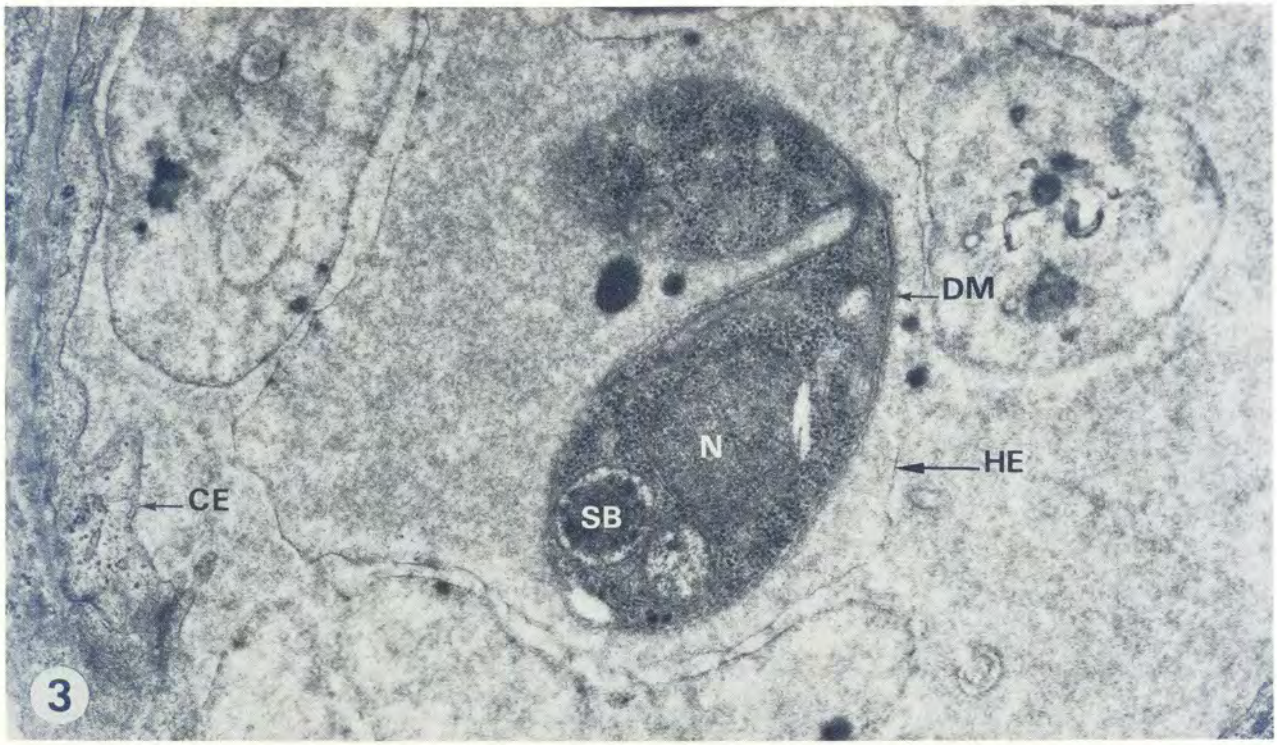


FIG. 1 Section through typical paired piriform merozoites. ×50 000
FIG. 2 Section through a trophozoite. Bold arrow indicates area in cytoplasm which appears devoid of ribosomes and usually is associated with a group of microtubules. ×62 500



