A STUDY OF THE GUT CELLS OF *BOOPHILUS MICROPLUS* IN RELATION TO *BABESIA BOVIS* DEVELOPMENT AND HOST IMMUNITY

by

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To My Father and Mother.

DECLARATION

I declare that the work presented in this thesis is to the best of my knowledge and belief original, except where acknowledged.

The material in this thesis has not been submitted either whole or in part for a degree at this or any other University.

Date: 22/3/84 .....

R.I.S. AGBEDE

ABSTRACT

Almost nothing is known about digestion in Boophilus microplus and the first aim of this thesis was to describe the cell types in the gut of nymph and female ticks and to follow gut development during feeding on the host. This provided the basic information for further studies on two important interactions between ticks and their hosts (cattle). That is (i) the development of Babesia bovis in the tick; (ii) regulation of tick numbers by host immunological reactions.

The cycle of B. bovis development in the tick starts in the gut and the aim was to identify the babesial stages and the gut cells in which they develop. The next aim was to study the histopathology of ticks on vaccinated hosts as recent work had identified the gut as one site of damage to ticks feeding on vaccinated animals. Finally, the use of cattle for vaccination trials against B. microplus is expensive, so a suitable laboratory model was tried.

A histological study of the gut cells in B. microplus was followed by histochemical identification of some of the cell products and transmission electron microscopy, with emphasis on the secretory cells. There were two small experiments on scanning electron microscopy of the digest cells and X-ray microanalysis of haematin granules. The scope was now extended to Babesia infection of the gut. Ticks were infected with B. bovis in vivo and in vitro. At suitable intervals thereafter, the gut was dissected and processed for both light and electron microscopy. The babesial infection was followed and the cell types invaded were identified.

Similar histological techniques were used to identify the site of immune mediated damage to ticks feeding on cattle vaccinated with tick antigen preparations, or damage to ticks feeding in vitro. In the laboratory model, rabbits were vaccinated with extracts of R. sanguineus

females and later challenged with tick infestations. Tick yields and histology of tick tissues were used to gauge the effectiveness of vaccination. Immuno-gel diffusion and electrophoresis tests demonstrated antibodies to tick antigens.

The following cell types were identified in the gut.

1. Digest cells specialised for intracellular digestion of host blood, were of two subtypes; one (dhl) which ingested by phagocytosis; and pinocytosis and the other (dhv) in which only pinocytosis occurred and ingested material was mostly lysed erythrocyte products. Ultrastructure of these digest cells was similar to that described by Raikhel (1975b) and Balashov & Raikhel (1976) for cells in nymphs of H. asiaticum. Ingested host blood and tissue fluids were taken into endosomes, these fused with the primary lysosomes and enzymic digestion gave rise to deposits of lipid, glycogen and residual bodies in the spent cells. Haematin granules in spent digest cells were detected using X-ray microanalysis.
2. Secretory cell type 1 (s_1) synthesised and secreted large spherical granules which stained for glycoproteins and may be a haemolysin.
3. Secretory cell type 2 (s_2) synthesised smaller, more complex granules which were released by apocrine secretion as a "colloidal" mass, and this may be an anticoagulant.
4. Basophilic cell (b) appeared to be the reorganised basal remnant of s_2 cells in which new cisternae of r.e.r. arranged in whorls and parallel arrays, synthesised granules of a different ultrastructure.

During slow feeding (nymphs and females) two generations of digest cells completed their development. Just before final rapid engorgement the third generation type 1 (dhl) began digestion. After engorgment type II (dhv) were the main cells involved. Secretory cells type 1 (s_1) were in low numbers throughout feeding but secretory cell type 2 (s_2), though

numerous, were active only just prior to final engorgement. During rapid feeding the basophilic cell had not yet developed secretory activity but the basal portion had a well developed basal labyrinth and numerous mitochondria and may be involved with transport of water and ions across the gut. After engorgement, the basophilic cells synthesised granules with a distinctive ultrastructure and this material may be egg proteins (vitellogenins).

In B. bovis infected ticks from in vivo and in vitro feeding, the Strahlenkörper (possibly sexual stages) were detected in the gut lumen, and the first infection of gut cells was found in a few digest cells type II (dhv), where the parasites were developing between the large food vacuoles. Fission bodies subsequently developed not in digest cells but in the basophilic cells which are the site of synthesis of egg proteins. These cells presumably provided the required nutrients for Babesia development and it is proposed that selection of basophilic cells is linked with the subsequent selection of the ovary and eggs for development.

Rejection of B. microplus ticks from vaccinated cattle differed from the rejection due to acquired resistance. The main difference was the complete destruction of gut cells in some ticks, with host leukocytes and erythrocytes escaping into the tick haemolymph. Significant damage was also found in secretory cell type 2, basophilic cells, malphigian tubules and cells of the male reproductive organs.

Vaccination of rabbits against R. sanguineus resulted in similar damage to the tick's gut and this laboratory model for vaccination proved successful.

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CHAPTER 1

GENERAL INTRODUCTION

1.1 TICK BIOLOGY AND THE ECONOMIC IMPORTANCE OF TICKS

The generally accepted classification of ticks is as follows:- Phylum: Arthropoda; Class: Arachnida; Order: Acarina or Acari; Sub Order: Ixodoidea. Three families are recognised: Argasidae (soft ticks), Ixodidae (hard ticks), and Nuttalliellidae, possessing characteristics intermediate between the first two families (Hoogstraal, 1956). Ixodidae comprise about 800 known species of ticks, whilst Nuttalliellidae is represented by only one species (Hoogstraal, 1973). Ixodid ticks are referred to as hard ticks because of their sclerotised hardened scutum, and ventral plates in the male. On the other hand, Argasid ticks are the leathery or soft ticks because they lack a scutum and the males have no ventral plates.

Ticks are obligate ectoparasites of vertebrates and attack members of all the major groups except fish. Ixodidae and Argasidae are world wide in their distribution, with ixodid ticks being recorded from all natural and climatic zones including regions far north of the Arctic circle and islands surrounding the Antarctic (Balashov, 1972).

The ticks used in the studies described throughout this thesis are: the cattle tick, Boophilus microplus (Canestrini); and the brown dog tick, Rhipicephalus sanguineus (Latreille), both ixodid ticks.

Argasid and Ixodid ticks differ in biology in many respects. One major difference is that Argasids feed rapidly and pass through numerous moults to reach the engorged female stage, whereas ixodids feed slowly (5-

8 days for most female B. microplus) and ingest large volumes of host blood, thus avoiding the frequent moults and host finding associated with argasids. Ixodid feeding behaviour is seen in its most extreme form in one-host ticks such as B. microplus. In this species, the tick remains on the host throughout its entire parasitic life, moulting to the nymph and to the adult while on the host and leaving the host only as a fully engorged female after 18-25 days (Wharton & Utech, 1969). In two-host and three-host ticks, juvenile instars drop from the host after engorgement and moult on the ground before reattaching to another host, and finally engorging as adults. Most female ixodid ticks imbibe sufficient blood during feeding to give an increase in weight of about 100-200 times, which allows the production of large numbers of eggs. Males feed intermittently or not at all and may mate on or off the host (reviewed by Oliver, 1974).

Economic losses due to ticks are caused in three ways:-

(1) By feeding in massive numbers, which can lead to anaemia, and heavy infestations with ticks have been notorious in this regard. Each female B. microplus ingests approximately 0.5 ml of blood, and a burden of 5000 ticks on cattle is not uncommon (Roberts, 1952). This blood loss results in unthriftiness, and sometimes death by exsanguination (Barnett, 1961). The puncture wounds themselves result in direct damage to hides and may cause dermatitis and loss of meat production through allergic responses to components of tick saliva; this skin damage can proceed to secondary infestation with screw-worm larvae (Roberts, 1970).

(2) By paralysing the host. The saliva of Ixodes holocyclus, for example, contains paralysing toxins that can be fatal to man, animals

and birds (Seddon, 1968 and reviewed by Murnaghan and O'Rourke, 1978). Even B. microplus, which is well adapted to its host causes toxic effects when ticks are present in large numbers (O'Kelly, Seebeck & Springell, 1971).

(3) By transmitting diseases. Ticks harbour and transmit pathogens including viruses (Hoogstraal, 1973), rickettsiae (Knyvett & Sandars, 1964), bacteria (Balashov, 1972), spirochaetes (Theiler, 1949), anaplasmas (Ristic, 1960), babesias (Smith & Kilborne, 1893), theilerias (Neitz, 1959), filariae (Worms, Terry & Terry, 1961), fungi and toxoplasma (Balashov, 1972) and even flagellates (Muratov & Kheisin, 1959).

1.2 TICK FEEDING AND DIGESTION

The mechanism of tick attachment and feeding was reviewed recently by Kemp, Stone & Binnington (1982). Feeding and digestion in the adult occurs in two main phases. Host tissue and tissue fluid are the main components of early feeding, and digestion of these host components permits the development of the reproductive tissue, salivary glands and other organs but particularly, growth of the cuticle to allow for body expansion during the final phase of rapid engorgement (Lees, 1952; Hackman & Filshie, 1982). This final engorgement is mainly on whole blood (Seifert, Springell & Tatchell, 1968).

Feeding is promoted by secretions from the salivary glands which pass out through the salivary ducts and into the host via the same channel through which food is ingested. The secretions fulfil a variety of functions. They contain several enzymes and pharmacological agents including possibly anticoagulants, and prostaglandins. Earlier work in

the 1920's by Russian authors (reviewed by Arthur, 1965) indicated that the tick saliva also contained haemolysins but, more recently no enzymes which might participate in the digestion of blood have been shown in tick salivary secretions (Tatchell, 1969a). Non-specific esterases were found in the cytoplasm of secretory cells in the salivary glands of Anopheles freeborni and Aedes aegypti, but not in the salivary secretions (Freyvogel, Hunter & Smith, 1968). However, Gooding (1975), in a review on digestive enzymes and their control in haematophagous insects, presented data which indicated that very few digestive enzymes have been found in the salivary glands or their secretions. Ticks therefore differ from mosquitoes in having no contribution from saliva in the digestion of the blood meal.

The salivary gland also secrete the cement which serves to anchor the mouthparts of some species of ticks to the host's skin. Some components of the salivary secretions have antigenic and other properties that set in motion host responses which are normally detrimental to the tick, but may in some cases assist in feeding (Tatchell & Moorhouse, 1968). In ixodids the salivary glands also serve as an osmoregulatory organ (reviewed by Kaufman & Sauer, 1982).

The weight of female ixodid ticks as mentioned earlier may increase 100-200 times during feeding, but the quantity of material ingested is much greater because concentration of the blood meal proceeds during feeding. The excess water and ions pass through the gut epithelium into the haemolymph from where they are returned to the host via specialised salivary gland cells (Kaufman & Phillips, 1973; Meredith & Kaufman, 1973; Kaufman & Sauer, 1982).

Thus while a great deal of attention has been paid recently to ixodid tick salivary glands, there is little new information on the role of the gut cells in osmoregulation or digestion. Changes in the gut epithelium of ticks as a result of blood feeding and digestion were summarised by Balashov (1972). Comparison with the earlier studies is difficult because they were made on different species and at different physiological stages of the tick. It is still not clear whether the cells which were described represent different cell types or different physiological stages of the same cell type. The ultrastructure of these cells in one argasid tick (Grandjean & Aeschlimann, 1973) and one argasid and one ixodid tick (Balashov & Raikhel, 1977,1978; Raikhel, 1974,1975,1978) has been studied. These studies have provided the first clear understanding of the details of tick digestion. Many problems remain; for example, how many secretory cell types are involved in blood digestion in ixodid ticks? Are there specialised cells for water transport to the haemolymph? What cells are specialised for digestion, and how do they arise? What is the sequence of development of these cells, and how does this relate to the feeding rate of the tick?

It is apparent that further study of the gut structure and function in ticks is required to answer some of these questions.

1.3 TRANSMISSION OF PATHOGENS BY TICKS

Ticks, like many other blood-feeding arthropods, are important vectors of vertebrate pathogens. The pathogens, protozoa and other micro-organisms are taken up in the blood meal into the gut; but unlike the situation in other haematophagous arthropods which contain several enzymes in the digestive tract (reviewed by Gooding, 1975) the pathogens are not exposed to a potentially hostile environment in the tick but lumen. They

1.4 OUTLINE AND AIMS OF THE PRESENT RESEARCH

The unifying topic of this thesis is the development of the tick gut. Secondary topics arising from the primary study are, the role of the tick gut in disease transmission, and in host immunity to ticks.

The first aim of my studies has been to apply more recent histological processing such as glycol methacrylate (G.M.A.) and ultrastructural techniques to the gut of B. microplus in order to study some of the questions raised earlier. From Section 1.2 it is clear that the histological modification in the gut epithelium caused by blood feeding and digestion in ixodid ticks is little understood. Furthermore, there is no fine structural information on the secretory cells. This prompted an investigation into the different cell types involved in the digestion of the blood meal in the gut of feeding nymphs and adult females of B. microplus.

Related to this is disease transmission by ticks to livestock, and babesiosis is of major interest because it is almost cosmopolitan in its distribution and affects nearly all domestic mammalian species and some wild ones.

Bovine babesiosis was first recorded in Australia in 1880 by Gilruth (1912a) and as there is only one vector in Australia, B. microplus, there is every reason to believe that the two species of Babesia present were in fact brought into the country with the vector from Java in 1872 (Seddon, 1952).

The cycle of development of Babesia in the tick B. microplus was studied by Riek (1964,1966). Various other workers in various parts of

the world have studied the life cycles of different Babesia species in various hosts (Friedhoff, 1969; Friedhoff & Smith, 1981; Shortt, 1973; Potgeiter, 1977), but no one study has been able to resolve all the details of the life cycle. Most of these studies did not include an examination of the cellular changes in the adult tick gut during the blood feeding and digestion phases. I therefore undertook an investigation into the development of infection in the tick with Babesia as it relates to the development of the gut.

One other topic, which at first sight may not appear to have much relationship to tick gut, is tick control. Chemical pesticides (acaricides) have been used traditionally all over the world to control ticks. In Australia, as in other parts of the world, use of acaricides is becoming increasingly costly and is jeopardised by the progressive development of resistant strains of ticks (Wharton & Roulston, 1970). Strategies less reliant on acaricides are required both for retaining the effective use of present acaricides and avoiding problems of environmental polution and food-stuff residues.

Vaccination using tick antigens to induce resistance to ticks in susceptible animals has been studied for many years but has not yet provided an alternative method for control. Recently, it has been shown that cattle could be vaccinated against ticks with a variable degree of success. Feeding and reproductive performances of Dermacentor andersoni infesting immunised guinea pigs and calves were significantly reduced (Allen & Humphreys, 1979), and the yield of cattle tick B. microplus from vaccinated cattle fell to one third of the control yield (Johnston, Kemp & Pearson, 1983). There is good evidence that an important part of the response of vaccinated cattle is directed against tick gut cells.

The importance of the gut for tick development, for vaccination against ticks, and Babesia transmission is the subject of this thesis. In fact, it is logical to approach these topics in one study because the tick gut cells are important sites for developing parasites and are also the first and possibly the only points of contact between the tick and the host immunological responses. These will be shown to be closely related topics. Light and electron microscope observations have been made on cellular changes during and after blood feeding, on the cell types selected by the developing parasites of B. bovis (Babes, 1889), and on the cells damaged as a result of contact with the antibodies and cells of the vaccinated hosts.

It is suggested here that so little work has been done on the tick gut because it is a difficult tissue for histological and physiological studies. There have been many illustrations of tick gut cells but very few photographs of good histological preparations. The cells of this tissue are delicate as indicated in the only two phyisological studies of tick gut (Barker, Guenther & Sauer, 1977; Kaufman, Kaufman & Phillips, 1981).

As cattle are expensive animals for use in vaccination experiments (where numerous antigens must be tested) attention has been focussed on the use of a laboratory animal model. Since B. microplus will not feed successfully on non-cattle hosts, a chapter of the thesis is devoted to an attempt to immunise rabbits against R. sanguineus using a crude antigen made from partially fed adult ticks. Comparisons are made between the effects of vaccination against B. microplus and R. sanguineus.

or in 0.1M phosphate buffered saline (containing 0.1M NaCl and 0.1mM $CaCl_2 2H_2O$ pH 7.2) and large pieces of gut or whole ticks were fixed either in Carnoys for 1 h at room temperature (Martin, Barnett & Vidler, 1964) or Karnovsky's fixative overnight at 4°C and then processed for methacrylate embedding (Ruddell, 1967 a,b; Feder & O'Brien, 1968). Semithin (1.0 µm) sections were cut on a JB-4 Sorvall microtome and stained either with modified H & E; haematoxylin, toluidine blue-phyloxinate (H & TB-P) (Turner, 1980) or Giemsa, and a number of observations made to cover adequately the progressive development of the gut.

2.2.2 Electron Microscopy

After dissection in Karnovsky's fixative, small pieces of gut were kept in fresh fixative (1° fixation) overnight at 4°C. Tissues were rinsed overnight in the phosphate buffer at 4°C, followed by a brief wash in phosphate buffer at room temperature. Post-fixation was in 1% osmium tetroxide in 0.1M phosphate buffered saline (containing 0.1M NaCl and 0.1 mM $CaCl_2 2H_2O$ pH 7.2) for 1 h at room temperature. Following a brief wash in the phosphate buffer, and two 15 minute changes of 30% ethanol, the pieces of gut were immersed in 1% uranyl acetate en bloc in distilled water for 1 h at room temperature. In most cases en bloc staining was omitted as such treatment could extract glycogen or render it unstainable (Glauert, 1975).

The gut pieces were then dehydrated through an ascending series of ethanol to propylene oxide, and embedded in a mixture of epon and araldite (Glauert, 1975). Sections were cut on an LKB UM III ultramicrotome. Semithin sections mounted on slides were stained either with 1% toluidine blue in 1% borax, or with Lee's modified methylene blue/basic fuschin or with 0.2% Martinotti's toluidine blue as described by Morzaria, Bland & Brock-

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1 TICKS

Two strains of *B. microplus* ticks were used. (i) The *Babesia*-free N strain from the Tick Fever Research Unit, Wacol, Brisbane, Australia (Stewart, Callow & Duncalf, 1982), was used for the studies on the histology and histochemistry of the gut (Chapter 3). (ii) The *Babesia*-free Yeerongpilly strain was used for the studies reported in Chapters 4, 5, 6, 7 and 8. Finally the RS 5 strain of *R. sanguineus* (Green, 1972) was used for the vaccination experiment in Chapter 9.

2.2 FIXATION AND EMBEDDING

2.2.1 Light Microscopy

2.2.1.1 Paraffin embedding: Ticks were collected from the donor calf at 10.00 h each day and were immediately fixed in aqueous Bouins, Helly's or formol acetic alcohol fixatives for 24 h at room temperature. The cuticle was punctured for rapid penetration of the fixative and the ticks were subsequently dehydrated and cleared through supercedrol (Binnington, 1970) and embedded in paraplast. Molliflex (BDH Chemicals - Microscope reagent) was used to soften the blocks before serial sections 3-5μm thick were cut using a Reichert-Jung Biocut Model 1130 with Ralph glass knives (Bennett, Wyrick, Lee & McNeil, 1976) made with Histo Knife Maker LKB2078. Sections were stained with Mayer haematoxylin and buffered eosin (H & E) or with Giemsa (Humason, 1979; Clark, 1973).

2.2.1.2 Glycol methacrylate (GMA) embedding: Nymphs and young adults less than 5 mm in length were processed whole but fed female ticks greater than 5 mm were dissected in Karnovsky's fixative (Karnovsky, 1965)

lesby (1976). The semithin sections were stained for 15 seconds on a hot plate set at 60°C. Ultrathin sections on grids were stained with 1% uranyl acetate in distilled water and with lead citrate (Reynold's method in Glauert, 1975) and were examined in a Philips EM 300 electron microscope.

2.2.3 Histochemistry

For histochemical studies, ticks were fixed in buffered neutral formalin or formol calcium. The Periodic Acid Schiff (PAS) stain of McManus (1948) and Pearse (1972) was used to locate polysaccharide groupings. Loss of PAS staining following the action of diastase (saliva) was taken as an indication of the presence of glycogen. Acidic mucopolysaccharides were identified using the Alcian Blue 8GX pH series, and Alcian Blue/PAS (Steedman, 1950). Haemoglobin was identified using Amido Black (Buffalo black) stain (Puchtler, Rosenthal & Sweat, 1964). For basic proteins, the Azo black (Chlorazol Black E) stain (Cannon, 1941) and the mercuric bromophenol blue staining method of Bonttag (Pearse,1972) were used. Lipids and lipoproteins were identified using frozen sections, stained with Sudan Black B and Scarlet R in ethylene glycol (Pearse, 1972).

2.3 MICROSCOPY

2.3.1 Light Microscopy

The microscope used for all morphological studies was a Carl Zeiss Einbau-Trafo 910137 fitted with 10X eye pieces; 25X 40X dry neofluar and 100X oil immersion neofluar objectives. The microscope was calibrated by placing a Carl Zeiss K8X micrometer graticule in one eye piece, and the linear value of each division on that graticule was estimated by comparing it with a Carl Zeiss micrometer slide placed on the microscope stage. The calculation was done according to the method of Sprent, Hoyte, Pearson & Waddell (1967).

2.3.2 Electron Microscopy

Two microscopes were used:

(i) A.E.I. Corinth Electron Microscope EM 275, and

(ii) Philips Electron Microscope EM 300.

2.4 PHOTOGRAPHY

2.4.1 Light Photomicrography

All photomicrographs were taken using a Carl Zeiss 4325202 microscope equipped with 25X, 40X, 63X and 100X planapo oil immersion objectives with a tube factor of 1.25, and with a Zeiss MC 63 photographic unit.

Kodak SO 115, TP 2415 and Ilford Pan F Black and White panchromatic films were used for photographing the sections and Ilfo print or Agfa glossy paper was used for printing. Colour micrographs were taken using Kodak Colour II and Kodak Ektachrome 50 professional film and printed commercially on Ilford Cibachrome paper.

2.4.2 Electron Photomicrography

The original electron micrographs were taken between 2,000-42,000 magnifications, and were enlarged from 2-5 times with a Durst Laborator S-45 Special Enlarger. Films were developed in Ilford PQ Universal developer and printed on Ilfobrom glossy single weight paper. The prints were developed by the Ilford Bromophen developer process.

2.4.3 Macroscopic Photography

A Pentax Asahi 35 mm Single Lens Reflex (SLR) camera was used for the picture of ticks on the ears of rabbits using Kodak TP 2415 Film, and a Wild M8 stereomicroscope with camera attachment and Wild photo Automat MPS 55, was used for the photo of red ticks using Kodak Ectachrome 50 professional film.

CHAPTER 3

DIGESTION IN B. MICROPLUS.

LIGHT MICROSCOPE STUDY OF THE GUT CELLS IN NYMPHS AND FEMALES

3.1 LITERATURE REVIEW

Cattle rearing, in many tropical and sub-tropical countries, is jeopardised by the tick *B. microplus* because it is an efficient vector of *B. bovis*, a major pathogenic protozoal disease (Mahoney, 1962; Ristic, 1970) and because its high reproductive rate and heavy infestation depress cattle growth (Sutherst & Utech, 1981). The importance of this and other ticks in disease transmission has led to numerous studies on the internal anatomy of ticks and the development of the disease agents within them.

In the early studies, emphasis was mainly on the general anatomy of ticks (Williams, 1905; Christophers, 1906). Subsequent papers gave some detail of the internal anatomy and histology (Nuttall & Strickland, 1908; Cornwall & Patton, 1941; Douglas, 1943), while others provided some details of the digestive system and digestion (True, 1932; Roesler, 1934; Hughes, 1954; Till, 1961; Chinery, 1964; Sonenshine & Gregson, 1970; Khalil, 1971;' Balashov, 1972; Grandjean & Aeschlimann, 1973). Other studies laid emphasis on development of parasites within the tick (Riek, 1964,1966; Friedhoff, 1969; Shortt, 1973; Mehlhorn & Schein, 1976,1977; Potgieter & Els, 1977; Mehlhorn, Schein & Warnecke, 1978; Kocan, Ewing, Holbert & Hair, 1982; Moltmann, Mehlhorn & Friedhoff, 1982a,b). In an excellent combination of these two fields of study, Fawcett, Büscher & Doxsey (1981a,b,1982b) and Binnington, Young & Obenchain (1983) have followed the development of the salivary glands of *R. appendiculatus* and the preferential selection by *T. parva* of the type 'e' cells of Acinus III for the development of the sporoblast and sporozoite stages.

The salivary glands have received most attention, starting at the turn of this century when Nuttall & Strickland (1908) detected the presence of anticoagulin in disected organs of the Argasid tick, Argas persicus (Oken). Anticoagulating properties were also found in the salivary glands of I. holocyclus (Ross, 1926; Kaire, 1966), Ixodes ricinus (Foggie, 1959) and O. moubata (Hawkins & Hellman, 1966). Anticoagulants in B. microplus were not found in saliva (Tatchell, 1969a), though a proteolytic enzyme inhibitor with anticoagulant properties was found in homogenates of B. microplus larvae (Willadsen & Riding, 1979). This enzyme inhibitor was antigenic, which suggested that it was secreted into the host via saliva. It is just possible that the inhibitor was introduced to the host immune system by regurgitation (Gregson, 1967; Tatchell & Moorhouse, 1968).

Recent studies by Balashov (1972), Coons & Roshdy (1973), Binnington (1978), Megaw & Beadle (1979), Fawcett et al. (1981a,b), and Kaufman & Sauer (1982) have greatly improved our knowledge of the structure and function of the tick salivary glands.

In recent years, there has been many fewer papers dealing with the structure and function of the tick gut than with the salivary glands. However, the development of secretory and digestive cells in the gut has been followed in argasid ticks by Tatchell (1964), Gurgis (1971), Aeschlimann & Grandjean (1973), Balashov & Raikhel (1977,1978), and in the nymphs of the ixodid tick H. asiaticum from fasting through to engorgement (Balashov & Raikhel, 1976).

3.2 INTRODUCTION

The literature cited suggests that *B. microplus* is an efficient vector of bovine babesiosis (Mahoney, 1962; Ristic & Kreier, 1981). The importance of this tick has led to numerous studies of its biology, but there has been little work on the digestive system.

In ticks, blood digestion is a slow intracellular process (Balashov, 1972). Some aspects of digestion in *B. microplus* have been studied. Kitaoka (1961) measured the rate of digestion of the blood meal, and the nitrogen, iron and sterol economy during oviposition, and O'Hagan (1974) studied the digestion of haemoglobins by the engorged female tick. Apart from these studies which were limited to the engorged females, the gut structure, function, and the cytological modifications of the gut epithelium caused by blood feeding and digestion are unknown.

The papers by Balashov & Raikhel (1976) and Raikhel (1974,1975,1978a) have clarified the process of intracellular digestion in *H. asiaticum*. However, their papers report little detail of the secretory cells and their function. In any case, digestion in the one-host tick *B. microplus* may also be specialised because they are adapted for continuous development on the host.

There are several reasons why we need to know the details of the gut histology of *B. microplus*, and when and where secretion and digestion occurs. Firstly, a degree of success not previously reported for vaccination against *B. microplus* was obtained by Johnston *et al.* (1983). The source of material for vaccination was an antigenic preparation from partly-fed female *B. microplus* and the contention at the moment is that at least some of the important antigens in the preparation come from the tick

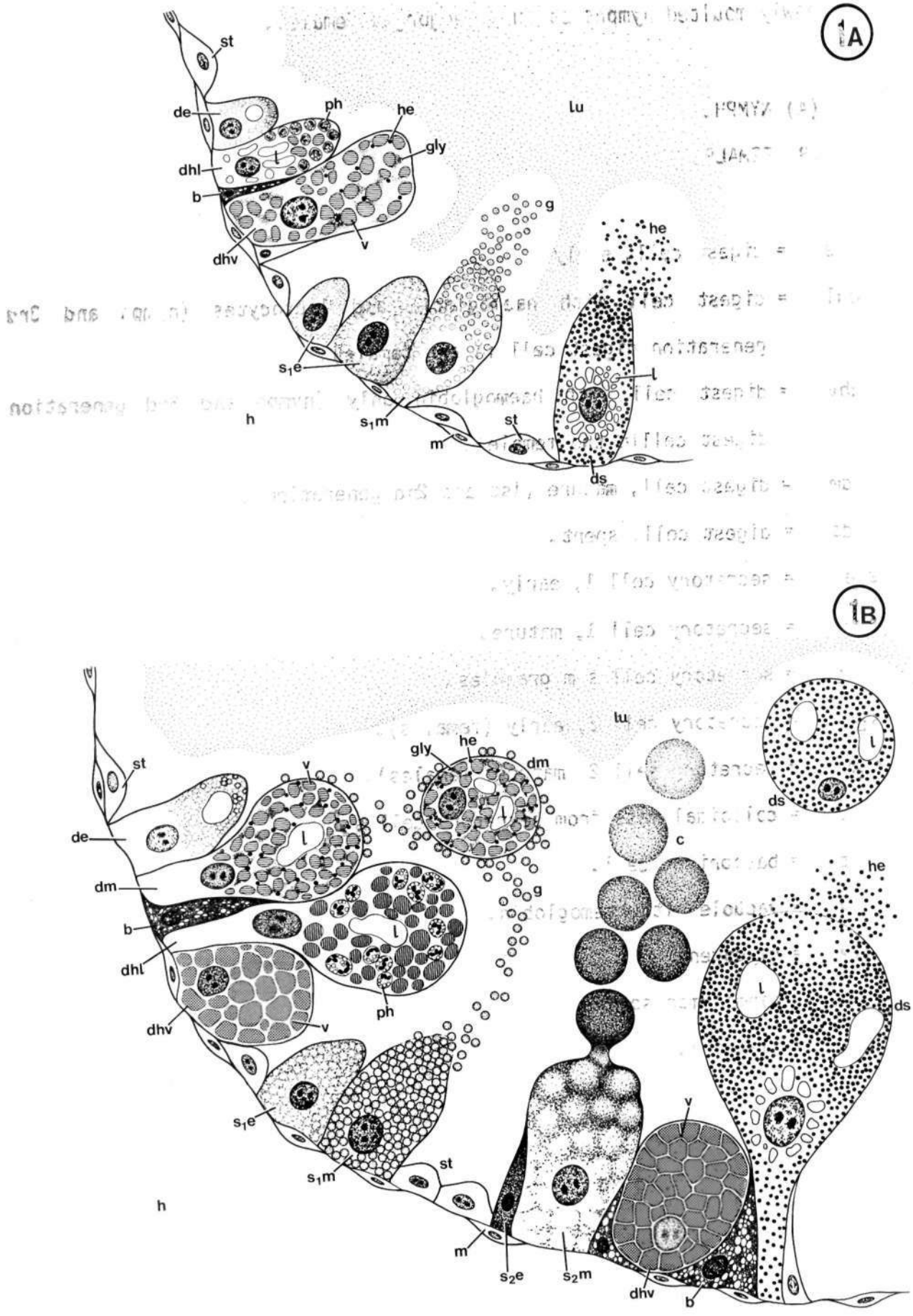
gut (Kemp, Agbede, Johnston, Pearson & Gough (1984), submitted for publication, and Chapter 8). Secondly the histology of normal gut development is needed for comparative histopathology with the gut of ticks from vaccinated animals which is the subject of Chapter 8. Finally the results in this chapter will form the basis for subsequent studies on the ultrastructure of the gut cells (Chapters 4, 5 & 6) and the development of B. bovis in the gut (Chapter 7).

In this chapter I have therefore followed digestion in B. microplus in the nymphs, through the period of moulting to the adult and subsequently to the engorged females.

3.3 MATERIALS AND METHODS

The ticks used were the Babesia-free N strain (Stewart et al., 1982). They were cultured at 30°C and 90-100% R.H. Ticks at various stages of development were collected from Hereford-cross calves (Bos taurus) purchased from a B. microplus-free zone.

Ticks were fixed at the following stages:- pharate nymphs, newly moulted nymphs (from engorged larvae incubated at 35°C and 70-80% R.H., until the nymphs emerged), feeding nymphs on days 8, 10, 12 and 13 after infestation as larvae, pharate females on days 14, newly moulted females (from engorged nymphs incubated at 35°C and 70-80% R.H. until females emerged) and females on days 15, 16, 17 and 19 after infestation as larvae. Because of variation in the tick's rate of development on the host, the length of females was measured as a guide to their stage of development (Roberts, 1968). Ticks were collected from the donor calf and fixed and processed for both paraffin and methacrylate embedding as described in Chapter 2.


1A
st
de
ph
he
lu
gly
dhl
b
g
v
dhv
he
s_1e
l
h
s_1m
st
m
ds
1B
lu
gly
he
dm
st
v
l
ds
de
c
dm
g
he
b
l
dhl
ds
ph
dhv
v
s_1e
v
s_1m
st
h
m
s_2e
s_2m
dhv
b

Five ticks were taken for each stage and at least three were sectioned and stained for analysis. Sections were cut in three planes; transverse, vertical and sagittal. Cell types were identified, including dividing cells, and counted in representative caecae to a total of at least 100 cells.

3.4 RESULTS

The development of cells from undifferentiated stem cells to a number of secretory and digestive cell types is shown diagrammatically in Figure 1, and the nomenclature detailed in Table 1.

TABLE 1. CELL TYPES IN THE GUT OF IXODID TICKS

This Thesis	Balashov (1972)*
Boophilus microplus	*Hyalomma asiaticum* and *Ixodes ricinus*
Stem Cell	Reserve cell
Digest cell Series including subtypes I and II of the 3rd generation	Digest cell 1 & 2
Basophilic cell	No equivalent
Secretory cell (s_1)	No equivalent
Secretory cell (s_2)	Secretory cell

* The cell types described by Hughes (1954), Till (1961), Chinery (1964) and Tatchell (1964) were discussed by Balashov (1972).

3.4.1 Description of Cells

3.4.1.1 *Stem cell* (Figs. 1 & 2). This cell is usually dome-shaped and is found in the early stages of each instar, although it is most numerous at the mid-stage of nymphal and adult development. The cell is undifferentiated and cytoplasmic organelles cannot be distinguished by light microscopy.

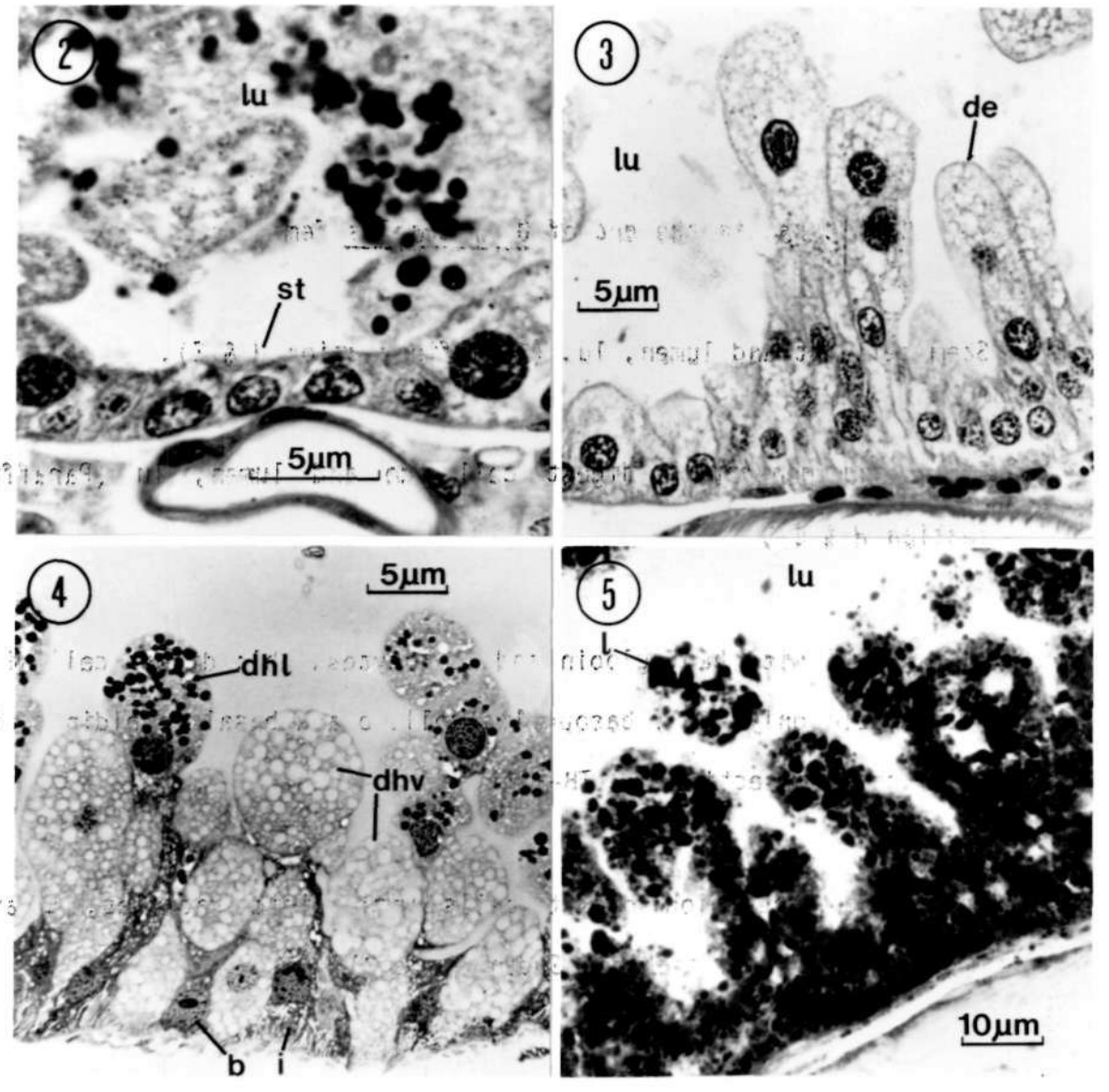
FIGS. 2-5. Cell types in the gut of *B. microplus* females:

2. Stem cell, st and lumen, lu. (Paraffin section H & E).

3. Early 2nd generation digest cell, de and lumen, lu (Paraffin section H & E).

4. Digest cell with haemoglobin and leukocytes, dhl; digest cell with haemoglobin only, dhv; basophilic cell, b and basal infoldings, i. (Methacrylate section H & JB-P).

5. Early 2nd generation digest cells with lipid vacuoles, L and lumen, lu. (Frozen section, Sudan Black B).


2
lu
st
5µm
3
de
lu
5µm
4
5µm
dhl
dhv
b
i
5
lu
l
10µm

3.4.1.2 Digest cell series. These cells are derived from the stem cell and intermediate stages are recognisable (Figs. 1, 3 & 4). The earliest stage (de) is larger than the stem cell and has a few small food vacuoles and some large vacuoles which stain for lipid (l) (Sudan Black B) (Figs. 1 & 5). The next stages (dl, dm) have small and large food vacuoles containing haemoglobin (Amido Black stain) and other soluble blood constituents presumably ingested by pinocytosis. The large vacuoles increase in size and number, possibly by accretion of the small vacuoles. During adult feeding two types of this cell become distinguishable, the first has many vacuoles containing host leukocytes and presumably soluble constituents of the diet (dhl), the other (dhv) develops later and has no leukocytes in the large food vacuoles (Figs. 1, 4 & 6). Haematin granules (he) accumulate in the cells as digestion progresses. At first they are small and yellowish, later becoming larger and darker in colour as described by Wigglesworth (1954). An unusual digest cell occurs in the newly moulted nymphs and adults and these appear to be derived from old digest cells which have shed their haematin.

Spent digest cells (ds) are full of haematin granules (Fig. 7) and some vacuoles which stain for lipid. Some are budded into the lumen where they either rupture or are excreted whole. Others, still attached to the basal lamina, rupture and release their haematin. The rectal sac contains free haematin granules, and occasionally an intact spent digest cell (ds).

3.4.1.3 Secretory cell type 1 (s_1). Two forms of this cell, an early (s_1e) and mature form (s_1m) can be distinguished. Both forms have a dark staining nucleus and homogeneous orange eosinophilic granules in the cytoplasm (P.A.S. stain, Figs. 1, 8 & 10), with the mature form (s_1m) having a greater density of granules and granules apparently being released into the lumen. The granules (g) are P.A.S. positive and diastase (saliva) fast, indicating the presence of a glycoprotein.

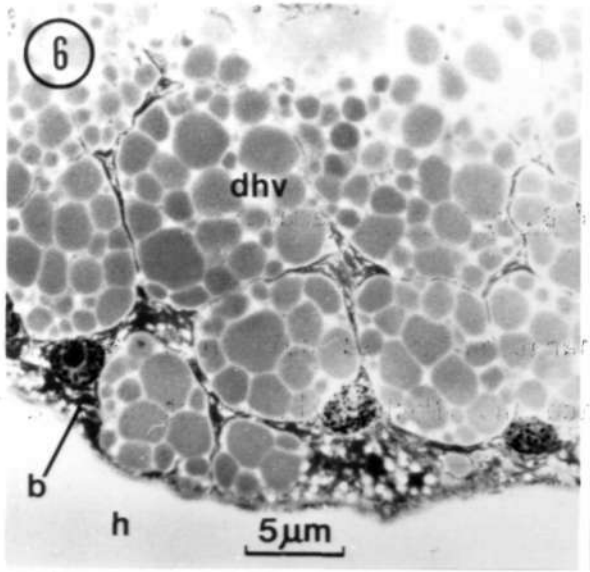
FIGS. 6-9 Cell types in the gut of *B. microplus* females:

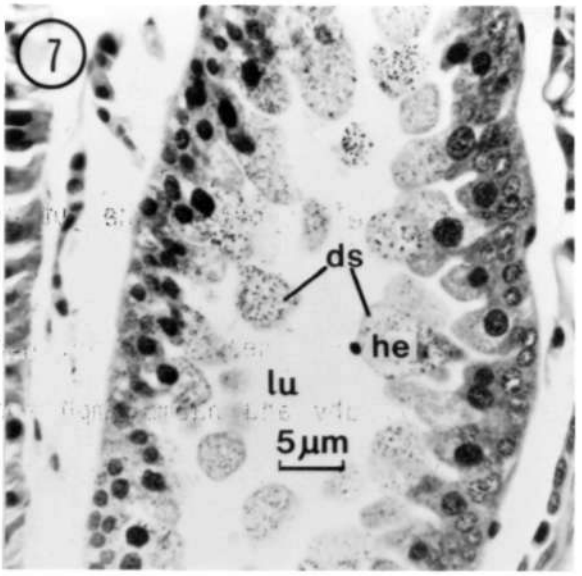
6 Basophilic cell, b; 3rd generation digest cell with haemoglobin only, dhv and haemolymph space, h. (Methacrylate section, H & TB-P)

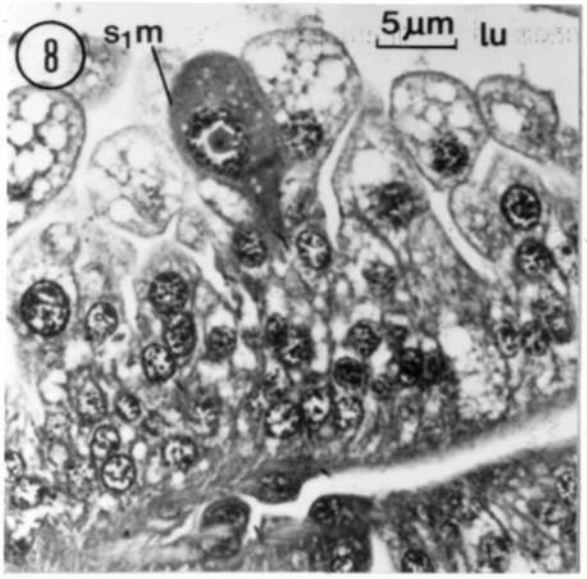
7 Spent digest cells, ds; haematin granules, he; lumen, lu. (Paraffin section, H & E)

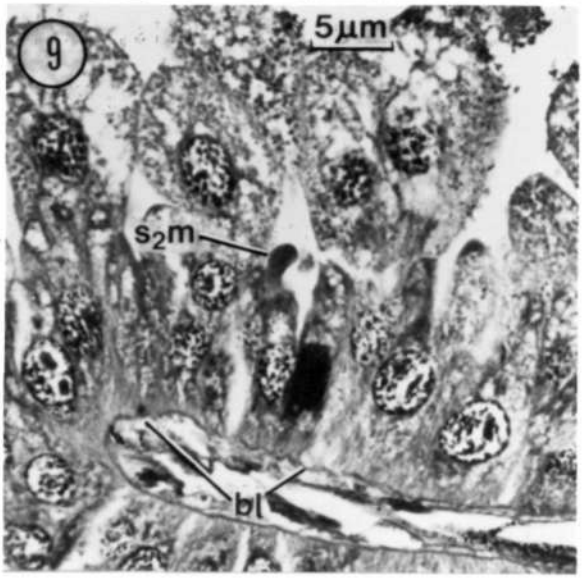
8 Mature secretory cell type 1, s_1m and lumen, lu. (Paraffin section, P.A.S.)