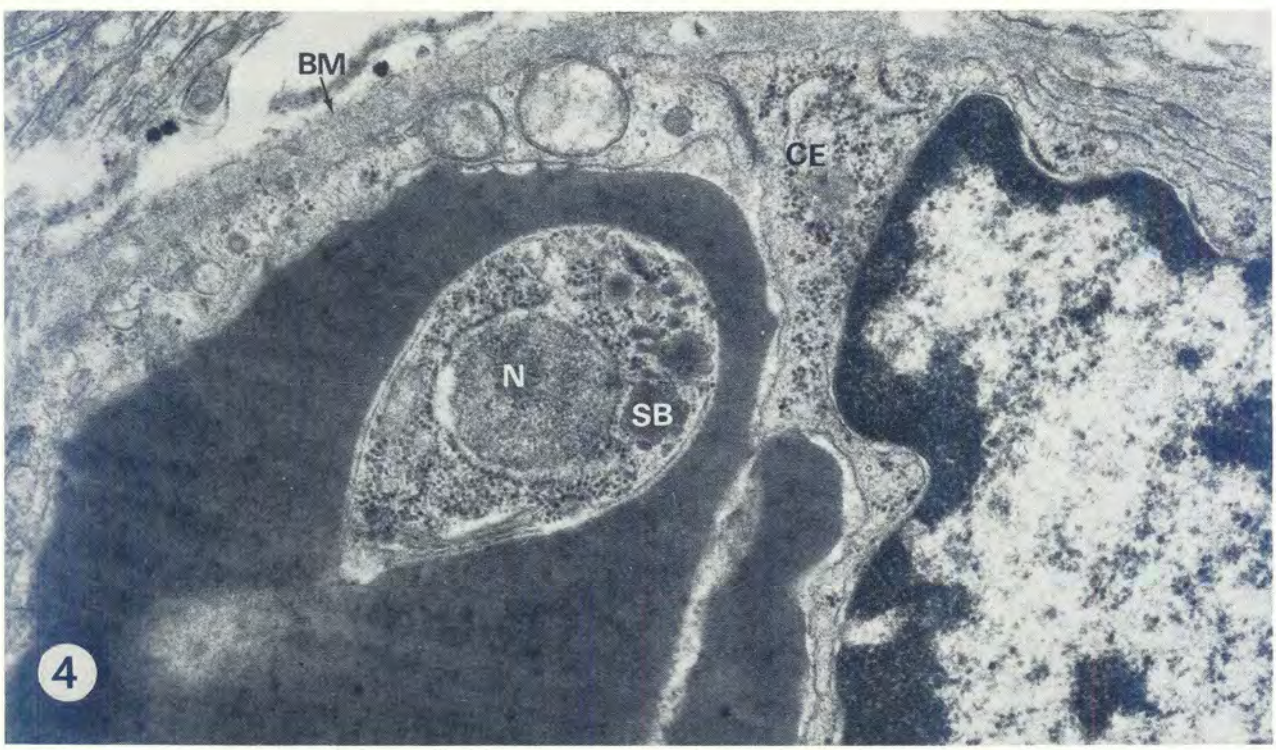
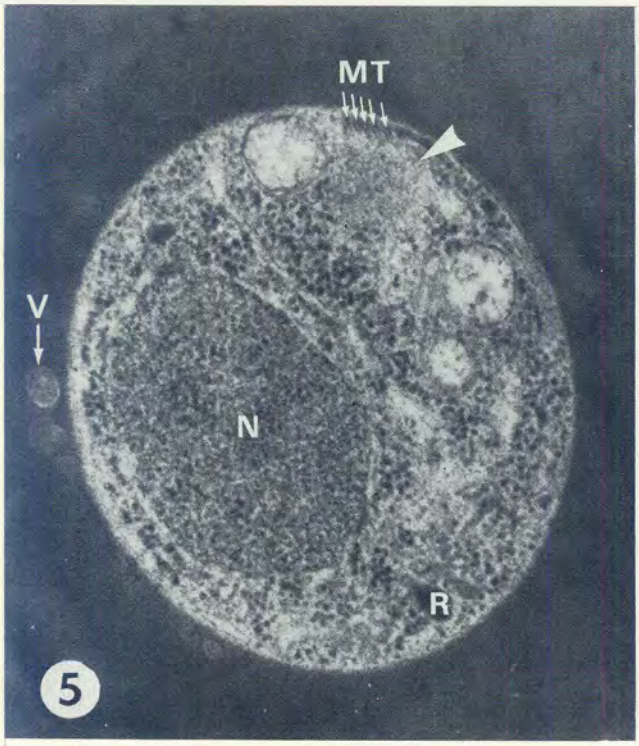
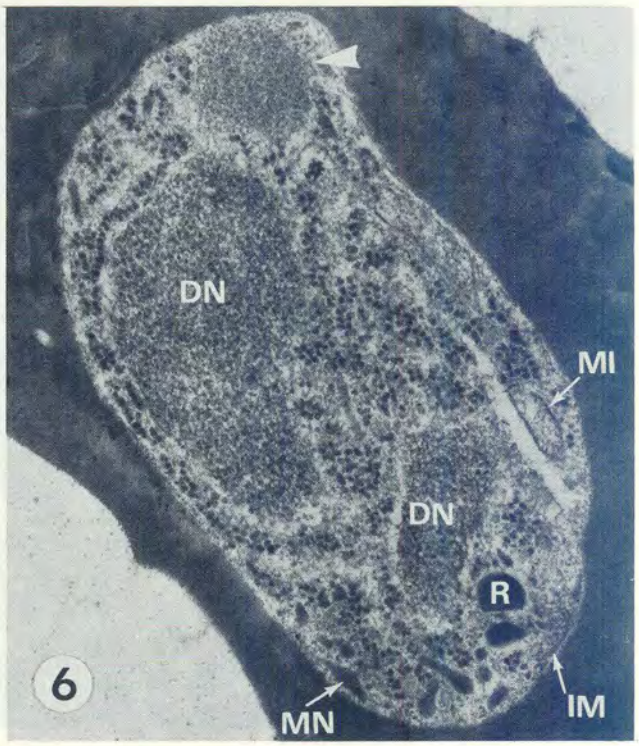