9 Mature secretory cell type 2, s_2m; and basal lamina, bl. (Paraffin section, Alcian blue/P.A.S.)


6
dhv
b
h
5µm


7
ds
he
lu
5µm


8
s1m
5µm
lu


9
5µm
s2m
bl

FIGS. 10-12 Histochemistry of the cell types in the gut of *B. microplus* females.

FIG. 10 Mature secretory cell type 1, s_1m; glycogen, gly; lumen, lu; haemolymph space, h. (Methacrylate section, P.A.S.)

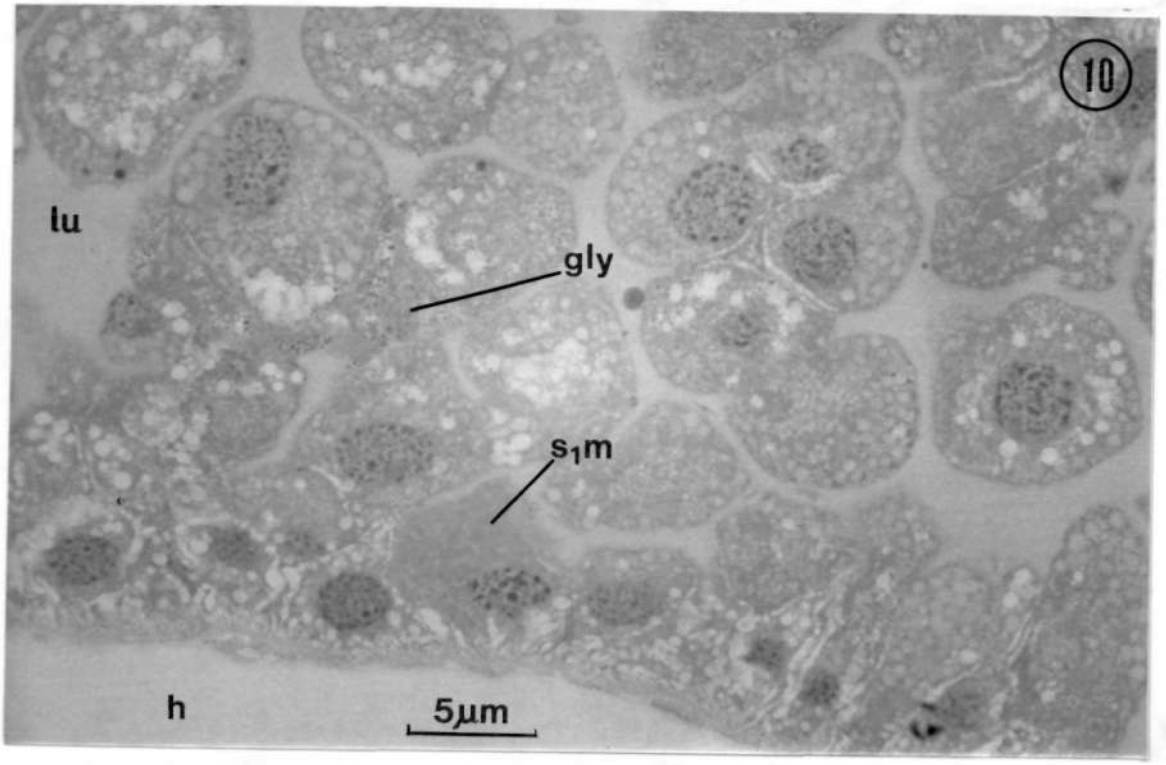
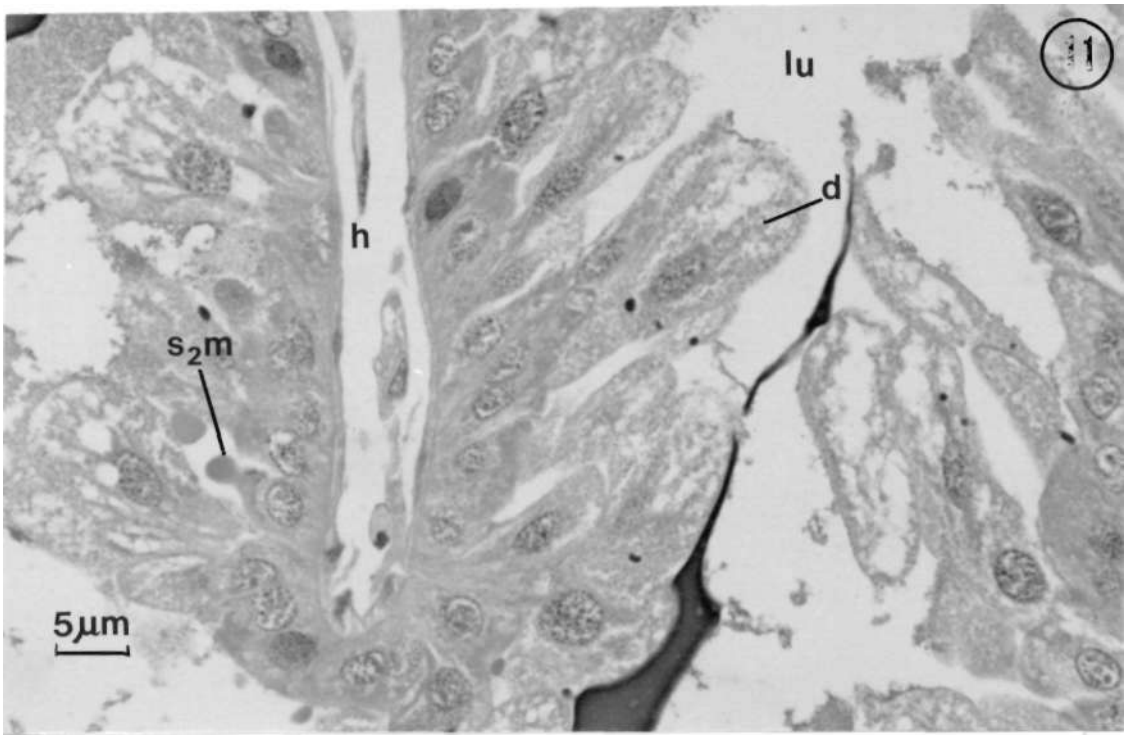
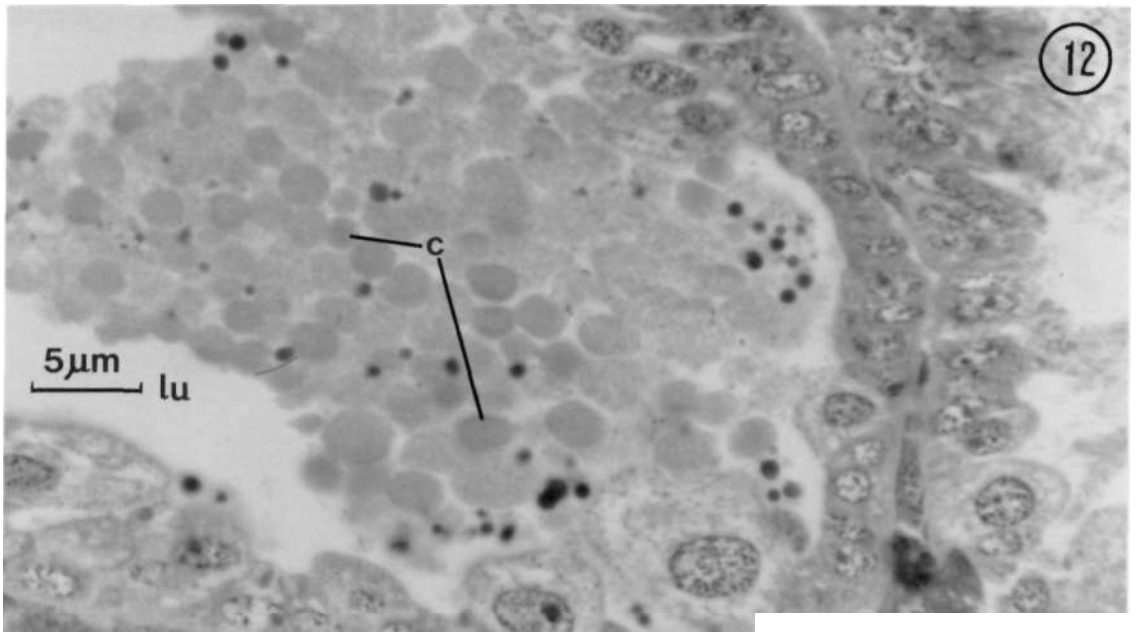

10
lu
gly
s_1m
h
5µm

FIG. 11. Mature secretory cell type 2, s_2m; lumen, lu; haemolymph space, h; digest cells, d. (Paraffin section, Alcian blue/P.A.S.)

FIG. 12. Lumen "colloidal mass" c, from secretory cells type 2; lumen, lu. (Paraffin section, Alcian blue/P.A.S.)


11
lu
d
h
s_2m
5µm


12
c
5µm
lu



3.4.1.4 Secretory cell type (s_2). This cell is found only in females, and in the immature form (s_2e) is a narrow dark cell with an intensely staining nucleus (H & E). The mature cell (s_2m) has a highly granular cytoplasm which it secretes as a "colloidal" mass (c) when the apical end of the cell buds off into the lumen (Figs. 1, 9 & 11). The "colloidal" mass (Fig. 12) is faintly P.A.S. positive, and also positive when stained in a combined Alcian Blue 8 GX and P.A.S. stains.

3.4.1.5 Basophilic cell. This is a triangular basophilic cell (b) which stains intensely blue to purple with haematoxylin (Figs. 1 & 6). The cell has extensive basal infoldings in the adult and undercuts adjacent digest cells. In the nymphs, the cell differentiates from the stem cell and is first observed late in the nymphal stage (day 12). In the adult female it is more densely staining and appears just before rapid engorgement (4-6 mm females) and persists until post-engorgement and death. In the females, the basophilic cell appears to be derived from the basal remnant of secretory cell type 2 (s_2m).

3.4.2 Gut Cell Development During Feeding of Nymphs

3.4.2.1 Pharate nymphs (day 6) and newly moulted nymphs (day 7). These two stages are very similar (Table 2 & Fig. 13). In the pharate nymph the gut lumen is surrounded by a thin unicellular layer of cuboidal digest cells and a few larger haematin containing digest cells which remain from the larval instar. Very few secretory cells are seen within this layer. There are no cells with discernible food vacuoles and no cells with nuclei in division. In the newly moulted nymph, the digest cell is again the main cell present, and now the cell has various sizes and shapes. Many have enlarging nuclei, corresponding to the enlarging intermediate cells of the digest cell series (Fig. 1A). Secretory cells are rarely seen within this layer.

3.4.2.2 Nymph (day 8 - slow feeding). The day 8 nymph has started feeding and the caecae present a striking change (Table 2 & Fig. 13); the cells are larger and possess larger nuclei. Stem cells enlarge from 11.1 ± 0.6 µm to become digest cells of 36.2 ± 5.5 µm (basal to apical measurements) and the nuclei have a corresponding increase in diameter from 7.4 ± 0.7 µm to 11.9 ± 0.5 µm. A few of the digest cells are cuboidal in shape, while others are dome-shaped and contain small food vacuoles which are fairly evenly distributed in the cells. Many of the cells have a few larger food vacuoles, unstained vacuoles (lipid) and a few small yellow to dark brown haematin granules. The most advanced digest cells (dhl) still have food vacuoles and have not yet become typical spent digest cells (ds, Fig. 1A). However, their presence suggests a progression from advanced digest cells to spent digest cells. Secretory cells are virtually absent (<1%). Stem cells are few but since some are in division there appears to be a new generation of stem cells forming. The lumina of the gut caecae are enlarging as more cells are produced.

3.4.2.3 Nymph (day 10). This stage has a uniform cell composition in all the caecae studied. A striking feature is the increased numbers of stem cells (Table 2 & Fig. 13). Most of these cells seem to be enlarging, some are dome-shaped and others becoming cuboidal as they expand. There are, in fact, all stages of cells intermediate between stem cell and digest cell. Some cells contain pale eosinophilic granules on the luminal border which appear to be small food vacuoles. The tick is feeding slowly and host blood cells and tissue fluid are seen in the lumen.

Secretory cells (s_1e) and (s_1m) are present in low numbers at this stage.

TABLE 2. CHANGES IN THE CELL COMPOSITION OF THE GUT DURING FEEDING OF NYMPHS AND FEMALES OF **B. MICROPLUS***

Stages of Development of The Tick	Percentage of Cell Types Occurring at Each Stage of Development						
	Stem Cells	Stem Dividing	Digest Cells	Spent Digest Cells	Secretory (s_1)	Secretory (s_2)	Basoph Cel
Pharate Nymph - Day 6	0	0	92	8	<1	0	0
Newly Moulted Nymph - Day 7	8	0	90	2	<1	0	0
Feeding Nymph - Day 8	14	3	78	4	1	0	0
Feeding Nymph - Day 10	39	2	50	8	1	0	0
Feeding Nymph - Day 12	21	0	53	26	0	0	0
Engorged Nymph - Day 13	0	0	90	3	0	0	7
Pharate Female I & II - Day 14	0	0	92	0	1	0	7
Adult Female - Day 15	2	0	90	1	0	7	0
Adult Female I - Day 16	40	1	50	1	0	8	0
Adult Female II - Day 16	23	3	60	6	0	8	0
Group 1 Females (3 mm) - Day 17	16	4	70	2	0	8	0
Group 2 Females (4-5 mm) - Day 17	14	0	74	0	2	8	2
Group 3 Females (5-6 mm) - Day 17	2	0	83	0	3	7	5
Group 4 Females (7-8 mm) - Day 17	0	0	Subtype I - 25 Subtype II - 61	0	0	0	14
Fully Engorged Female - Day 19	0	0	Subtype I - 26 Sybtype II - 36	0	0	0	38

* 100 cells counted at ech stage of development.

FIGURE 13. SUMMARY OF GUT CELL DEVELOPMENT

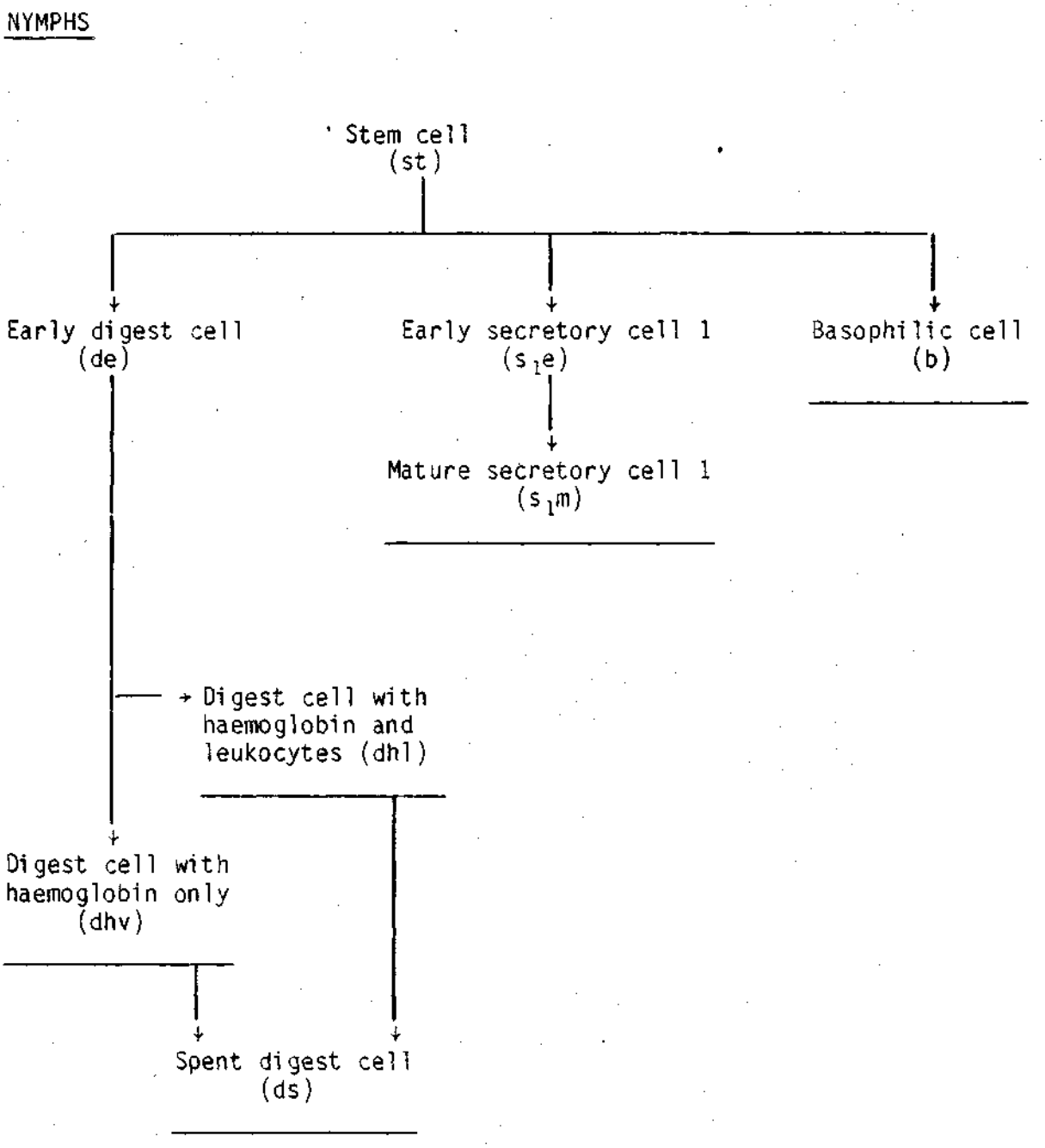
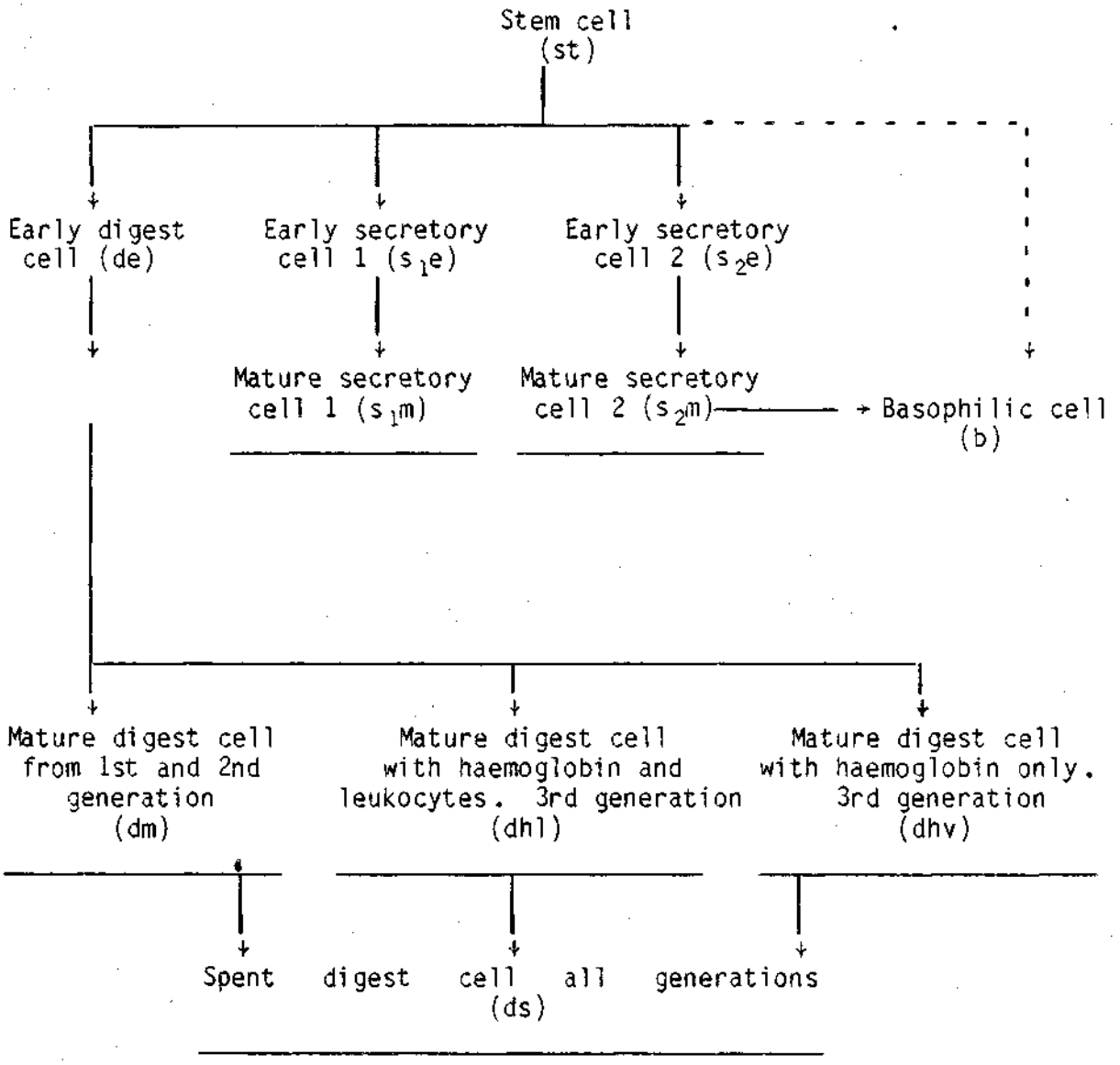


FIGURE 13. CONTINUED

FEMALES



3.4.2.4 Nymph (day 12). The different caecae are again uniform in structure and no dividing nuclei are seen (Table 2 & Fig. 13). The lumen is large as the tick is engorging rapidly. Erythrocytes in the ingested blood appear clumped and the distinct erythrocyte morphology is no longer discernible due to paler staining of the cell cytoplasm. It is noticeable that secretory cell type 1 (s_1) is absent and stem cells are declining in numbers.

Digest cells are the most numerous cells and are very actively ingesting host material. These cells contain many small to large food vacuoles. They include all stages between early digest cells and spent digest cells. Some cells are like spent digest cell (ds) with many haematin granules, but still contain a few medium sized food vacuoles. Many spent digest cells (ds) are also present, and most are still attached to the basal lamina.