FIG. 3 Section through a pair of merozoites in a haemolyzed erythrocyte. Note double membrane of inner layer of pellicle. ×25 000
FIG. 4 Section through a merozoite. Note dark staining spherical bodies. ×25 000





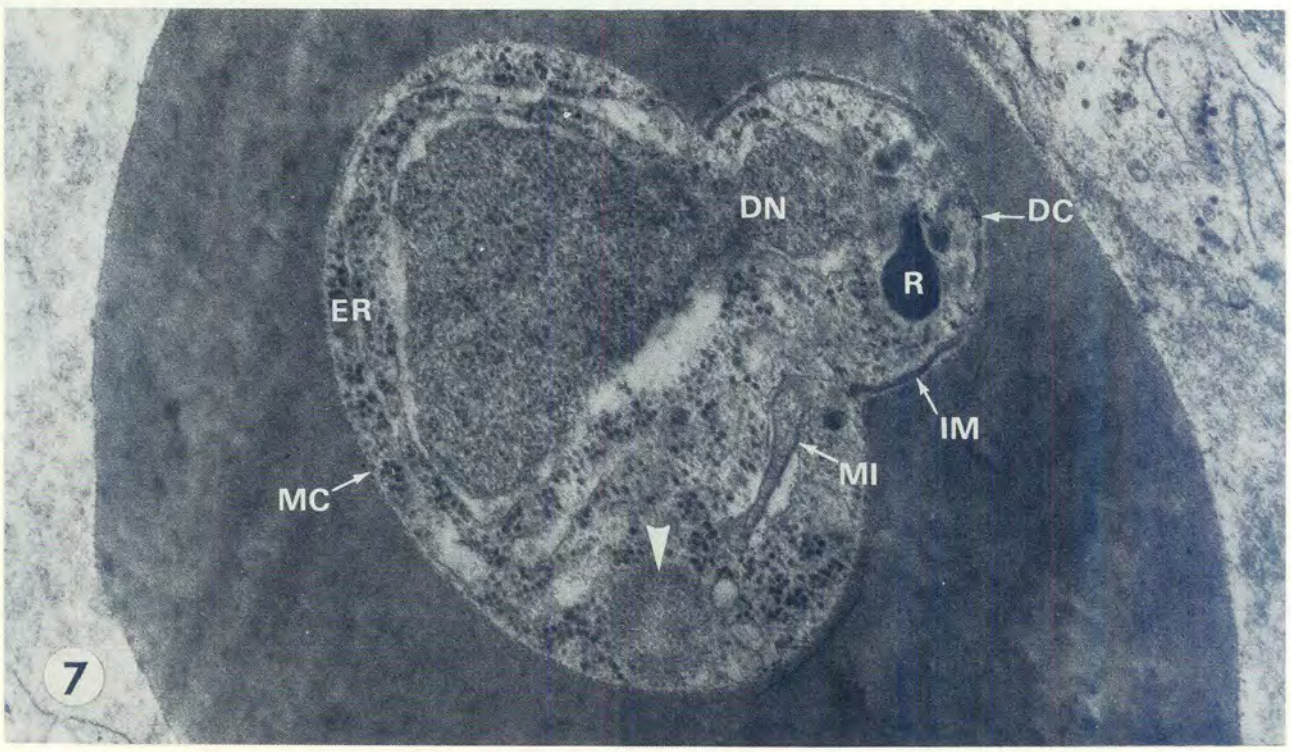


FIG. 5 Section through an intermediate stage showing tangentially sectioned microtubules along periphery of parasite. Bold arrow indicates specific area associated with microtubules. ×45 000
FIG. 6 Section through dividing form showing segment of inner layer, rhoptries and micronemes. Note dividing nucleus. ×44 000
FIG. 7 Section through dividing form. Note thick inner membrane of pellicle of developing daughter cell (merozoite) and nuclear material extending into the daughter cell. ×50 000

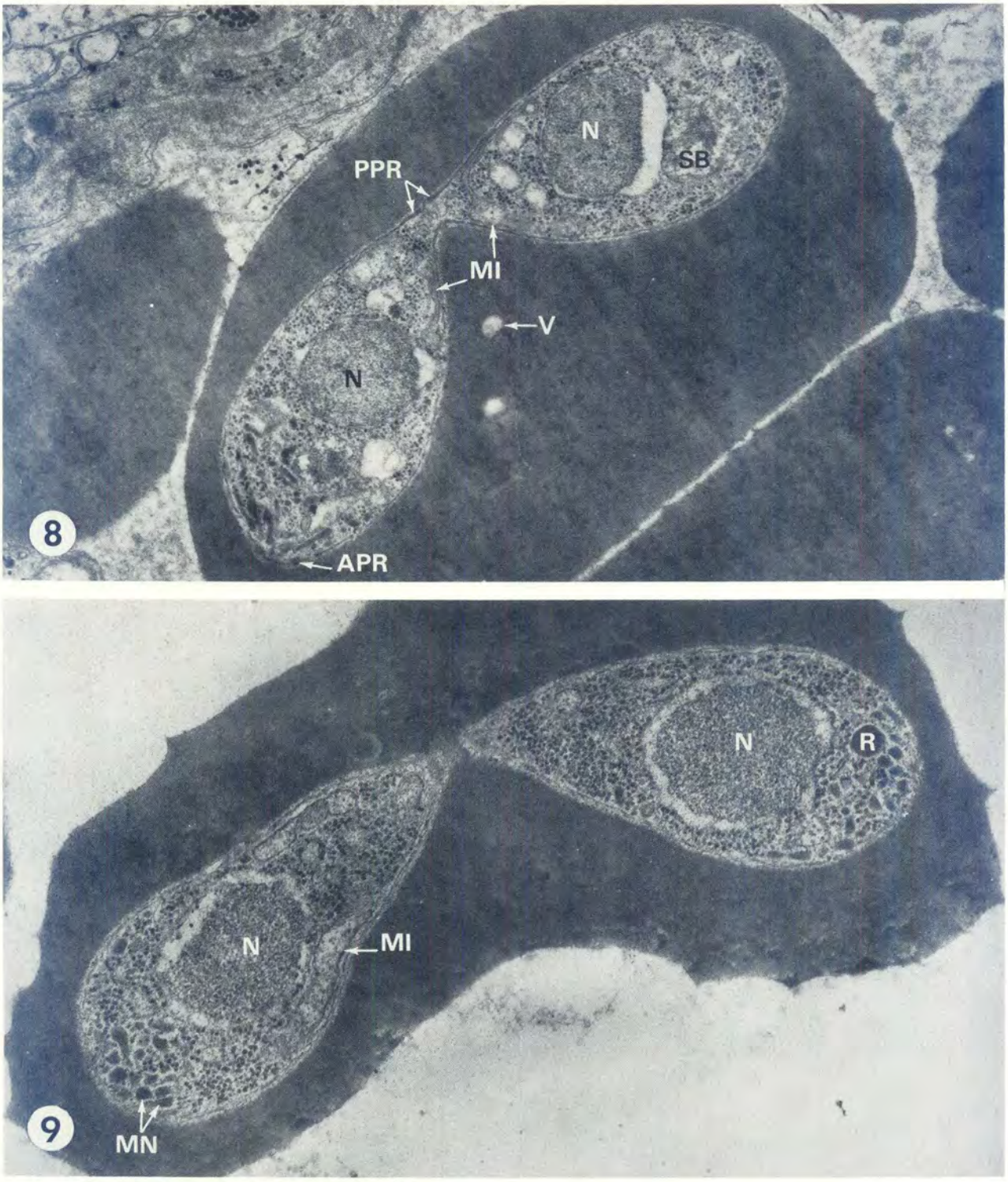