3.4.2.5 Fully engorged nymph (day 13). The lumina are larger still and filled with blood. Stem cells are absent, most having apparently transformed into the digest cells and into much smaller numbers of basophilic cells (Table 2 & Fig. 13). The digest cells are all packed with food vacuoles, most of which are large and stain heavily with eosin. Some cells contain haematin granules and digestion appears to be progressing rapidly. The density of the food vacuoles in the digest cells is varied; some cells are tightly packed and are very darkly stained, others are less packed, have empty spaces between them and are less heavily stained. Digest cells (dhv, dhl) have enlarged to such an extent that in many caecae, there is no lumen visible (Fig. 1A).

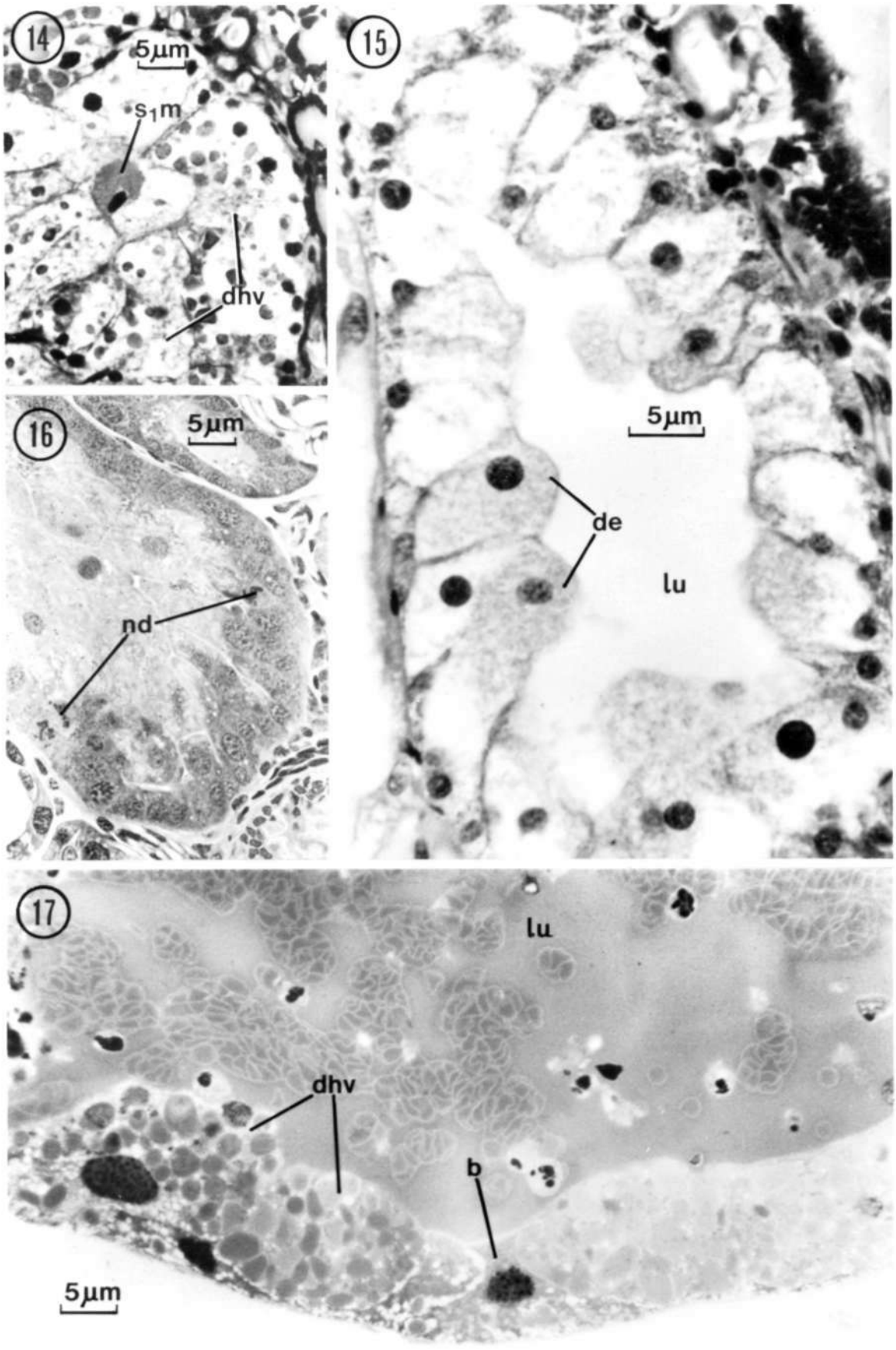
FIGS. 14-16. Cell types in the gut of *B. microplus* females.

14. Pharate adult female I. Secretory cell type 1, s_1m; digest cell, dhv. (Paraffin section, H & E)

15. Pharate adult II. Digest cell of the 1st generation, de; lumen, lu. (Paraffin section, H & E).

16. Nuclear division in stem cell, nd. (Methacrylate section, H & TB-P).

17. Third generation digest cell, dhv; basophilic cell, b; lumen with host erythrocytes and leukocytes, lu. (Methacrylate section, H & TB-P).


14
5μm
s_1m
dhv
15
5μm
de
lu
16
5μm
nd
17
lu
dhv
b
5μm

Secretory cells are absent, but for the first time basophilic cells (b) appear with their cytoplasm darkly stained by haematoxylin. The nuclei of these cells lie just above or on the basal lamina, and the cytoplasm appears to extend laterally thus separating adjacent digest cells from the basal lamina.

3.4.3 Gut Cell Development During Feeding of Females

3.4.3.1 Pharate female I (day 14). The digest cells remain the most numerous cell during the moult (Table 2 & Fig. 13), but active digestion has left only a few recognisable food vacuoles in the cytoplasm. Digest cells from the previous two days are no longer recognisable and may have shed their haematin into the lumen (see later). A few secretory cells are seen, but not in all caecae, and the basophilic cell is still present (Fig. 14).

3.4.3.2 Pharate female II (day 14). The cell numbers here are the same for pharate female I, but the cells are more advanced. The food vacuoles have disappeared from the main cell present, the digest cell, which does not appear to be active in this stage (Fig. 15). Secretory cells are present and basophilic cells although present are few.

3.4.3.3 Unfed adult female (day 15 - moulted off host). The gut is similar to that of the pharate adult female II (Table 2 & Fig. 13). The early digest cell (de) is the main cell throughout all caecae. It is cuboidal and is free of staining food vacuoles (Fig. 1). A few spent digest cell (ds) which contain large and numerous haematin granules are present in the lumen. Between the digest cells there appear for the first time a few early secretory cells type 2 (s_2e) which stain intensely with haematoxylin. A few stem cells are present but there are no secretory cell type 1 (s_1e).

3.4.3.4 Adult female (day 16 - slow feeding). Some female ticks moulted earlier than others on the host, resulting in ticks of different sizes by day 16. They were separated into two populations (I & II) on the basis of size, and five ticks from each population were sampled (Table 2 & Fig. 13).

(a) Day 16, I. These ticks are essentially the same as day 15 unfed females, except that the lumina here contain what appears to be tissue fluid and blood leukocyte fragments, suggesting that feeding has begun. However, digest cells (de) have not yet extended into the lumen. There is no evidence of food vacuole formation but a few digest cell contain small yellowish haematin granules. Stem cells are present and possess enlarging nuclei. The secretory cell type 2 (s_2m) is enlarging also and stains lightly with haematoxylin. Cell division is apparent, presumably giving rise to the much greater number of stem cells compared with day 15 females.

(b) Day 16, II. These ticks were also picked on day 16 and show a slightly different picture to day 16, I ticks, with evidence of more cell activity (Table 2 & Fig. 13). Stem cells enlarge from 18.0 ± 1.7 µm to digest cells of 64.6 ± 8.1 µm (basal to apical measurements) and the nuclei have a corresponding increase in diameter from 9.6 ± 0.2 µm to 15.1 ± 1.1 µm. Digest cells are present in various sizes (Fig. 3), digestion is progressing and lipid is being stored in the cells (Fig. 5). The lumina have some yellowish to dark brown haematin granules presumably ejected from a few earlier spent digest cells. The lumina also contain many host erythrocytes showing that these ticks have started feeding on whole blood. Secretory cell type 2 (s_2m) is present and enlarging.

3.4.3.5 Adult female (day 17 to fully engorged). By day 17, females very different in size are found because of the different individual rates of development. Five ticks each of various sizes were sampled on this day to accommodate these differences in physiological development (Roberts, 1968; Wharton & Utech, 1969).

(a) Group 1; 3mm Females (day 17, 2 days before engorgement). The picture here is uniform. Many mitotic divisions (Fig. 16) are characteristic of this stage and digest cells apparently of the third generation are abundant (Table 2 & Fig. 13). Some of these cells are small, and have "empty" cytoplasm, others are larger and have started ingesting the blood meal, and contain small food vacuoles (de). Digest cells are either attached to the basal lamina or are free in the lumen and contain numerous haematin granules. A few intermediate stages exist with a combination of food vacuoles and small haematin granules.

Secretory cell type 2 (s_2e) are still enlarging and appear to be stretching towards the lumen.

(b) Group 2; 4 - 5 mm Females (day 17 - collected 10.00 h, 1 day before engorgement [Wharton and Utech, 1969]). More differentiated cells are present than in the gut of 3.00 mm females of the previous stage (Table 2 & Fig. 13). Stem cells are still present, but some have started to develop into digest cells (de). Secretory cells type 2 (s_2m) have started to bud off cytoplasm into the lumen, and the first few basophilic cells (b) can be identified. Secretory cell type 1 (s_1m) are present and have orange to red granules in the cytoplasm (Fig. 10) (P.A.S. stain).

The tick has just completed the slow phase of feeding and digestion. Presumably most of the remains of this first phase of digestion are excreted because there are no spent digest cells (ds), and no haematin granules in the lumen.

Digest cells (de) are abundant and bulge further into the lumen. The cells have numerous large vacuoles which stain for lipid and a few food vacuoles but no haematin granules. The tick is now ready to begin the stage of rapid feeding.

(c) Group 3; 5 - 6 mm Females (day 17, collected 10.00 h 1 day before engorgement). This is more advanced than the 4-5 mm ticks. Digest cells (de) are larger and spreading as if being stretched by the enlarging lumen as the tick begins to take in large quantities of host blood. The host erythrocytes are still intact in the lumen and a few leukocytes are present, mainly neutrophils. The digest cell (de) are present in various shapes and sizes. The largest which are club-shaped, begin to bulge into the lumen and contain very small food vacuoles. Some cells have clear cytoplasmic vacuoles which stain for lipid. No haematin is visible in these cells and spent digest cells (ds) are absent (Table 2 & Fig. 13).

Some of the secretory cells type 1 (s_1m) are bulging into the lumen where they appear to be discharging their red to orange granules. A few more secretory cells type 2 (s_2m) are beginning to bud off the "colloidal" mass into the lumen and basophilic cells (b) are more numerous.

(d) Group 4; 7 - 8 mm (day 17, collected 10.00 h, 1 day before engorgement). The lumina of these ticks are larger than in the previous group. Basophilic cells (b) are squeezed between the enlarging digest

cells (dhl, dhv) with extensions of their cytoplasm separating much of the digest cells (dhv) from the basal lamina. Methacrylate sections show these basophilic cells to have deep basal infoldings (Fig. 4).

Digest cells (dhl) of the third generation appear to have ingested many leukocytes from the blood meal whereas the lumina contain very few leukocytes (Fig. 4). Spent digest cells (ds) are again absent.

3.4.4 Fully Engorged Tick (day 19)

Ticks fully engorged can be collected on days 18-23 (Roberts, 1968). The fully replete ticks were taken on day 19 and were not more than 24 h older in physiological age than the Group 4 females of day 17. Basophilic cells (b) again deeply undercut the digest cells (dhv) and appear to extend towards the lumen as the digest cells grow. They contain a few small granules which could not be identified as haematin in light microscopy. Digest cells of the third generation now have two subtypes at this stage (Fig. 1-4 & 13; Tables 2). The first (dhl), as in group 4 (day 17), has many host leukocytes in the food vacuoles as well as whole erythrocytes and presumably the non-cellular components of host blood. Cells of the second type (dhv) contain mainly the non-cellular components of the diet in large vacuoles. This second type may contain most of the haemoglobin as cell lysis has occurred in the lumen. A range in size of the digest cells could reflect the different ages of the cells. In the fully engorged ticks the gut caecae stretch, such that in places, only a thin layer of flattened cells line the gut lumen (Fig. 17).

3.5 DISCUSSION

Balashov (1972) described three basic cell types in the gut of the ixodid tick Hyalomma asiaticum; namely, reserve cell, digest cell and

secretory cell. He proposed that all gut cells have a common origin, the undifferentiated reserve cell being the initial type from which the other cells differentiate. In addition, secretory and digestive cells are irreversibly specialised. When the functional activity of these cells end, the cells degenerate. Nevertheless, one exception was noted and he observed a few secretory cells containing large haemoglobin food vacuoles, i.e. secretory cells transforming to digestive cells. Other investigators also believed that the three different cell types listed above comprise the midgut of ticks (Roesler, 1934; Hughes, 1954). Still others believed that the gut wall consists of one cell type, the form and structure of which may vary during different digestion phases and that these cells perform secretory and food digestion functions (Till, 1961; Tatchell, 1964; Chinery, 1964; Guirgis, 1971; Khalil, 1971).

Results from the sequential histological and histochemical studies in this investigation confirm Balashov's (1972) opinion that cells with different functions arise from the stem cell. The name "Stem Cell" is more apropriate than "Reserve cell" which is the translation given in Balashov (1972) since "reserve cell" is a term which can be confused with the later stages of digest cells in which reserves of food are stored. Digest cells differentiate from the stem cells, and my observations of their development shows that there is a series of intermediate stages. When fully developed, two types of digest cells are to be observed. The first (dhl) is responsible for rapid digestion during slow feeding and growth. Phagocytosis is evident from the presence of host leukocytes in food vacuoles and pinocytosis is suspected because of ingestion of host fluids. The second digest cell type (dhv) is involved with the digestion of the blood meal taken in rapidly during the final stages of engorgement. It has been suggested (Balashov, 1972; Balashov and Raikhel,

1976) that these cells are specialised for haemoglobin digestion. However, there is no direct evidence for specialisation. The presence of haematin granules in the digest cells of the first type (dhl) shows that these cells also can digest haemoglobin.

The most interesting discovery in this study is the presence of two secretory cell types in the gut of B. microplus. Only one type has been found in other ticks (Tatchell, 1964; Balashov 1972) and it has been suggested from histochemical studies that these cells contain anticoagulants or haemolysins, rather than digestive enzymes. Secretory cell type 1 (s_1) in the present investigation was found to contain probably glycoprotein granules which form dense aggregates within the cell. The rapid development of secretory cell type 1 (s_1) can be easily missed because the mature cell appears to rupture and release its granules into the lumen (holocrine secretion). The development of secretory cell (s_1) is essentially the same in the nymphs and the cell is present in two stages of the tick's development. The first stage is at the time of attachment, but larger numbers are found in the second stage, just prior to final rapid engorgement.

The timing of these events suggests that a haemolysin may be secreted by cell (s_1). In this study, haemolysis of ingested red blood cells occurred rapidly in the first phases of digestion, and again after rapid engorgement. The mechanism is not known, but according to Hughes (1954) in the tick I. ricinus it is not a simple lysis but a complete digestion of the red cell envelope, which disappears entirely. This is true also for B. microplus. Tatchell (1964) also observed a saliva-fast, P.A.S. positive material in the gut lumen of A. persicus. This material was secreted by cells of the developing epithelium and he concluded that the secreted material was a hemolysin.

Secretory cell (s_2) is found only in females and mature cells are produced only at the 5 to 8 mm stage of development. In contrast to secretory cell (s_1) the cytoplasm of these cells is highly vacuolated. The secretory material has been described as "colloid-like" (Hughes, 1954) and forms loosely aggregated spheres in the cytoplasm of these cells. The "colloidal" mass may be an acid mucopolysaccharide, possibly an anticoagulant and is released by merocrine secretion with the apical two thirds of the cell budding off into the lumen (Fig. 8). There is inevitably a mixing of the (s_1m) and (s_2m) secretions in the lumen and some of these materials are apparently taken in with host fluids by the developing digest cells. Further evidence for two distinct secretory cell types is the presence of both cells in the same caecae at the same time.

Another cell type which has not apparently been noted previously by light microscopy is the basophilic cell (b). It occurs in the adult female gut during the rapid phase of feeding and beyond, and in the nymphal gut to a lesser extent. The presence of the basophilic cell (b) during rapid feeding as well as its large basal surface area which is increased further by extensive basal infoldings, suggests it may have some role in transporting digested materials, water and ions across to the haemolymph. It has been shown by Coons *et al.* (1982) that when the tick *R. sanguineus* drops from the host the basophilic cell synthesises vitellogenin for oocyte development. The origin of these basophilic cells in the females is uncertain, but their appearance in some of the methacrylate sections of *B. microplus* suggest that they are derived from the basal remnants of the secretory cell (s_2) (Chapter 5). Such an origin would be in keeping with the observation made by Tatchell (1964) on the gut of *A. persicus*. Thus for most of the cells of the gut the proposal of Balashov (1972) is correct, that cells derived from stem cells are irreversibly specialised, but the s_2 cell is an exception.

Having identified and described the gut cells, the dynamics of digestion can now be clarified. The first generation of enlarging digest cells and secretory cells (s_1) form in the pharate adult. On attachment the secretory cells (s_1) presumably discharge their contents into the lumen and red cell lysis is demonstrable at this stage (day 15). Digestion is rapid and digest cells mature through a series of stages. The second generation of digest cells arise from division of stem cells soon after feeding begins (day 16), and digestion progresses as the tick grows to 3 mm in length. Cell divisions are numerous at the 3-4 mm stage, giving rise to two types of digest cells, which make up the third generation. When females reach 4-5 mm in length they are beginning rapid engorgement, filling the lumen with host blood which the third generation of cells will digest. The first type of digest cells (dhl) from the third generation appear earlier and may be specialised to phagocytose leukocytes, while the second type (dhv) apparently ingest mainly haemoglobin. Both continue to function after the tick has dropped from the host. A few new secretory cells (s_1) and numerous secretory cells (s_2) differentiate at the same time as the third generation digest cells. Materials from (s_1) and (s_2) often fill the lumina before rapid feeding begins and as a result, the material can be ingested, together with host blood, by the digest cells.

Having established when the three generations of digest cells arise, it is possible to follow the progress of intracellular digestion during the development of stem cells through to the spent digest cells. The haemoglobin and other host material accumulates in large vacuoles which grow in size possible by accretion of smaller ones. As intracellular digestion proceeds, breakdown products are formed. The digest cells contain lysozomal enzymes (Raikhel 1978). The most conspicuous end

product is haematin. Lipid accumulates in vacuoles particularly around the nucleus and glycogen also accumulates in small deposits, but is quickly utilised by the ticks. As the digest cells that have completed digestion of their food vacuoles become spent digest cells, cytoplasmic contents are discharged into the lumen (nymphs) or the whole cell is shed into the lumen and may then be excreted (adults).

This study also provides new information on the origin of the first generation of digest cells in the newly moulted nymphs and females. In the engorged nymphs no stem cells are observed as over 90% of the cells are hypertrophied digest cells which are packed with food vacuoles. By the late pharate adult stage the digest cells which were numerous on the previous day have disappeared. The sudden appearance then, in newly moulted adults, of many first generation digest cells without food inclusions and with no apparent nuclear divisions, suggest that many of the previous day's digest cells have shed their contents and reverted to early digest cells. Using light microscopy, it is difficult to be certain at any stage that no stem cells are present, but it becomes clear when division of stem cells is giving rise to a new generation of cells. The reversion of older digest cells to early digest cells is not the case in the later adult gut where the evidence is for a one-way progression from stem cell, to the digest cell series.

It has been suggested that an essential part of adult digestion is the budding of digest cells into the lumen where they gain access to unabsorbed blood (Lees 1952; Hughes 1954; Till 1961; Balashov 1972). However, Tatchell (1964), working with A. persicus, states that no budding occurs during digestion and this was confirmed for Argas arboreus (Guirgis, 1971). Since the digest cells in B. microplus bulge into the

lumen and can have very narrow connections to the basal lamina, it is difficult to establish without complete serial sections whether they are actually detached. The point has not been clarified in this thesis but it has been noted that haematin-containing spent digest cells occur in the rectal sac, apparently still intact, indicating that free-floating cells would occur in the gut lumen. However, they may be shed cells rather than actively digesting ones.

3.6 SUMMARY

Histology of normal development in the gut of B. microplus showed that during feeding of nymphs and adults, the gut consists of five cell types, stem cell, digest cell, secretory cells type 1 and 2 and basophilic cell. The stem cell differentiates into any of the other cell types, although in the females the basophilic cell is probably derived from the basal remnant of secretory cell type 2.

The digest cells mature through a series of stages and have three generations during feeding of female ticks on the host. The final generation has two distinct cell types, the first type is thought to be capable of both phagocytosis and pinocytosis. Cells of the second type are predominant at the end of feeding, and may be specialised to ingest by pinocytosis only. Digestion occurs mainly after feeding has stopped. The final stage of the digest cell series is the spent digest cell which discharges its content into the gut lumen or is excreted whole.

The basophilic cell has structures which suggest that one of its functions is to transport digested materials, water and ions across the gut.

Secretory cell type I secretes a glycoprotein which may be a haemolysin and secretory cell type 2 secretes the gut "colloid" mass, an acid mucopolysaccharide, which may function as an anticoagulant.

Intracellular digestion leads to the breakdown of host blood and storage of lipid and glycogen in the digest cells.

CHAPTER 4

SCANNING AND TRANSMISSION ELECTRON MICROSCOPY OF THE DIGEST CELLS IN THE MIDGUT EPITHELIUM OF **B. MICROPLUS**

4.1 LITERATURE REVIEW

I have found no reports in the literature of observations made with the scanning electron microscopy (SEM) of the tick gut. The organ is very delicate and has been disected out for physiological experiments in apparently only one study (Barker et al., 1977). Grandjean & Aeschliman (1973), in the first transmission electron microscope study (TEM) of the tick gut cells, identified only two cell types in the gut of O. moubata, stem and digest cells. They suggested that in the gut, only one cell type, the digest cell, differentiated from the stem cell and the structure and shape of the digest cell changed considerably at the different stages of digestion. Observations by Raikhel (1975), and Balashov & Raikhel (1977,1978) using O. papillipes did show, however, that there were three different types of cells (stem, digest and secretory) in the gut epithelium.