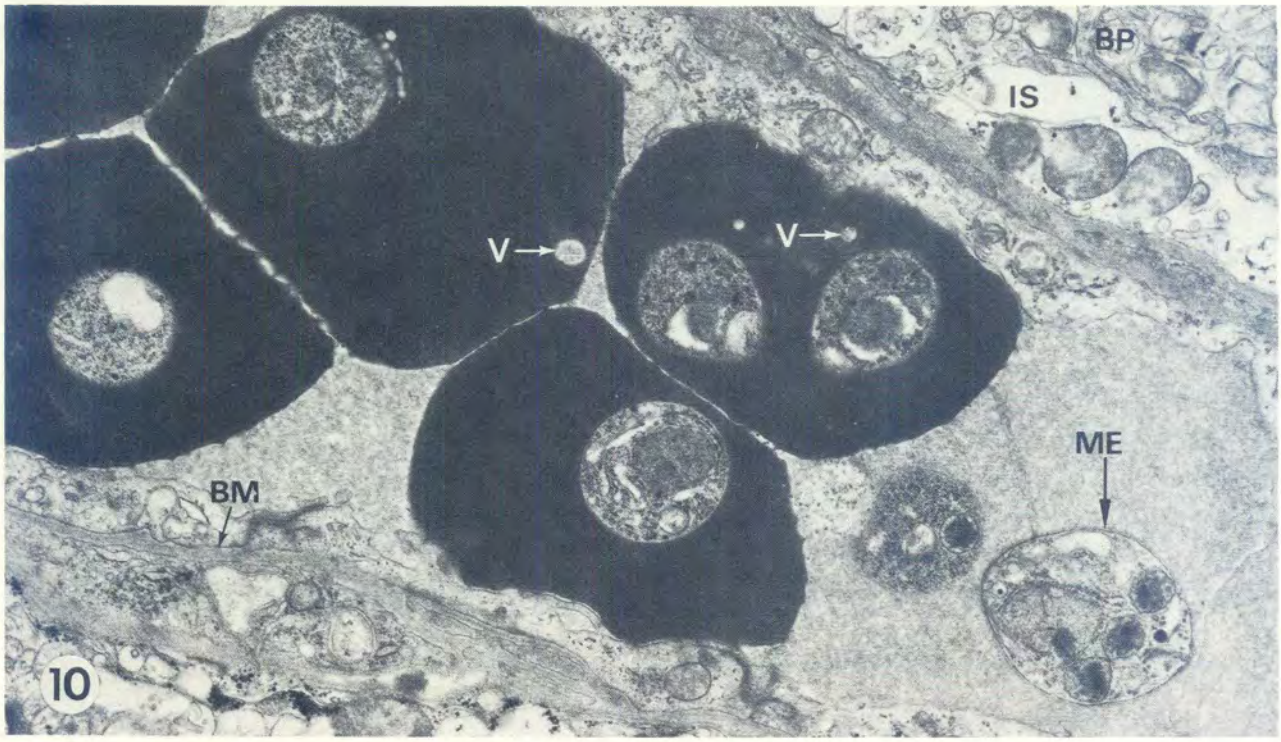