In the nymphs of the ixodid tick H. asiaticum, Raikhel (1975), Balashov & Raikhel (1976) found a higher level of specialisation. The cuboidal stem cells had features which characterised them as typical undifferentiated cells. Microvilli and basal infoldings were poorly developed. Their cytoplasm was dense and contained mainly free ribosomes and few other organelles. After the tick attached to the host, rapid differentiation of stem cells occurred. This gave rise to secretory and digest cells.

They found that the digest cells differentiated from stem cells into two different types. Type 1 cells rapidly enlarged and protruded into the midgut lumen. A characteristic feature was their ability to take up blood components by phagocytosis. Pinocytotic structures were represented by coated vesicles and tubular invaginations of the plasma membrane which opened on the cell surface in small pits. The coated vesicles, tubular structures and phagocytosis vesicles transported blood into the endosomes (food vacuoles), which increased in size and number until they filled the cytoplasm of the cells. The presence of whole host leukocytes in many endosomes suggested to them that phagocytosis predominated in these type 1 digest cells. Using ultracytochemical studies, Raikhel (1975,1978a,b) showed that intracellular digestion occurred actively in type 1 cells while the endocytosis of blood components was still continuing. The majority of inclusions filling the cytoplasm of these digest cells showed a positive reaction for acid phosphatase, and were considered to be heterolysosomes in various stages of hydrolysing the blood components. As digestion progressed there was a rapid increase in the number of lipid and glycogen inclusions, together with haematin. When the activity of the type 1 cells was over, the cytoplasm became filled with various types of residual bodies and lipid inclusions. The cells then began to undergo degeneration.

Type II digest cells developed from a new generation of stem cells after the nymphs of H. asiaticum had been feeding for 3-4 days. They suggested that type II cells were specialised to take in haemoglobin and other non-cellular components of the diet exclusively by pinocytosis, because the homogeneous endosomes in these cells lacked the host cellular or granular components of the type 1 cells. The development of the type II cells was associated with the first ingestion of large numbers of

erythrocytes which were lysed in the gut. During the uptake of haemoglobin, the cortical cytoplasm of these cells contained abundant coated vesicles and tubular invaginations which contained electron-opaque material similar to that in the gut lumen. The tubular invaginations terminated in rounded expansions containing dense material, and were believed to be forming heterophagosomes (endosomes) which accumulated haemoglobin. The endosomes increased in size by the acretion of several of these tubular invaginations and small endocytic vacuoles, and the vacuole contents appeared to be condensed because larger vacuoles were more darkly stained and were located deeper in the cell cytoplasm. In the type II cells intracellular digestion occurred after feeding had stopped, and during the preparation for moult off the host.

Raikhel (1975,1978) further observed that during differentiation of both types of digest cells the formation of synthesising organelles occurred. The central parts of the digest cells contained rough endoplasmic reticulum (r.e.r.), the cisternae of which were distended. Some cisternae were seen to produce dense drop-like inclusions. Golgi complexes were also located in these zones. Staining for acid phosphatase showed that the synthesis and accumulation of this enzyme occurred in the rough endoplasmic reticulum and that the dense bodies which were formed in the Golgi were in fact the primary lysosomes. These were the main structures which transported lysosomal hydrolytic enzymes to the endosomes which formed as a result of endocytosis.

No more recent ultrastructural study has been reported on the digest cells of ixodid ticks.

4.2 INTRODUCTION

Transmission electron microscopy (TEM) has confirmed the presence of microvilli and pinocytotic vesicles on the luminal plasma membranes of digest cells (Balashov & Raikhel, 1976, 1978). For a number of reasons, this surface is very important in the interaction between the tick and its host. From the tick's side, the plasma membrane has the structural molecules necessary for the rapid growth of the cells into the lumen for phagocytosis of host cells and for pinocytotic vesicle formation. It must have receptor molecules to recognise host material and initiate intracellular ingestion and it probably has enzymes for the transfer of specific ions or molecules across the membrane. From the host side, the plasma membrane molecules are potentially antigenic and can come into direct contact with the host immune effector mechanisms (Chapter 8). Access to this membrane for experimental purposes would be valuable, for example, for locating antigens or receptor molecules. This was found to be possible despite the delicate nature of the gut epithelium as can be demonstrated by scanning electron microscopy.

The end product of haemoglobin digestion in ticks has been described as haematin by Wigglesworth (1954), Balashov (1972), Akov (1972), and Gooding (1972). Balashov (1972) reported also that serum albumin and globin are completely assimulated during digestion in H. asiaticum. Chiefly the globin was assimulated from haemoglobin, while most of the prosthetic haem group (3.38% of haemoglobin) was discharged with faeces. In B. microplus this haem presumably accumulated in the spent digest cells (ds) which either disintegrated and released the haematin into the lumen (nymphs), or the whole cells may be shed and excreted (adults, Chapter 3). A definite amount of haem (containing blood iron) is apparently vitally necessary for tick development (Balashov, 1972). O'Hagan (1974),

reported that about 10% of the haem released from the haemoglobin was transferred to the eggs by the engorged female B. microplus, and from the eggs to the larvae.

Studies in Chapter 3 indicated that there were two types of digest cells involved in digestion in B. microplus as had been shown in H. asiaticum by Balashov & Raikhel (1976). Much more study is needed on digestion in ticks. In this chapter a brief comparison is made with results from H. asiaticum, and scanning EM, X-ray microanalysis (XMA) was used on small haematin granules as a preliminary attempt to identify iron residues of haemoglobin digestion.

4.3 MATERIALS AND METHODS

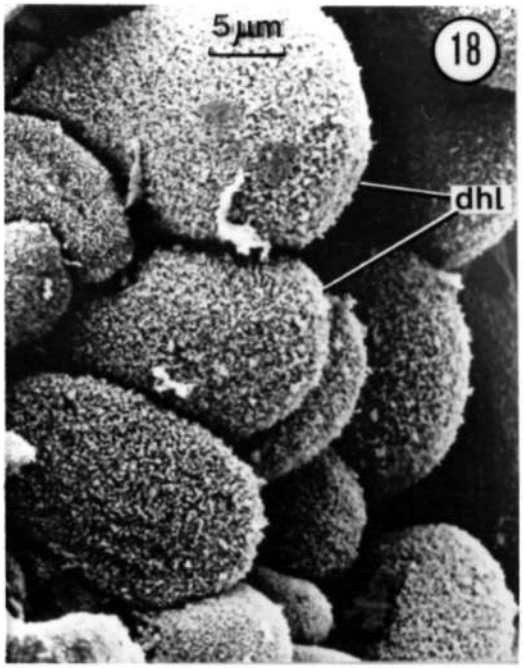
4.3.1 Scanning EM

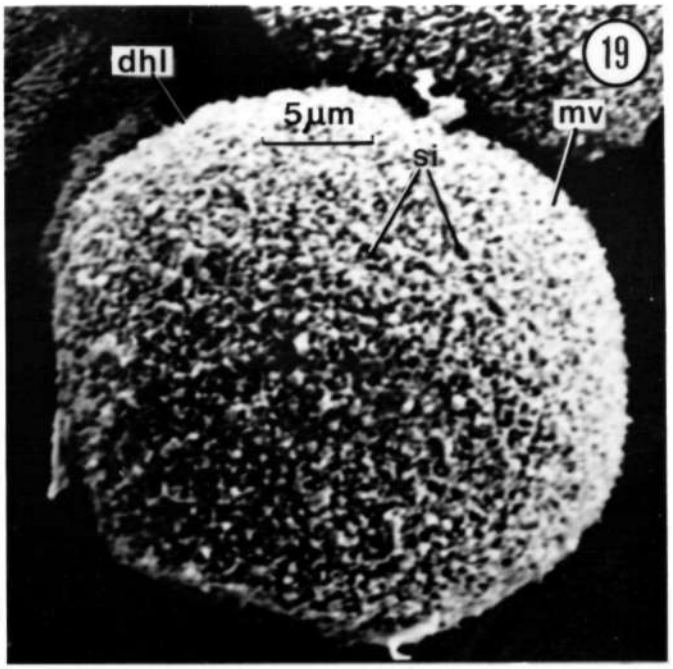
A 5 mm female tick was dissected at 4°C in 0.1M phosphate buffer (containing 0.1M NaCl and 0.1mM $CaCl_2$ $2H_2O$ pH 7.2). Pieces of the midgut caecum 1.5 mm long were carefully cut open and flushed in buffer, before being transferred into the Karnovsky's fixative (Chapter 2) overnight at 4°C. The tissues were then rinsed twice in the phosphate buffer at 12 hour intervals, before dehydration through graded alcohols, 50%, 70%, 90%, 100% and amyl acetate before being transferred into the critical point drying apparatus (DCP-1 Denton Vacuum, Inc) for 20 min. To prevent loss of tissues, the pieces were placed in a white porous cup before dehydration. Dried tissues were mounted on stubs using carbon dag (colloidal graphite) before being coated with gold using a Polaron Sputter Coater (Polaron Equipment Ltd, SEM coating unit E.500) for 10 min.

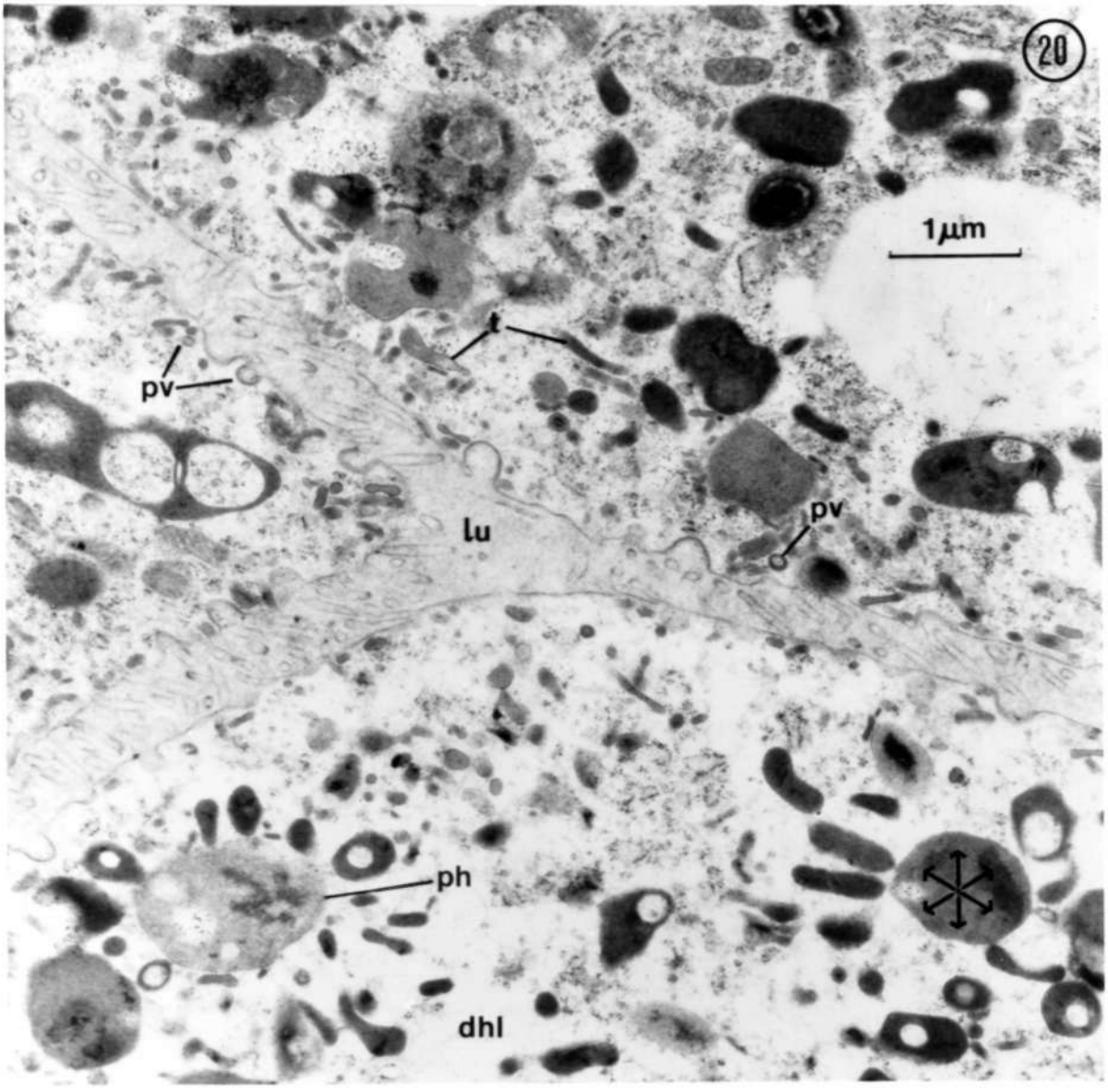
FIG. 18. S.E.M. of lumen surface of digest cell, dhl.

FIG. 19. S.E.M. of lumen surface of digest cell, dhl; microvilli, mv; surface identations, si.

FIG. 20. T.E.M. of digest cells type 1 dhl; lumen, lu; pinocytotic vesicles, pv; large spherical endosomes (food vacuoles), ph; tubular endosomes, t; *, accretion of endosomes. No <u>en bloc</u> staining.


5µm
18
dhl


dhl
19
5µm
mv
si


20
1µm
pv
t
lu
pv
ph
dhl

4.3.2 Transmission EM

Five 6 mm females (day 17) and five fully engorged females (day 19) were collected from the donor calf at 10.00 h and were processed and fixed immediately in Karnovsky's fixative as described in Chapter 2. En bloc staining was omitted. The pieces of gut tissues were processed and subsequently embedded in a mixture of epon and araldite (Chapter 2).

4.3.3 X-ray Microanalysis

Two 5-6 mm female ticks were fixed in 10% formalin, processed through supercedrol (Binnington, 1970) and embedded in paraplast. Serial sections 3 µm thick were stained in H & E, PAS and Amido Black B to study the details of cell structure. Two slides were dewaxed in xylene (two changs of 5 min each) and rinsed in absolute alcohol (three changes of 1 min each). The glass slides were then cut down to fit the tops of the scanning EM specimen stubs. The slides were attached to the stubs using carbon dag and the specimens coated with carbon using a Dynavac scan coat to a thickness of about 100A°.

The sections and the dissected, everted gut specimens were then viewed in a Joel 100C Scanning Electron Microscope, model JSM 35 CF fitted with X-ray analysis attachment and X-ray analysis was performed on small discrete granules within spent digest cells (ds). The electron micrographs were taken under an accelerating voltage of 10 kv.

4.4 RESULTS

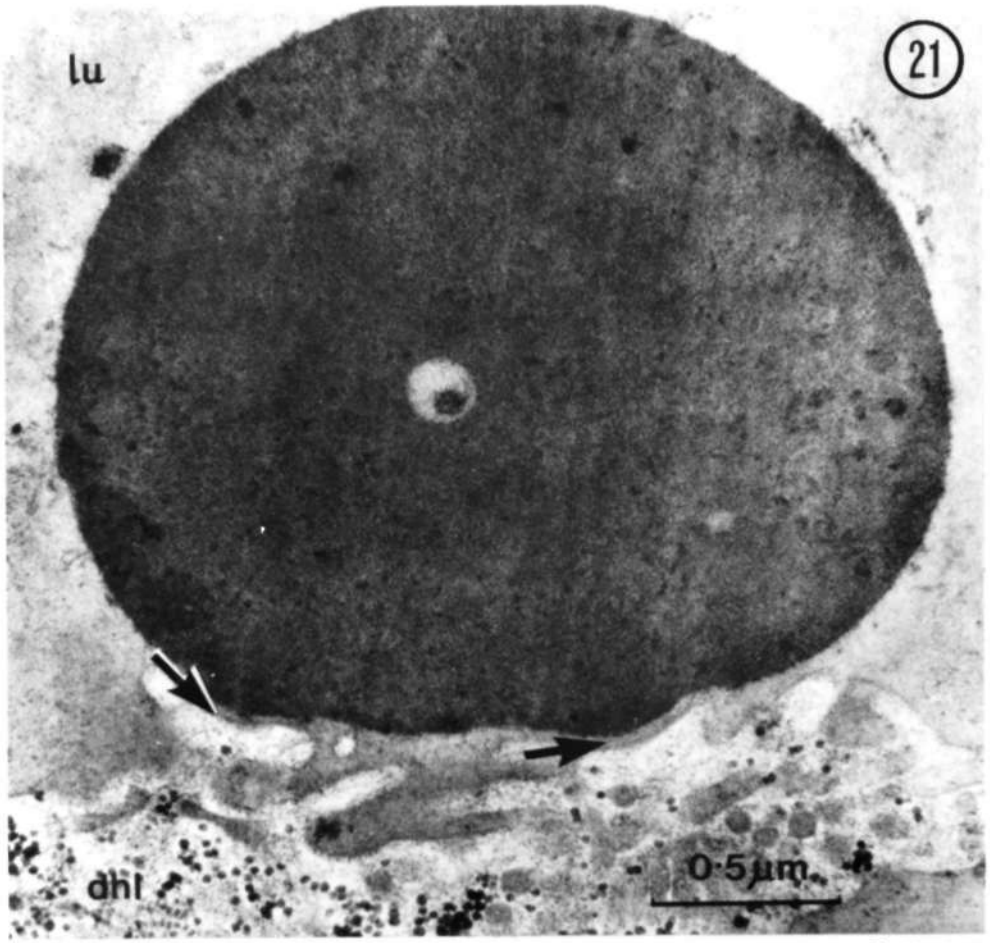
4.4.1 Scanning EM

Secondary electron signals provided the topographical details of the lumen surface of the gut epithelium (Figs. 18 & 19). The digest cells had bulged into the lumen, each cell separate from its neighbouring digest

FIG. 21. T.E.M. of digest cell, dhl; →, contact of microvilli with host erythrocyte; lumen, lu. No en bloc.*

FIG. 22. T.E.M. of digest cell, dhl; cytoplasmic projection engulfing erythrocyte, cpr; erythrocyte, rbc. No en bloc.

* For brevity, here and later, this notation is used to indicate that the material was not stained en bloc prior to sectioning.


lu
21
dhl
0·5µm

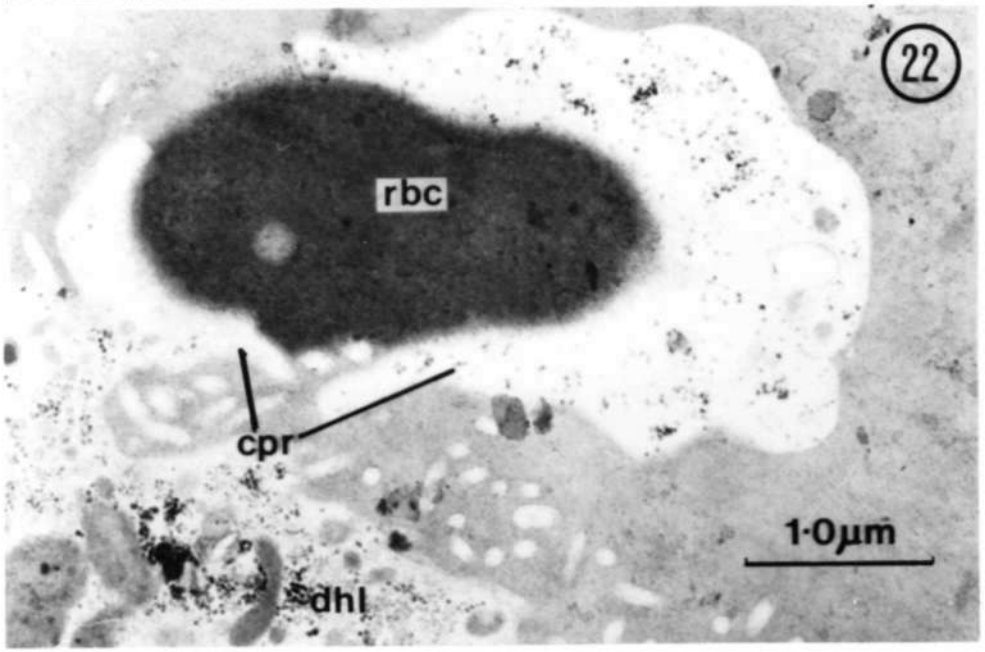
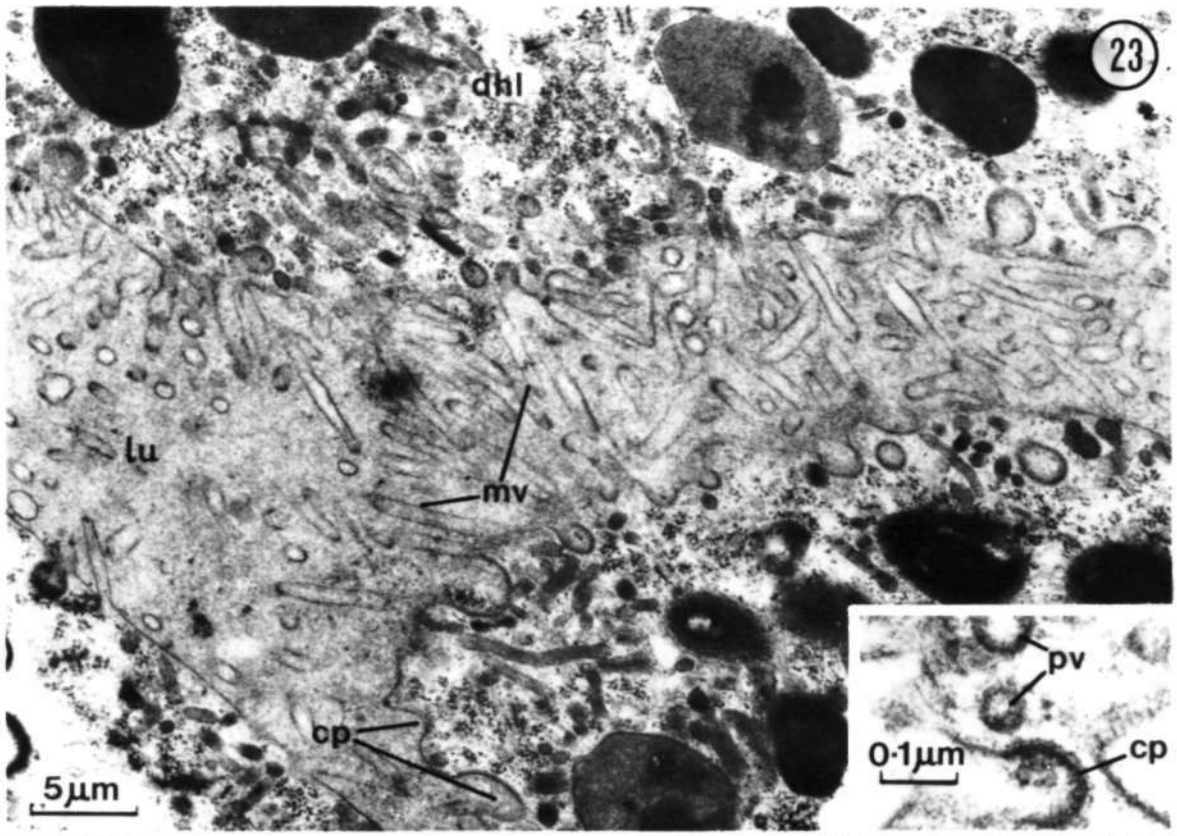
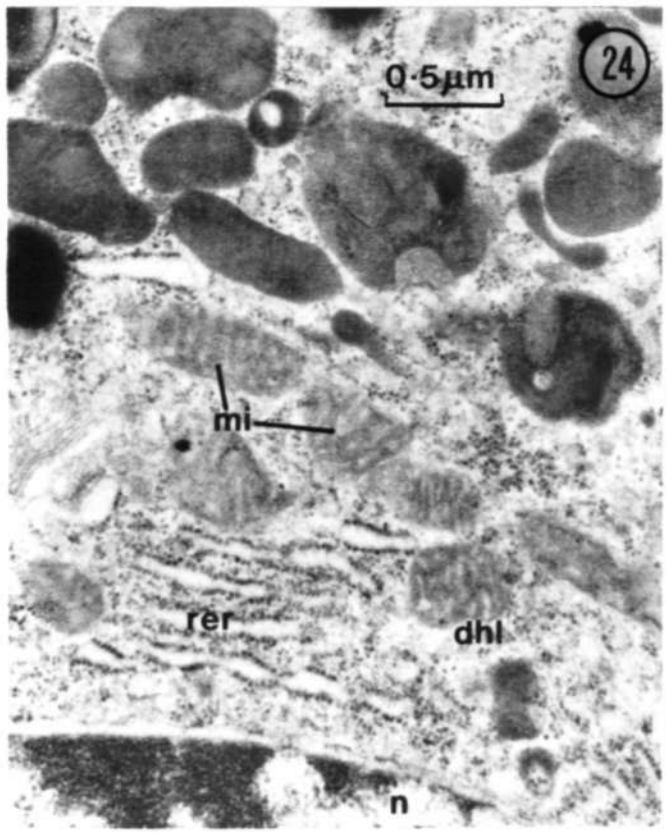

22
rbc
cpr
1·0µm
dhl

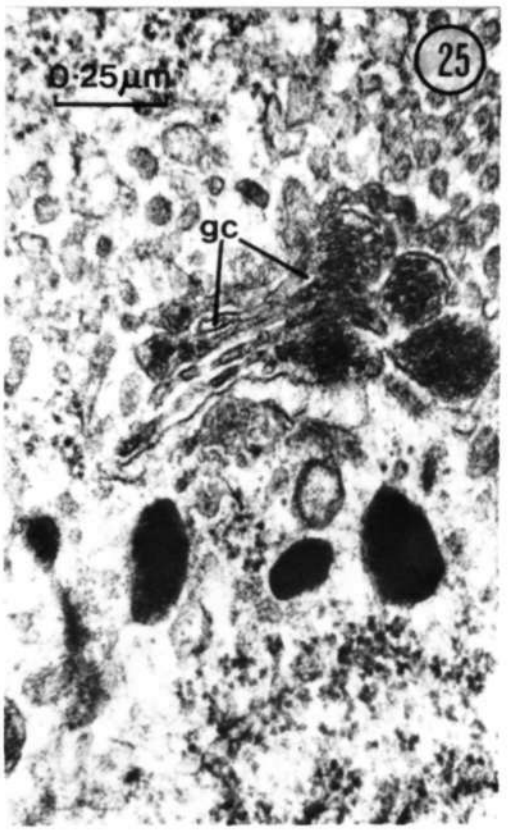
FIG. 23. T.E.M. of digest cell, dhl; microvilli, mv; lumen, lu; coated pits,cp; inset is enlargement of coated pits. en bloc.

FIG. 24. T.E.M. of digest cell, dhl; nucleus, n; distended rough endoplasmic reticulum, r.e.r.; mitochondrium, mi. No en bloc.

FIG. 25. T.E.M. of Golgi apparatus, gc in digest cell. en bloc.


23
dhl
lu
mv
cp
5µm
pv
0·1µm
cp


0·5µm
24
mi
rer
dhl
n


0·25µm
25
gc

cells and the luminal surfaces were covered with small microvilli. Maximum enlargement for this preparation revealed identations on the surface of the cells (Fig. 19) which could have been pinocytotic coated pits.

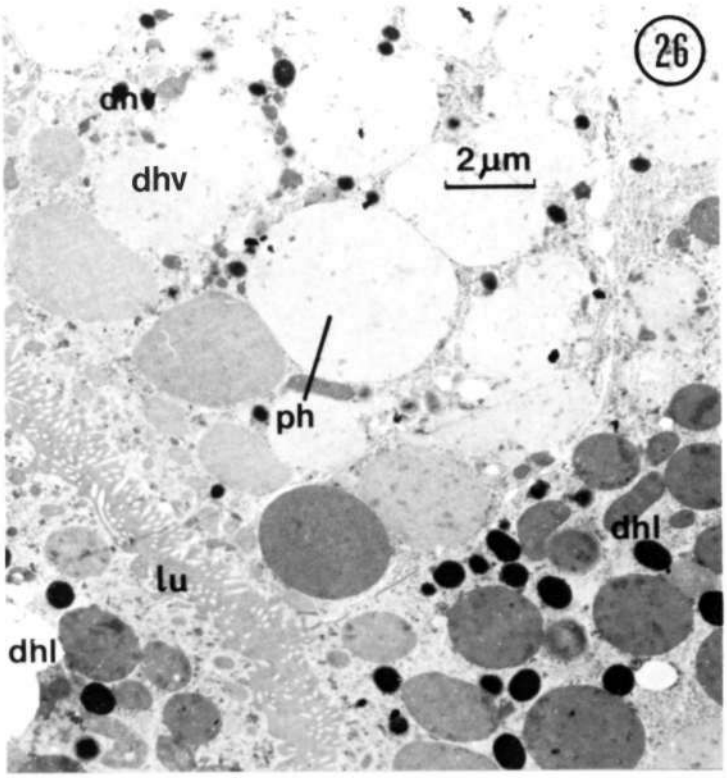
4.4.2 Transmission EM

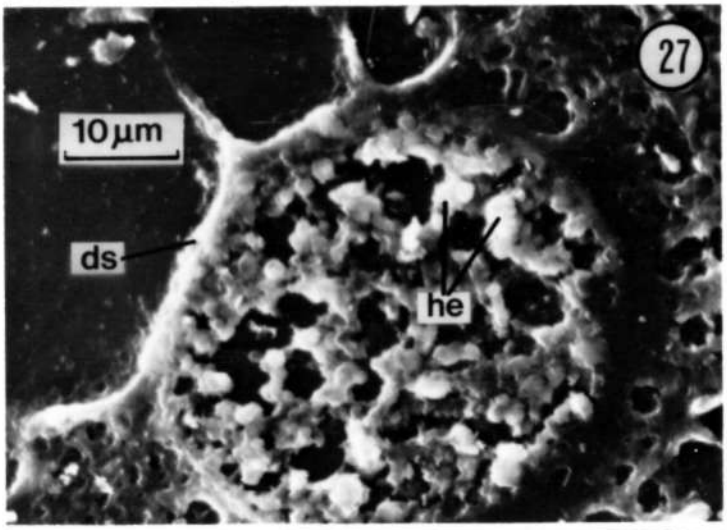
The cells observed in SEM were the digest cells type 1 (dhl). Two types of 3rd generation digest cells (type 1 dhl, and type II dhv) were observed at light microscopy (Chapter 3 and section 4.1), and the TEM showed the ultrastructural features of the cells. The type 1 cell (dhl, Fig. 20) are involved in the intracellular digestion of blood just prior to and during the rapid final phase of engorgement (Chapter 3). A characteristic feature of these cells was their ability to take up blood by both phagocytosis and pinocytosis. The host leukocytes, their fragments, other fragments of host tissue and whole red blood cells were engulfed by phagocytosis. The internalisation of large particles, for example a red blood cell, was observed. Initial firm contact was with the microvilli(Fig. 21) then cytoplasmic projections on the surface engulfed the r.b.c. (Fig. 22) before internalisation into an endosome (or receptosome). These cells also had coated pits on the luminal plasma membranes. The coated pits had double membranes and signs of condensation of material on both sides of the membranes of the pit was observed (Fig. 23) indicating that the cells were capable of pinocytosis. Both the coated vesicles and other endosomes presumably fused to form the very large endosomes (Fig. 20). These cells also contained some distended rough endoplasmic reticulum, Golgi complexes and mitochondria between the endosomes (Figs. 24 & 25). The microvilli on the surface of these cells were of variable size and were located far apart, possibly due to phagocytosis and the numerous coated pits on the surface plasma membrane.

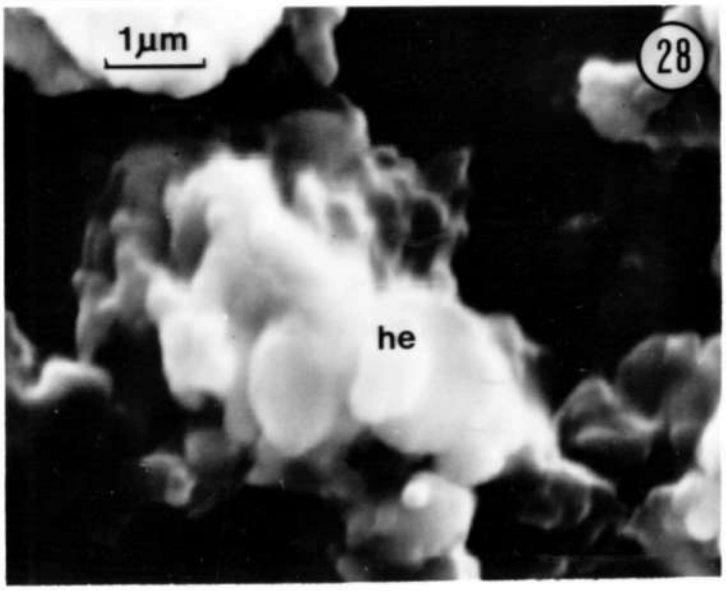
FIG. 26. T.E.M. of digest cell (type II), dhv; digest cell (type I), dhl; large homogeneous endosomes, ph: lumen, lu. No *en bloc*.

FIG. 27. S.E.M. of spent digest cells, ds; haematin granule, he.

FIG. 28. Enlargement of haematin granules, he, used for X-ray microanalysis.


26
dhv
dhv
2µm
ph
dhl
lu
dhl


27
10µm
ds
he


1µm
28
he

Type II cells had abundant coated pits on the luminal plasma membrane, but they had no structures on the surface which could be interpreted as being involved with phagocytosis. The cytoplasm of these cells were filled with homogeneous endosomes of varying sizes and appeared to be just beginning their activity when the tick dropped from the host (Fig. 26).

4.4.3 X-ray Microanalysis

Secondary electrons provided the topographical details of the sections on glass slides. On one section a spent digest cell (ds) was located in the lumen (Fig. 27). Characteristic X-ray pulses that were emitted by the small granule in the cell (Fig. 28), were detected by a solid state detector and were processed through a multichannel analyser (Tracor Northern, TN 2000 X-ray analyser) and the characteristic spectrum was displayed on a colour video monitor, and the qualitative element identification printed out as shown in Table 3 A & B, with the following heading and abbreviations.

(K = Shell line for 2° electrons)

KA = K alpha (first characteristic X-ray emitted during electron beam bombardment)

KB = K beta (second characteristic X-ray emitted during electron beam bombardment)

Energy = Specific energy for the particular X-ray line

Area = Calculated area under the spectral curve.

TABLE 3 TICK SPENT DIGEST CELL HAEMATIN GRANULE.
QUALITATIVE ELEMENT IDENTIFICATION.

(A) Peak listing: glass slide with section

No.	Energy	Area	Element	Line
1	1.033	383	NA	KA
2	1.259	270	MG	KA
3	1.743	30,236	SI	KA
4	2.314	590	S	KA
5	3.316	859	K	KA
6	3.698	4,916	CA	KA
7	4.027	584	CA	KB
8	6.403	709	FE	KA

TICK SPENT DIGEST CELL BACKGROUND CONTROL.
QUALITATIVE ELEMENT IDENTIFICATION

(B) Peak listing: glass slide only

No.	Energy	Area	Element	Line
1	1.031	1,385	NA	KA
2	1.259	654	MG	KA
3	1.743	61,144	SI	KA
4	*	*	S	*
5	3.316	1,500	K	KA
6	3.696	8,957	CA	KA
7	4.028	982	CA	KB
8	*	*	FE	*

* Not detectable above background.

The most obvious result was the detection, above background levels, of Fe and S in the granule being analysed (Table 3 , A). The areas under the spectral curves for analysis of the slide and the section was smaller than for slide alone due to the higher magnification of the specimen compared to the slide alone. This was necessary so as to concentrate on particular granules (Fig. 28). This gave a greater opportunity for detecting control amounts of Fe & S on the slide, which, in fact, could not be detected.

4.5 DISCUSSION

Using the SEM, numerous microvilli were observed on the surface of the digest cells. Microvilli are columnar projections of digest cells and have variable dimensions, arrangement and functions. Generally they are considered to be a device to increase the cell surface area and hence efficiency of transport through the cell membranes. Recent studies have revealed that the intestinal microvilli of mammalian tissues acively move by the actin-myosin mechanism (Mooseker & Tilney, 1975) and evidence is accumulating to indicate that many other types of microvilli are also motile. Moreover, certain types of microvilli such as those of gustatory cells, gut endocrine and epidermal mast cells are believed to serve as the receptor sites of the cells (Fujita, Tanaka & Tokunaga, 1981). The results from TEM indicated that the microvilli in the dhl cells of the tick gut might be involved with receptor mediated endocytosis (phagocytosis). They may have a variety of receptors for distinct molecules or classes of molecules. Coated pits are also specialised structures, and are believed to contain, on the cytoplasmic face of the pits, clathrin as the major scaffolding protein (Pearse, 1975). Clathrin is a 180,000-dalton nonglycosylated protein. The clathrin coat is thought to serve two principal functions: to select or exclude molecules from the

coated pit, and to provide a structural scaffold for the invagination of a vesicle (Pearse, 1976; Pearse & Bretscher, 1981). Coated vesicles were particularly large and numerous on the tick digest cells.

The results from the TEM of the digest cells agrees with the earlier reports by Balashov & Raikhel (1976). In B. microplus the endosomes fused with the primary lysosomes and enzymic digestion finally gave two types of residual bodies in the gut cells, haematin granules and dense unidentified tubular structures. Both of these were stored in the cell cytoplasm. TEM has so far confirmed the impression from Chapter 3 and from Balashov & Raikhel (1976) that the type II digest cells are not capable of phagocytosis. Experimental verification is required, because this is significant for studies on digestion and the invasion of B. microplus gut cells by B. bovis which is the subject of Chapter 7.

It has been possible to detect iron in the spent digest cell haematin granules using X-ray microanalysis. This supports the belief that the so-called haematin granules are the end-product of haemoglobin digestion in the ixodid tick gut. The presence of large amounts of sulphur is puzzling. It may result from digestion of blood proteins or it could be deposits of sulphur containing end-products of cell membrane digestion, for example glutathione.

A possible source of error could stem from scanning an actively digesting cell rather than a spent digest cell since endocytotic vesicles would contain iron. This is not the case here as sections from this tick had already been stained using H & E, P.A.S. and Amido Black B, and observations had established the presence of only spent digest cells in the lumen.

Although considerably more samples would be required to come to firm conclusions, the feasibility of the technique has been demonstrated. Greater accuracy could be achieved with ultra-thin sections and TEM X-ray analysis, where the granule types could be identified more accurately. In fact, it would be of interest to analyse food vacuoles, because there is a suggestion that concentration of the host blood takes place here (Section 4.1 and Chapter 6) and this could be expressed in terms of iron concentration.

4.6 SUMMARY

Scanning electron microscopy confirmed the presence of microvilli on the digest cells, but more important, it showed that the gut could be everted to expose the lumen surface of the cells to molecular analysis.

X-ray microanalysis demonstrated the presence of iron and sulphur in the haematin granules of spent digest cells, confirming the identification of these granules as deposits of the end products of blood digestion.

Transmission electron microscopy confirmed the presence of two types of 3rd generation digest cells. A characteristic feature of type I cells was their ability to take up blood by both phagocytosis and pinocytosis. Type II cells had abundant coated pits on the luminal plasma membrane, and were shown to take up blood by pinocytosis only.

CHAPTER 5

ULTRASTRUCTURE OF THE SECRETORY CELLS IN THE MIDGUT OF B. MICROPLUS

5.1 LITERATURE REVIEW

Grandjean & Aeschlimann (1973) in a study on the histology and ultrastructure of the cell types in the gut of O. moubata did not identify secretory cells, although at the beginning of blood digestion, they observed lysis of host erythrocytes in the immediate vicinity of the midgut epithelium. Haemolysins had earlier been suspected by Hughes (1954) in I. ricinus and by Tatchell (1964) in A. persicus. Balashov (1972) confirmed the presence of a secretory cell in the gut of the argasid tick O. papillipes and the ixodid tick H. asiaticum.

The only reports on the ultrastructure of a secretory cell in the gut of ticks are by Balashov & Raikhel (1976,1978). The secretory cells had a dense cytoplasm with abundant cisternae of rough endoplasmic reticulum, numerous mitochondria and Golgi complexes. The Golgi included narrow elongated cisternae and vesicles containing homogeneous moderately dense material. The above organelles occupied the central parts of the cells, while the apical parts were filled by numerous secretory inclusions. Small dark granules were located between the secretory inclusions. The surface of the secretory cells had various sized invaginations of the plasma membranes which were thought to have been formed after the release of the secretory inclusions. As blood entered the midgut, disintegration of these cells occurred and secretory material mixed with the host blood. The released material was a glycoprotein "colloidal" substance and was suspected to possess haemolytic enzymes.

Another subject which is appropriate for this chapter is the extraovarian synthesis of yolk proteins or vitellogenins, as it is likely that the tick gut cells are involved. This subject has been extensively studied in the insects with reviews by Wyatt & Pan (1978); Engelmann (1978,1979); Hagedorn & Kunkel (1979), but relatively little work has been done on ticks.

There is controversy in literature as to the sites of extraovarian synthesis of vitellogenin in ticks. Balashov (1972) discussing the different functions of the gut cells in H. asiaticum stated that besides the digestive function, the midgut served as a major depot of nutritional reserves, due to the intracellular storage of protein, lipid and carbohydrates (Chapter 4). He further speculated that since the fat body was poorly developed in ixodid ticks, many corresponding metabolic processes, for example synthesis of yolk proteins, probably occurred in the midgut cells. His views were supported, without further evidence by Diehl (1970), and Grandjean & Aeschlimann (1973). Jenni (1971), using an autoradiographical technique showed that midgut cells of O. moubata synthesised at least some of the extraovarian vitellogenin. Obenchain & Oliver (1973), however believed that the fat body was the only site of extraovarian vitellogenin synthesis. Attention was further drawn away from the midgut cells when fat body cells of R. sanguineus but not midgut cells after in vitro incubation with ^{14}C-amino acids released labelled proteins which had similar electrophoretic mobilities to haemolymph vitellogenins (Araman, 1979). Recently however, Coons et al. (1982), using immunocytochemical and ultrastructural studies, identified a cell in the midgut of ovipositing female R. sanguineus which had extensive synthetic machinery and appeared to form much of the egg proteins. This cell type is very similar to the basophilic cell described in Chapter 3 and the ultrastructural features will be discussed later in this chapter.

5.2 INTRODUCTION

The light microscope study reported in Chapter 3 showed that five cell types appeared to be involved in the digestion of the blood meal. Two of these are secretory cells, that is cells s_1, s_2. These results differed to some extent from the earlier reports. Firstly, the presence of two secretory cell types (s_1, s_2) where earlier reports quoted in Section 5.1 showed only one secretory cell type in the gut of argasid and ixodid ticks. Secondly, the basophilic cell was not recorded by Grandjean & Aeschlimann (1973), Raikhel (1974, 1978a) or by Balashov & Raikhel (1976). However, as noted, Coons *et al.* (1982), investigating the extraovarian sites for vitellogenin synthesis, described what is probably the equivalent of the basophilic cell in *B. microplus*.

The ultrastructural study in this chapter was intended to confirm (i) the separate identity of the two secretory cell types (s_1 and s_2) with emphasis on their origin, formation of the granules and possibly the mechanism of secretion, and (ii) to confirm the presence of the basophilic cell and study its structure in relation to function.