FIG. 8 Section through a pair of merozoites at the completion of the division process. Note attachment of 2 merozoites at their posterior poles. ×25 000

FIG. 9 Section through a pair of merozoites. Note absence of spherical bodies, usually in a position anterior to the nucleus. ×37 500



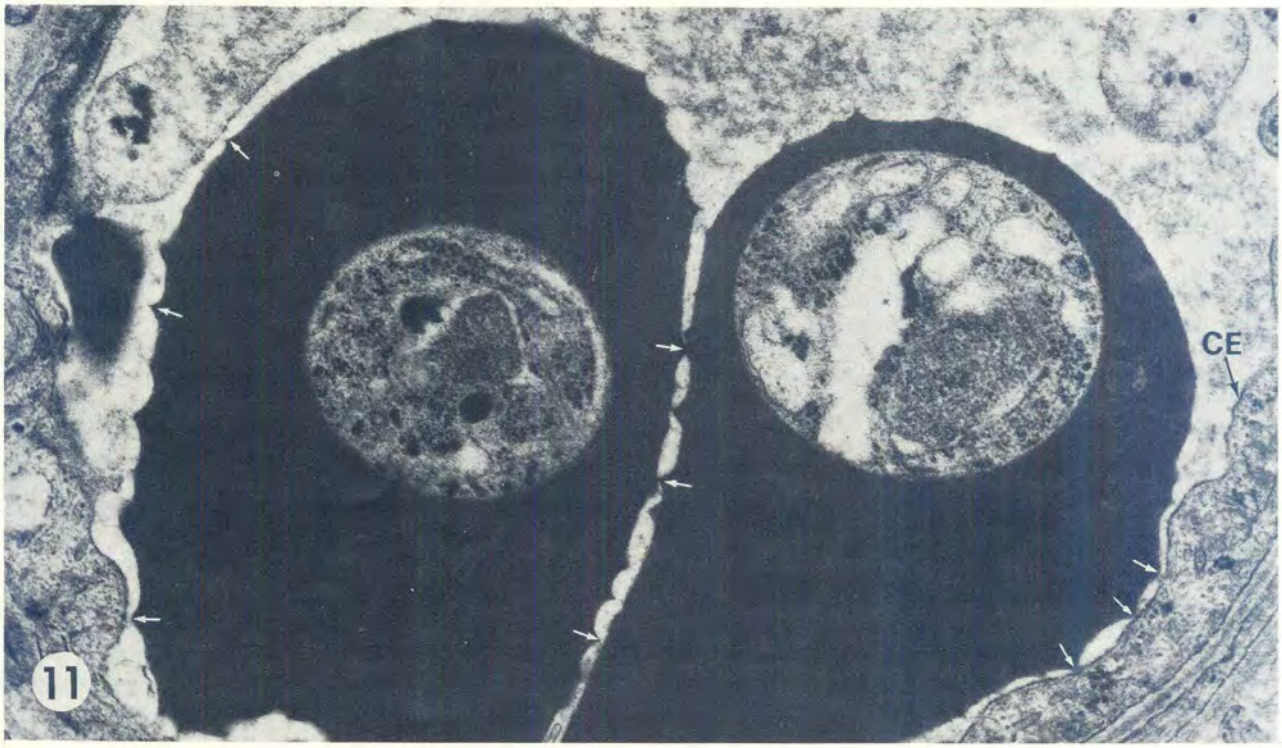


FIG. 10 Low magnification of erythrocytes in capillary. Note extracellular merozoite. ×12 500
FIG. 11 Section through parasitized erythrocytes in capillary. Note the projections radiating from the infected cell surfaces as indicated by the arrows. ×25 000