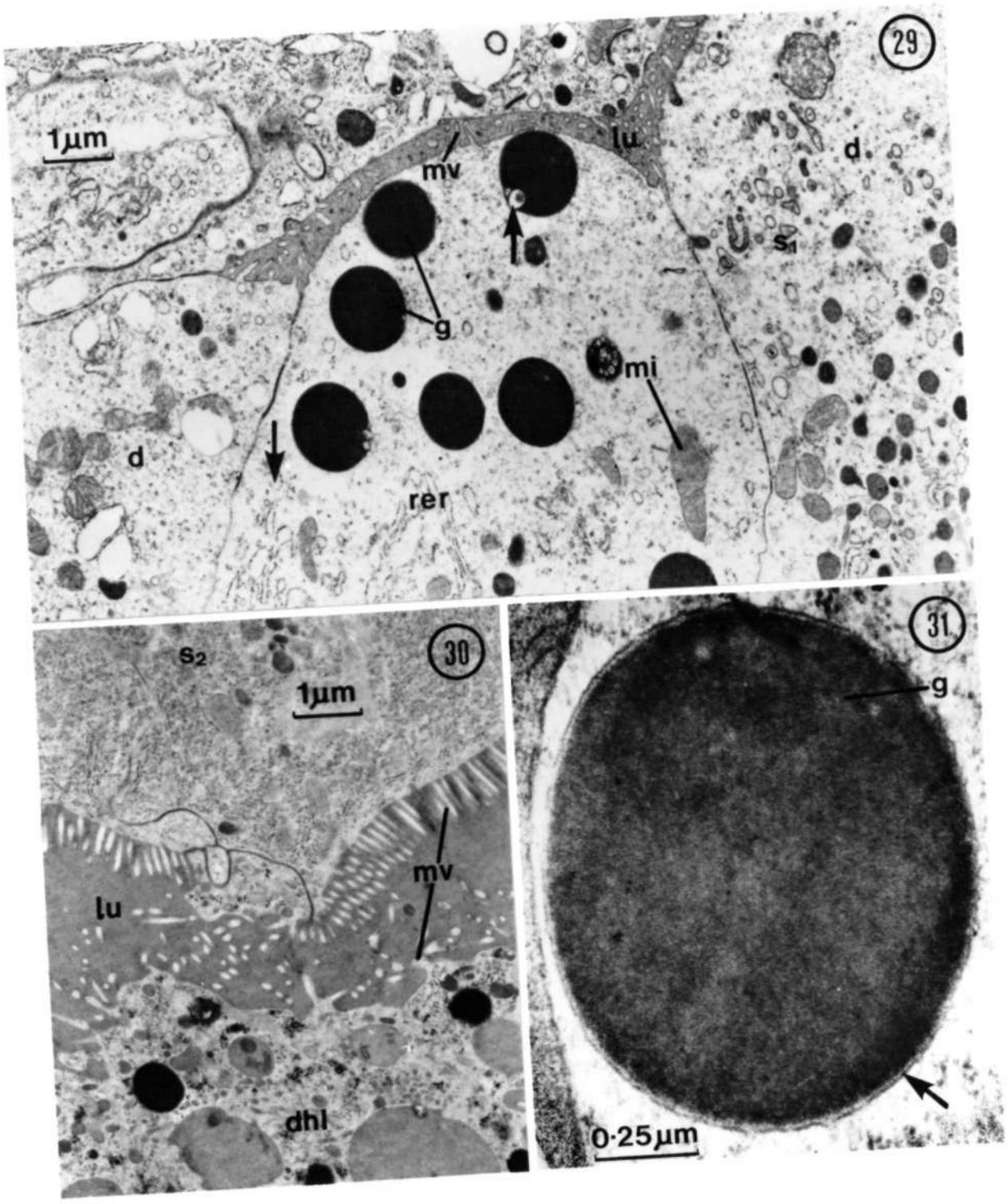
5.3 MATERIALS AND METHODS

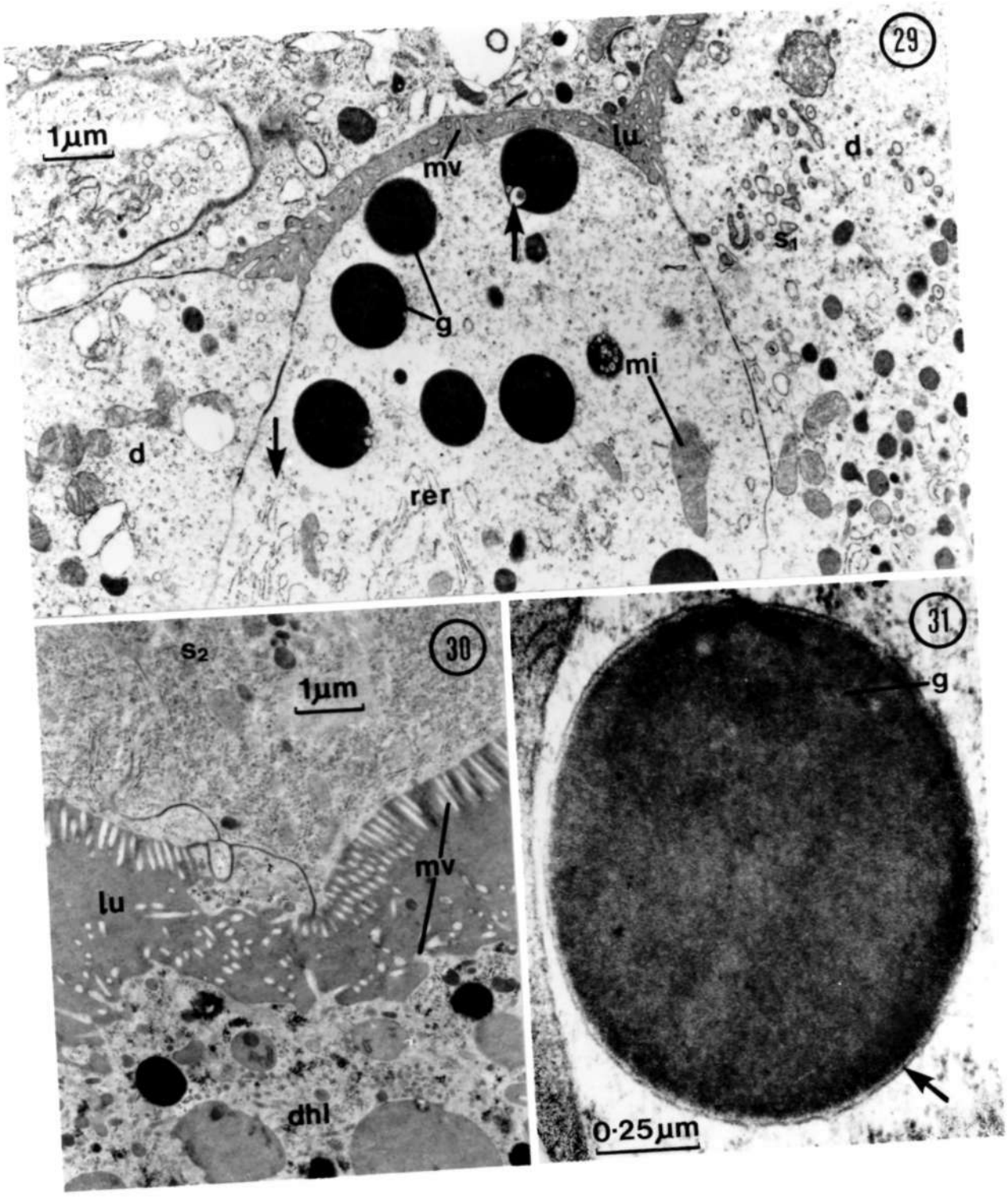
Five 6.0 mm females (day 17) and twelve fully engorged females (day 19) were collected from the donor calf at 10.00 h. The engorged ticks were incubated at 28±1°C and > 90% relative humidity and for four days post-incubation. Three ticks were taken each day and processed for electron microscopy. The 6 mm ticks and the engorged ticks were fixed in Karnovsky's fixative and the pieces of gut tissues were processed and subsequently embedded in a mixture of epon and araldite (Chapter 2). *En bloc* staining was omitted in some ticks.

FIG. 29. T.E.M. of secretory cell type 1, s_1; secretory granules, g; digest cell, d; microvilli, mv; lumen, lu; mitochondria, mi; distended rough endoplasmic reticulum, rer; upper arrow indicates electronlucent vesicles at margin of granule; lower arrow indicates vesicles budding off rer. en bloc.

FIG. 30. T.E.M. of digest cell, dhl; secretory cell type 2, s_2; microvilli, mv; lumen, lu. No en bloc.

FIG. 31. T.E.M. of secretory cell type 1 granule, g; arrow indicates double membrane. en bloc.


29
1µm
lu
mv
d
s1
g
mi
d
rer
30
s2
1µm
mv
lu
dhl
31
g
0·25µm


29
1µm
lu
mv
d
s1
g
mi
d
rer
30
s2
1µm
mv
lu
dhl
31
g
0·25µm

5.4 RESULTS

As was expected from earlier results, the sections contained many maturing secretory cell type 2 (s_2) and a few mature secretory cell type 1 (s_1).

5.4.1 Secretory Cell Type 1 (s_{1_e}, s_{1_m})

The s_1 cell is columnar to cuboidal, has a slightly convex luminal surface and has sparsely distributed microvilli (Fig. 29), which were much fewer than on the s_2 cells or the digest cells (Fig. 30). There were also no phagocytic or pinocytotic vesicles on the luminal surface. The nucleus occupied the central part of the cell, and the cytoplasm was packed with homogeneous densely-staining round granules. The cytoplasm also contained patches of organised rough endoplasmic reticulum which were concentrated around the nucleus. Mitochondria were scattered throughout the cytoplasm, but were absent towards the apical parts of the cell (Fig. 29), and there were no haematin granules or extensive infoldings of the basal lamina.

Cells that contained mainly mature granules had relatively empty cytoplasm. The granules measured on average 1.74 ± 0.10 µm and were bounded by double membranes (Fig. 31). A slight bulge in the granules was evident where a group of electron-lucent vesicles occurred at the margin (Figs. 29 & 32). Similar vesicles appeared to be budding off the r.e.r. (Fig. 29). By light microscopy mature cells were observed to be detached in the lumen and appeared to be disintegrated, with rapid release of the secretory granules.

FIG. 32. T.E.M. of secretory cell type 1 granule, g; arrow indicates electron-lucent vesicles at margin. en bloc.

FIG. 33. T.E.M. of unidentified secretory cell, s; microvilli, mv; mitochondria, mi; small secretory granules, g; rough endoplasmic reticulum, rer. No en bloc.

FIG. 34. T.E.M. of two secretory cells type 2, s_2m; granules, g; rough endoplasmic reticulum, rer; basal lamina, bl. No en bloc.

FIG. 35. T.E.M. of secretory cell type 2, s_2; lipid-like granules, l; arrow indicates glycogen deposit. No en bloc.

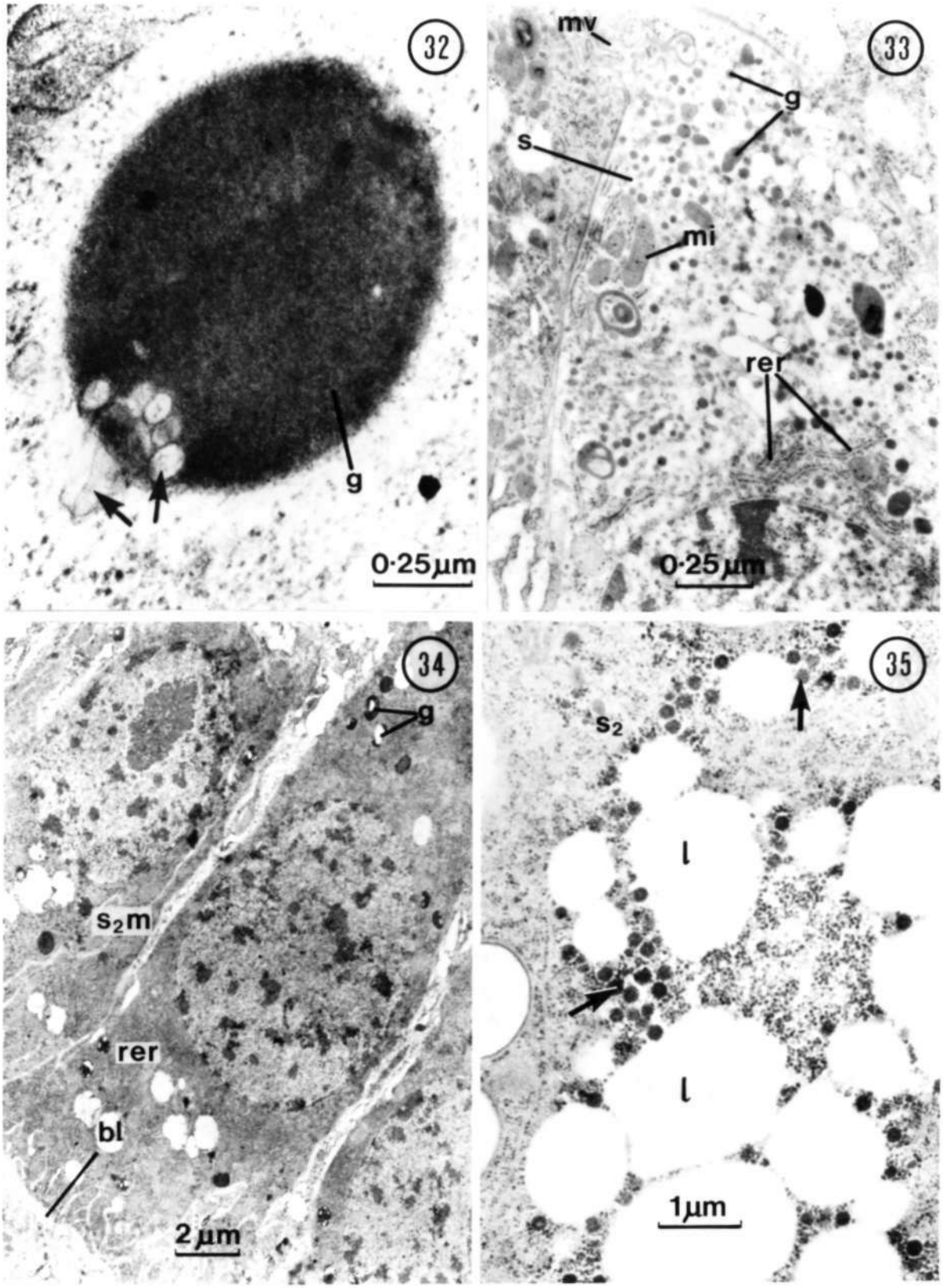
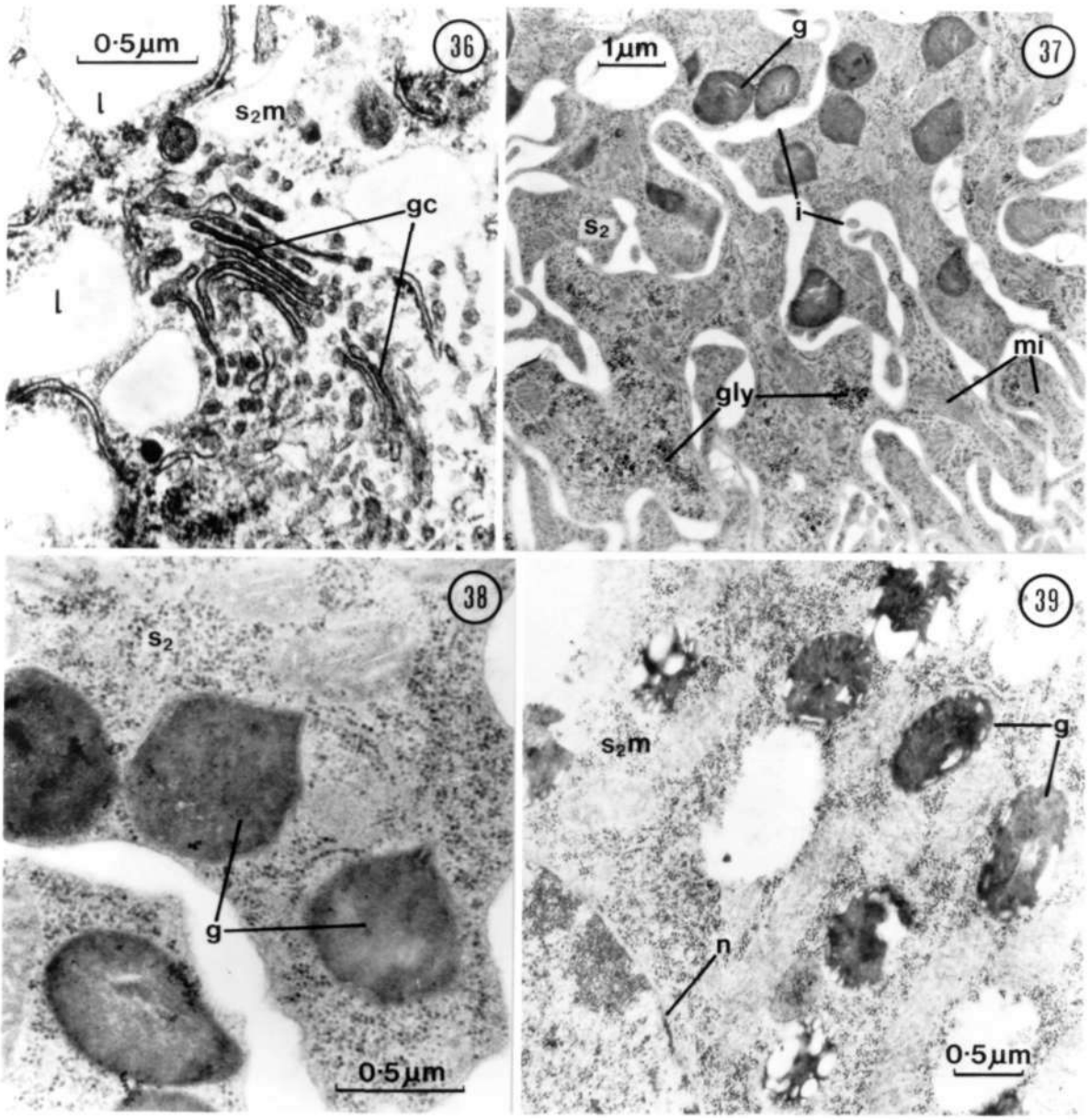

32
g
0·25µm
mv
33
g
s
mi
rer
0·25µm
34
g
s2m
rer
bl
2µm
35
s2
l
l
1µm

FIG. 36. T.E.M. of secretory cell type 2, s_2m; Golgi complex, gc; lipid-like granules, l. en bloc.

FIG. 37. T.E.M. of secretory cell type 2, s_2; deep infoldings of basal labyrinth, i; mitochondria, mi; glycogen, gly; secretory granules, g. No en bloc.

FIG. 38. T.E.M. of secretory cell type 2, s_2; granules with broad projections, g. No en bloc.

FIG. 39. T.E.M. of secretory cell type 2, s_2m; at the level of the glycogen and lipid accumulations. Nucleus, n; secretory granules, g. No en bloc.


0·5µm
36
l
s_2m
gc
l
1µm
g
37
s_2
i
mi
gly
s_2
38
g
0·5µm
39
s_2m
g
n
0·5µm

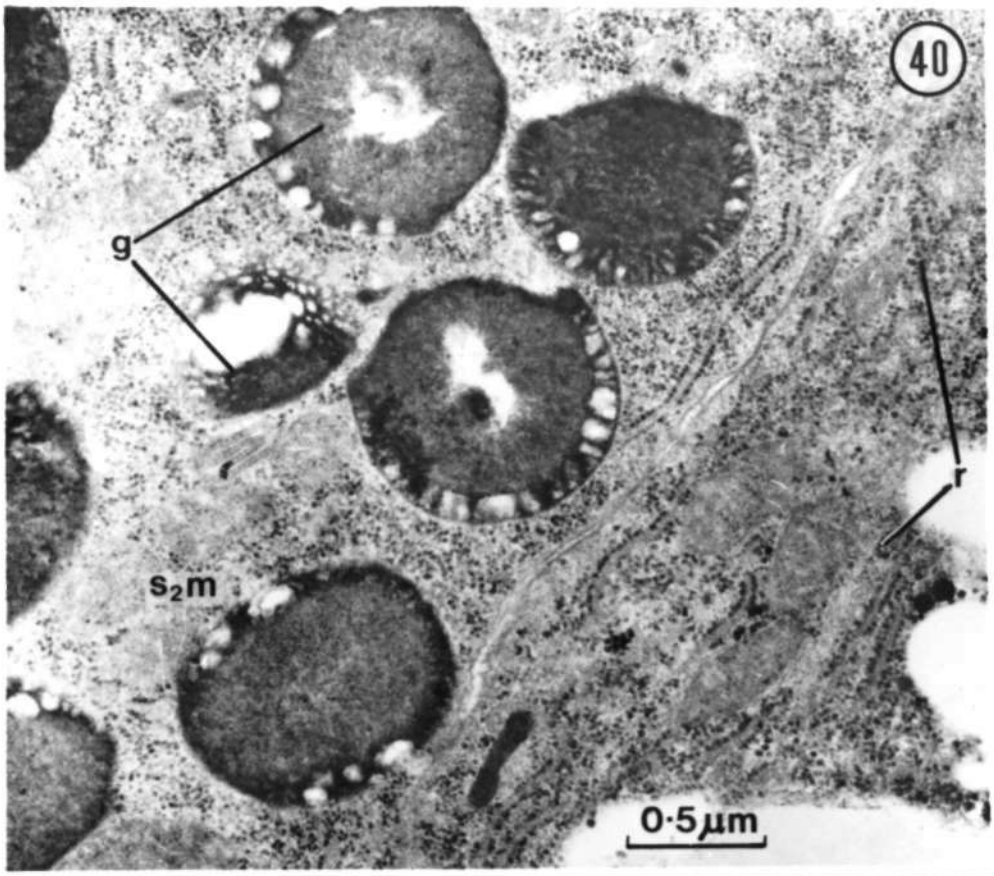
The possibility of another secretory cell type that was not observed by light microscopy is noted here (Fig. 33). The cell was small and pear-shaped and contained numerous small dense granules in the cytoplasm, and the luminal border had a tuft of long thick microvilli. Mitochondria and patches of organised r.e.r. were present in the cytoplasm.