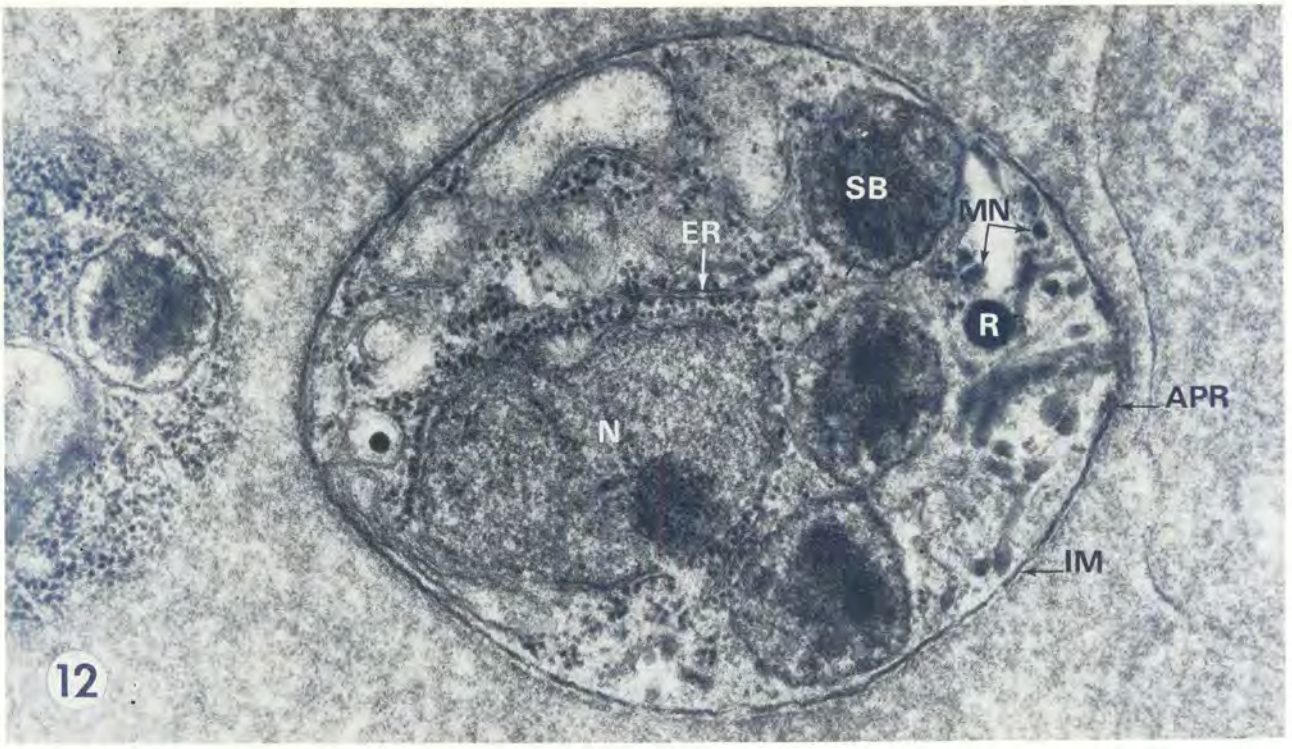


FIG. 12 High magnification of merozoite encountered extracellularly as seen in Fig. 10. ×50 000

Unlike the larger amoeboid or elongated trophozoites of *B. bigemina*, the trophozoites of *B. bovis* are on the whole relatively small and mostly spherical in shape. The homogeneously staining areas and associated microtubules observed in the cytoplasm of the trophozoites could not be identified.

The transformation process from merozoite to trophozoite involves the obvious cytological changes described earlier. It is feasible to assume that all the changes do not take place instantaneously and that there must be intermediate stages in terms of cytological structures observed. In this respect it is interesting to note that Bannister, Butcher, Dennis & Mitchell (1975) found that the merozoite of *Plasmodium knowlesi*, after adhering to an erythrocyte, takes 1 min to complete its invasion and that the trophozoite is formed after 10 min.

The same argument holds good for the stage in the development when the trophozoite begins to divide to form 2 merozoites. The point to be made is that it remains extremely difficult to differentiate between merozoites and trophozoites, particularly when only thin sections of the different developmental stages are studied. Thus the difficulty of differentiation must be taken into account when the structural detail of the stage which represents a trophozoite as described here is considered.

Intra-erythrocytic multiplication of *B. bovis* takes place in the same manner as described for *B. bigemina* (Potgieter & Els, 1977). Unlike merozoite formation in the case of *Babesia microti*, where the process is described as "budding" (Rudzinska & Trager, 1977), the entire parent cell of *B. bovis* is incorporated into the 2 developing merozoites, a process which by definition is not true budding (Levine, 1973); it can only be referred to as a form of schizogony as described for *B. bigemina* (Potgieter & Els, 1977).

Hote (1976) favoured the concept that *Babesia argentina*, *Babesia berbera* and *Babesia colchica* should be regarded as junior synonyms of *B. bovis*, and in view of his reasoning it is not surprising to find that the ultrastructural observations made in this study agree in most respects with those made by Wright (1972) during his investigation of intravascular agglutination in the cerebral cortex of calves infected with *B. argentina*. The only significant ultrastructural difference noticed was that none of the parasites examined in this study was multinucleate. The aggregations of nuclear material described by Wright (1972) resembled the spherical bodies observed in this study.

The stellate appearance of the infected erythrocytes and the apparent changes to their membranes, which have been held responsible for their presumed stickiness, were observed in both studies. The latest information seems to indicate, however, that the phenomenon of intravascular coagulation appears to be much more involved in that a markedly altered and activated coagulation system is present in cattle infected with *B. bovis* (Goc dg:r & Wright, 1977).

Other common features such as the transmission, life cycle and chemotherapy of Austrialian *B. argentina* and South African *B. bovis* (Potgieter, 1977) strongly support the concept that *B. argentina* should be regarded as synonymous with *B. bovis*.

LIST OF ABBREVIATIONS USED IN ALL MICROGRAPHS

APR—Anterior polar ring
BM—Basement membrane
BP—Brain parenchyma
CE—Capillary endothelium
DC—Daughter cell
DM—Double membrane
DN—Dividing nucleus
ER—Endoplasmic reticulum
HE—Haemolyzed infected erythrocyte
IM—Inner membrane

IS—Interstitial space
MC—Mother cell
ME—Merozoite
MI—Mitochondrion
MN—Microneme
MT—Microtubules
N—Nucleus
PPR—Posterior polar ring
PS—Perinuclear space
R—Rhoptry
SB—Spherical body
V—Vesicle

References

ANON—Annual report: Department of Agricultural Technical Services, 1/7/74–30/6/75, South Africa, 1976.

BANNISTER, L. H., BUTCHER, G. A., DENNIS, E. D. & MITCHELL, G. H., 1975. Structure and invasive behaviour of *Plasmodium knowlesi* merozoites *in vitro*. *Parasitology,* 71, 483–491.

GOODGER, B. V. & WRIGHT, L. G., 1977. *Babesia bovis* (*argentina*): Observations of coagulation parameters, fribinogen catabolism and fibrinolysis in intact and splenectomized cattle. *Zeitschrift für Parasitenkunde,* 54, 9–27.

HOTE, H. M. D., 1976. The tick-fever parasites of cattle. *Proceedings of the Royal Society of Queensland,* 87, v–xiii.

JOHNSTON, L. A. Y. & CALLOW, L. L., 1963. Intracerebral inoculation and brain biopsy in cattle. *Australian Veterinary Journal,* 39, 22–24.

LEVINE, N. D., 1973. Protozoan parasites of domestic animals and of man. Minneapolis: Burgess Publishing Company.

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,* 44, 123–128.

POTGIETER, F. T. & ELS, H. J., 1977. The fine structure of intra-erythrocytic stages of *Babesia bigemina*. *Onderstepoort Journal of Veterinary Research,* 44, 157–168.

RUDZINSKA, M. A. & TRAGER, W., 1977. Formation of merozoites in intra-erythrocytic *Babesia microti:* An ultrastructural study. *Canadian Journal of Zoology,* 55, 928–938.

WRIGHT, I. G., 1972. An electron microscopic study of intravascular agglutination in the cerebral cortex due to *Babesia argentina* infection. *International Journal for Parasitology,* 2, 209–215.