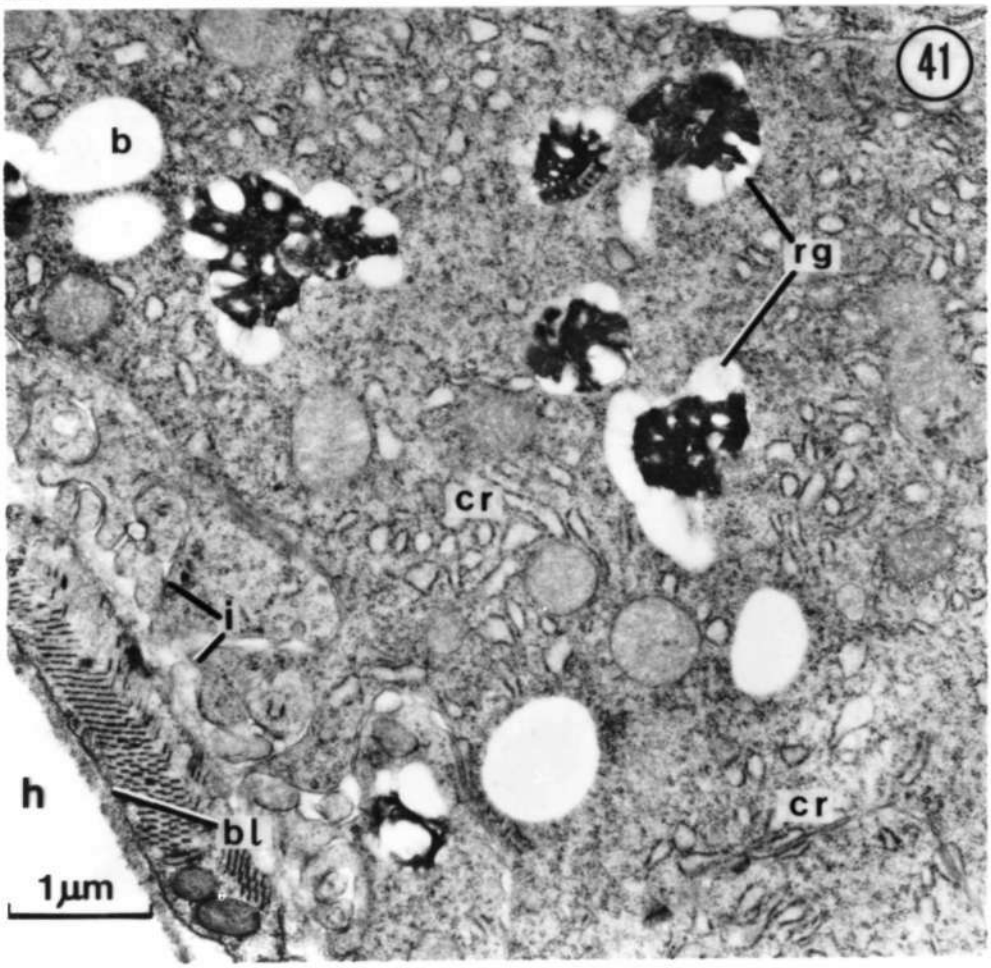
5.4.2 Secretory Cell Type 2 (s_2e, s_2m)

The early s_2 cell (s_2e) had darkly staining cytoplasm which was basophilic in light microscopy. On the luminal border, the cell had numerous, closely spaced and uniformly sized microvilli, but no pinocytotic vesicles (Fig. 30). Usually the cell tapered to a narrow rounded tip. The cytoplasm was filled with developing parallel arrays of organised rough endoplasmic reticulum whose cisterns were swollen and were concentrated around the nucleus (Fig. 34). Large lipid-like granules and glycogen deposits accumulated below and above the nucleus (Fig. 35) but there were no haematin granules. Around the nucleus, but in close association with the rough endoplasmic reticulum, there were small parallel, flattened saccules and vesicles which contained a homogeneous substance of moderate electron density which appeared to be vesicles of the Golgi complex (Fig. 36). The basal region of the cell had infoldings of the plasma membrane forming a basal labyrinth (Figs. 34 & 37). Numerous mitochondria were present in the labyrinth, but also in the other parts of the cell. Electron-dense granules accumulated within the folds of the basal plasma membrane, and up to the level of the nucleus but not beyond. The granules were roundish and had small broad projections and were smaller (0.94 ± 0.05 µm) than the s_1 granules (Figs. 37 & 38).

FIG. 40. T.E.M. of secretory cell type 2, s_2m; apical granules, g; free ribosomes, r. No *en bloc*.

FIG. 41. T.E.M. of basophilic cell developing from secretory cell type 2; remnant s_2 secretory granules, rg; poorly organised rough endoplasmic reticulum, cr; basal lalbyrinth, i; haemolymph space, h; basal lamina, bl. *en bloc*.


40
g
r
s_2m
0·5µm


41
b
rg
cr
i
h
bl
cr
1µm

In the mature s_2 cell (s_2m) the rough endoplasmic reticulum appeared to break down and become less organised and many free ribosomes abounded in the cytoplasm. The lipid vacuoles were larger with the glycogen present in clusters around the lipid deposits. The granules had also enlarged and now occupied a more apical position closer to the lumen. At the level of the lipid and glycogen deposits the granules became closely associated with the lipid, and appeared to have been coated with an uneven layer of lipid on the outer surface (Fig. 39). When these granules were near the tip of the cell, they had become larger and more rounded with their centres less electron-dense than the edges (Fig. 40). These mature granules were released together with the whole apical tip of the cytoplasm and they appeared as large spheres in the gut lumen, which form part of what has been referred to as the gut "colloidal" mass (Chapter 3).

5.4.3 Subsequent Development of s_2m to Basophilic Cell

The basophilic cells appeared to develop from the remaining basal portion of the s_2m cell which had budded off their granules into the lumen (see 5.4.2). During the period of rapid tick feeding, the basophilic cells grew and they underwent some changes in internal structure. Details of this reorganisation and its possible relevance to water transport across the gut is the subject of Chapter 6.

After engorgement, the basophilic cells could still be recognised because some of them had a few remaining s_2m granules (Fig. 41). The basophilic cells enlarged and stretched towards the lumen, thus maintaining contact with the digest cells which were expanding into the lumen due to increased numbers of food vacuoles (endosomes). The basal labyrinth contracted and the r.e.r. started to become reorganised and distended (Fig. 41). Also present in association with the widened

cisterns of the r.e.r. were structures which had vesicles similar to Golgi complexes. Closely associated with these vesicles were new, electron dense, secretory granules. The granules occurred in aggregates and their ultrastructure and position in the cells was different from the earlier granules (Fig. 42). Mitochondria were present, but there were no lysosomes or haematin granules. The secretory granules matured and some secretory material released into the extracellular space (exocytosis) could be observed by day 4 post incubation (Fig. 43), by which time it was observed that the ticks had started to lay eggs.

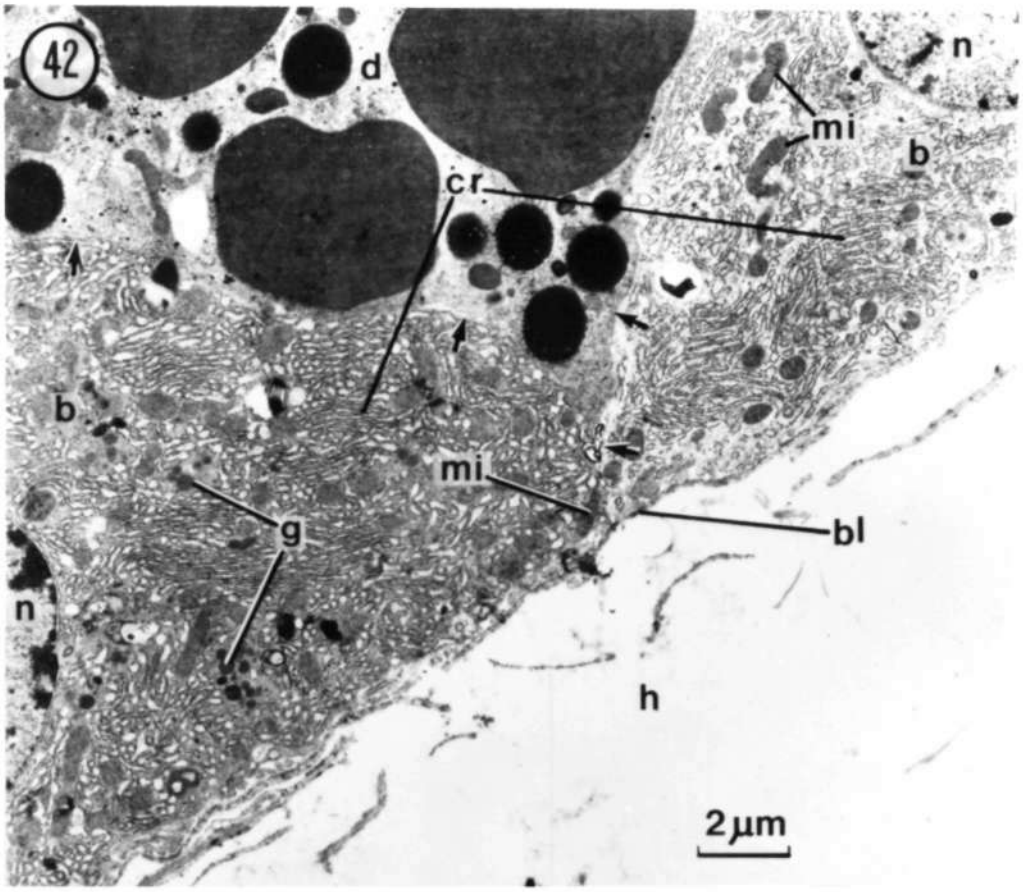
5.5 DISCUSSION

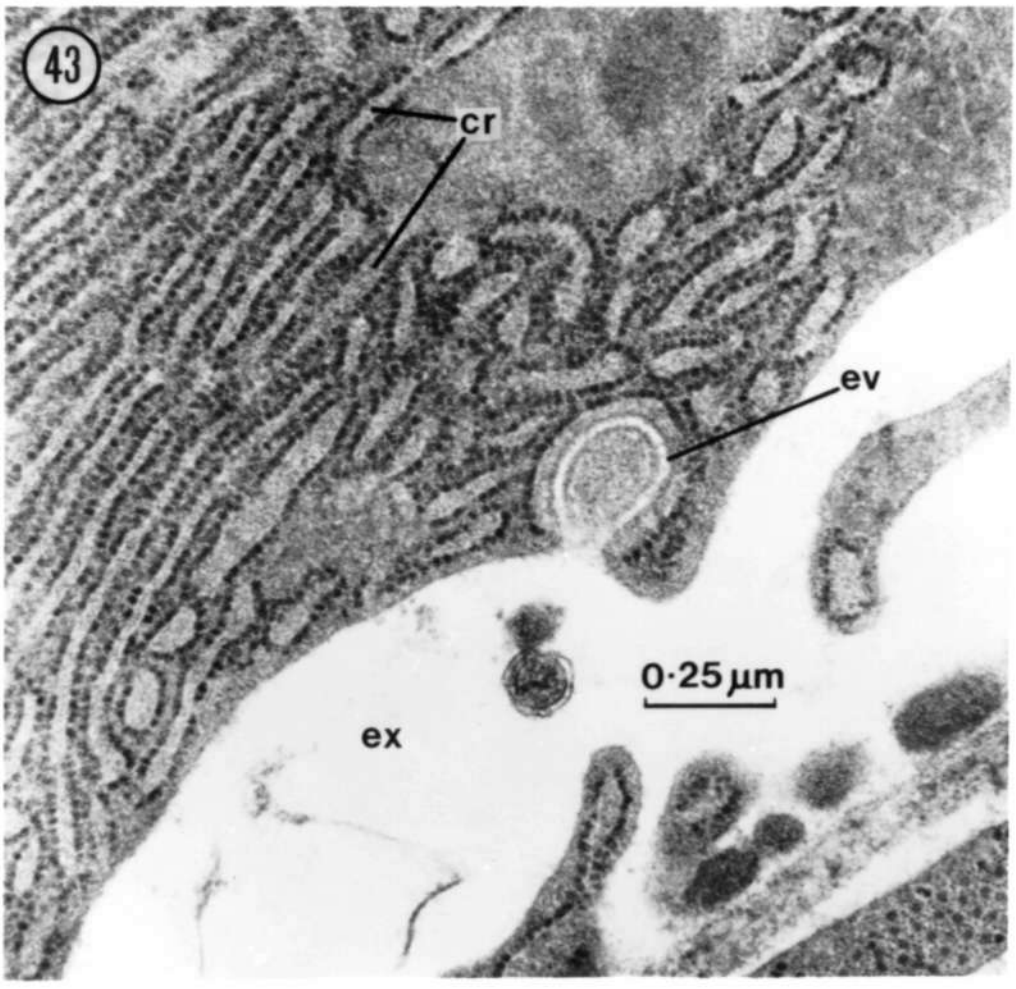
The ultrastructural study by Balashov and Raikhel (1976) suggested that the same cell secreted the P.A.S. positive material and the gut colloid mass which was demonstrable only when stained with Alcian blue/ P.A.S. stains. However, the results from this ultrastructural study showed that the granules of the s_1 and s_2 secretory cells were different and the two cell types could sometimes be found together in one caecum. The cells differed in size, shape, and ultrastructure. The s_1 cell cytoplasmic organelles were virtually depleted by the time the granules matured, whereas the s_2 cells still retained their organelles although they were less well organised than in the early stage. This evidence confirmed the suggestion in Chapter 3 that there are two distinct secretory cell types.

The presence of small vesicles at the edge of the large s_1 granules, similar to the budded vesicles from the r.e.r. suggest that the origin of the added vesicles was from the r.e.r. The granules have been associated with the release of haemolytic enzymes (Chapter 3). The s_2 granules were unusual, both in shape and formation. The results suggested that the

FIG. 42. T.E.M. of two basophilic cells, b and a digest cell, d; new secretory granules, g; organised cisternae of rough endoplasmic reticulum, cr; basal lamina, bl; digest cell, d; nucleus, n; mitochondria, mi; haemolymph space, h; arrows indicate boundary of basophilic cell. No en bloc.

FIG. 43. T.E.M. of basophilic cell 4 days post engorgement; possible exocytosis vesicle, ev; organised rough endoplasmic reticulum, cr; extracellular space, ex. No en bloc.


42
d
n
mi
b
cr
b
mi
g
bl
n
h
2 µm


43
cr
ev
0·25 µm
ex

electron dense material from the r.e.r. was packaged in the Golgi. The granules later became associated with the lipid and glycogen accumulations before being secreted. The staining properties suggested the colloid mass might be an anticoagulant (Chapter 3).

The possibility of a third secretory cell type requires further investigation. It is possible the cells with their small dense granules are immature secretory cell type 1, however it has been observed that some of the granules appear to be released into the lumen.

There has been considerable speculation on the origin and transformation of gut cells in ticks. Tatchell (1964) was sceptical about the existence of distinct secretory cells in A. persicus and he suggested that after differentiation from the stem cells, secretory cells could secrete and, after a phase of inactivity, had the potential to later revert to digest cells. On the other hand Balashov (1972), studying O. papillipes, referred to secretory cells which had a cytoplasm in which the apical half of the cells were highly vacuolated, and the basal half contained numerous basophilic, fibrillar structures (r.e.r.). He believed that the apices of these secretory cells broke away into the lumen together with part of the cytoplasm including the nucleus, and that the retained proximal parts of the cells soon degenerated. For B. microplus, the sequence suggested by Balashov has been found to be true only for s_1 cells.

The origin of the s_2 cell also appeared to be from the stem cell, but the release of granular material and subsequent transformation of the cell to basophilic cell was quite different. The evidence for transformation is as follows: in the replete females, specialised digest cells and

basophilic cells were the only cells present in the gut. The basophilic cells occupied a position in the gut which was the same as the former s_2 cells and there were no stem cells present at this stage from which basophilic cells could arise (Chapter 3). The similar ultrastructural characteristics of the s_2 cells and the basophilic cells particularly the presence of residual s_2 granules in the basophilic cells, is the final evidence that the basal remnants of the s_2m cells transformed to basophilic cells. The function of the basophilic cell during final rapid engorgement is discussed in Chapter 6.

After the tick dropped from the host, the *r.e.r.* of the basophilic cells became reorganised and the cells synthesised new granules which were different from the s_2m granules. The ultrastructure of these cells is now very similar to the cells in ovipositing R. sanguineus which synthesised vitellogenin (Coons et al., 1982). The material secreted by the basophilic cells in B. microplus might also be vitellogenin. This material is probably secreted first into the extracellular spaces between the basal infoldings of the cell which are in contact with the haemolymph, and is presumably then incorporated into the developing ova. The mechanism of secretion from the basophilic cell is unknown; possibly membrane bound granules are released into the extracellular spaces where they rupture and their contents dissolve, or material could be released by exocytosis of microvesicles.

Further discussion of this cell in relation to parasitisation by B. bovis is the subject of Chapter 7.

5.6 SUMMARY

The ultrastructural study of secretory cells type 1 and 2 and the basophilic cell, confirmed the separate identities of the two secretory cell types.

Secretory cell type 1 synthesised and secreted large uniformly electron-dense spherical granules.

Secretory cell type 2 synthesised smaller, irregularly shaped, and more complex granules.

The basophilic cell was shown to be the reorganised basal remnant of secretory cell type 2. A few of the cells still had characteristic s_2 granules left in the cytoplasm. The new cisternae of rough endoplasmic reticulum, which were arranged in whorls and parallel arrays, synthesised granules with a different ultrastructure and position in the cell. It was considered that the new secretory material was egg proteins which were released into the haemolymph, and transported to the ovary.

The presence of another secretory cell type with small spherical granules was shown, but more evidence is needed to prove that this was not an immature secretory cell type 1.

CHAPTER 6

LIGHT AND ELECTRON MICROSCOPE STUDY OF THE BASOPHILIC CELL DURING FINAL ENGORGEMENT: POSSIBLE ROLE IN WATER AND IONIC BALANCE OF THE TICK

6.1 LITERATURE REVIEW

Female ixodid ticks, while feeding on the host, can increase their body weight more than 100 fold (Lees, 1946a; Balashov, 1972). The mammalian blood ingested is a relatively dilute fluid and considerably hyposmotic to the tick tissues (Tatchell, 1969a), so to balance its own fluids and to gain greater nutrient value from the blood meal, ticks are continually excreting water and ions from ingested blood. The engorged weight of D. andersoni for example, represents only about 20% of the total weight of blood imbibed (Kaufman & Phillips, 1973). This ability to concentrate the blood meal greatly increases the fecundity of female ticks and the mechanisms involved have received more attention in recent years than most other aspects of tick physiology (Kaufman & Sauer, 1982).

In ticks, as in other animals, there appears to be two mechanisms for excreting water and ions, each with its own structural and physiological characterstics. In argasid ticks this function is performed by the coxal organ which is a pressure filtration-resorption system, as in the mammalian kidney (Bone, 1943; Lees, 1946b; Kaufman et al., 1981; Kaufman & Sauer, 1982) and as such has an ultrastructural appearance which is rather similar to the kidney. The filtration membrane of the O. moubata coxal gland has, between the pedicels, pore slits covered by a fine diaphragm (Hecker, Diehl & Aeschlimann, 1969) and this diaphragm is believed to give the characteristic filtration properties of the organ. Resorption takes place in the proximal tubule whose cells have the typical structural features of transporting epithelia, namely deep infoldings of the basal

and lateral plasma membrane with numerous mitochondria in the infolded region of the cell. In the alternative mechanism for water excretion, diffusion of water occurs along an osmotic gradient. This may be a passive process, or in the standing gradient hypothesis, sodium is actively pumped out of the cells (into the plasma membrane infoldings) creating the necessary concentration gradient for water to follow. Structural features of the cells would again be typical of transporting epithelia (Kaufman & Sauer, 1982).

Gregson (1967) was the first to suggest that the excess fluid taken in by ixodid ticks might be excreted via the salivary glands rather than by transpiration through the cuticle (Lees, 1946a). Tatchell (1967, 1969b) observed the regulatory role of the salivary glands in B. microplus and showed that the concentration of Na^+, K^+, Ca^{++}, Mg^{++} and Cl^- in the whole tick was equilibrated to stable values during the rapid phase of engorgement. He also calculated the blood meal volume of the tick on the basis of the tick's haemoglobin content. By comparing the blood meal volume with the net weight gain of the tick during the feeding period, Tatchell also calculated the total volume of excreted saliva, the weight of which was approximately equal to the final engorged weight of the tick. From the calculated volume of saliva, and measurements of ion concentrations, he demonstrated that excretion of excess Na^+ and Cl^- from the blood meal occurred entirely via the saliva. The studies also showed that salivation became important for fluid balance in B. microplus only during the rapid phases of engorgement, which means that the engorging tick must eliminate within a relatively short time the excess water as well as ions taken in with the blood meal.

These original studies focussed attention on the structure and [illegible] functions of ixodid salivary glands (reviewed by Sauer,

1977; Kaufman & Sauer, 1982). In terms of structure, Meredith & Kaufman (1973) were the first to identify at least one of the cell types (water cell) in the salivary glands involved in water excretion. Subsequent information and additions to the proposed mechanism of excretion were provided by Megaw (1976), Diehl, Kaufman, Aeschlimann & Guggenheim (1977); Megaw & Beadle (1979). Recently, Fawcett et al. (1981a,b) showed in ultrastructural studies that changes in acinus III of R. appendiculatus salivary glands provided valuable clues to the secretory process. Specifically they observed that the 'f' cells after a brief period of intense secretory activity underwent extensive changes in external form and internal organisation to become what had previously been called "vacuolar cells". These transformed 'f' cells, together with the water cells (renamed ablumenal interstitial cell) both contributed to the formation of an elaborate plasma membrane labyrinth and to the excretion of water.

It is surprising that rather little is known about fluid transport across the gut of ticks because rapid removal of large volumes from the haemolymph via the coxal or salivary glands, presumably means that a similar process is taking place from gut lumen to haemolymph.

6.2 INTRODUCTION

The literature reviewed above provides good evidence for the rapid, active transport of water and ions through the coxal and salivary glands. Presumably a similar process must be taking place across the gut to furnish the excretory organs with excess water and ions, especially during the final rapid phase of engorgement. Only two papers have dealt specifically with the ionic mechanisms of fluid transport across the gut (Barker et al., 1977; Kaufman et al., 1981), but it is not known which gut

cells are involved (Grandjean & Aeschlimann, 1973). It was noted by Belozerov & Tymopheev (1973) that the basal region of some gut epithelial cells in Dermacentor marginatum had infoldings which were characteristic of water and ion transporting cells, however they made no further comment on this point. This chapter deals with the structure of the gut in relation to water and ion transport with particular attention being paid to the basophilic cell and to cell to cell junctions.

6.3 MATERIALS AND METHODS

The Babesia-free Yeerongpilly strain of B. microplus was used and female ticks were sampled from the donor calf at 10.00 hrs on days 17, 18 and 19 post infestation as larvae.

Five ticks were taken on each day and processed for electron microscopy as described in Chapter 2.

6.4 RESULTS

Females sampled on days 17, 18 and 19 post infestation showed the progression in development of secretory cell type 2 and the changes taking place as the cell transformed to the basophilic cell (Chapter 5). The early secretory cell type 2 (s_2e) was narrow and spindle shaped with a basophilic cytoplasm rich in ribosomes and cisternae of r.e.r. The basal labyrinth was not particularly extensive and the extracellular spaces not usually distended (Figs. 44 & 45).

Before the onset of final rapid engorgement, the secretory granules which formed in the base of the cells appeared to move to the tips of the cells and the apical portion of the cells, with their granules, then budded of into the lumen to form the "colloidal mass". The basal

FIG. 44. Section of gut wall (light microscopy) showing two early secretory cells type 2, s_2e; lumen, lu; digest cells, d. (Paraffin section, Alcian blue/P.A.S.).

FIG. 45. T.E.M. of basal region of an early secretory cell type 2, s_2e; cisternae of rough endoplasmic reticulum, cr; arrows indicate free ribosomes; basal lamina, bl; basal labyrinth, i. No *en bloc*.

FIG. 46. T.E.M. of part of basophilic cell with extended basal labyrinth, i; extracellular space, ex; mitochondria, mi; glycogen, gly; basal lamina, bl; muscle cell, m; haemolymph, h. No *en bloc*.

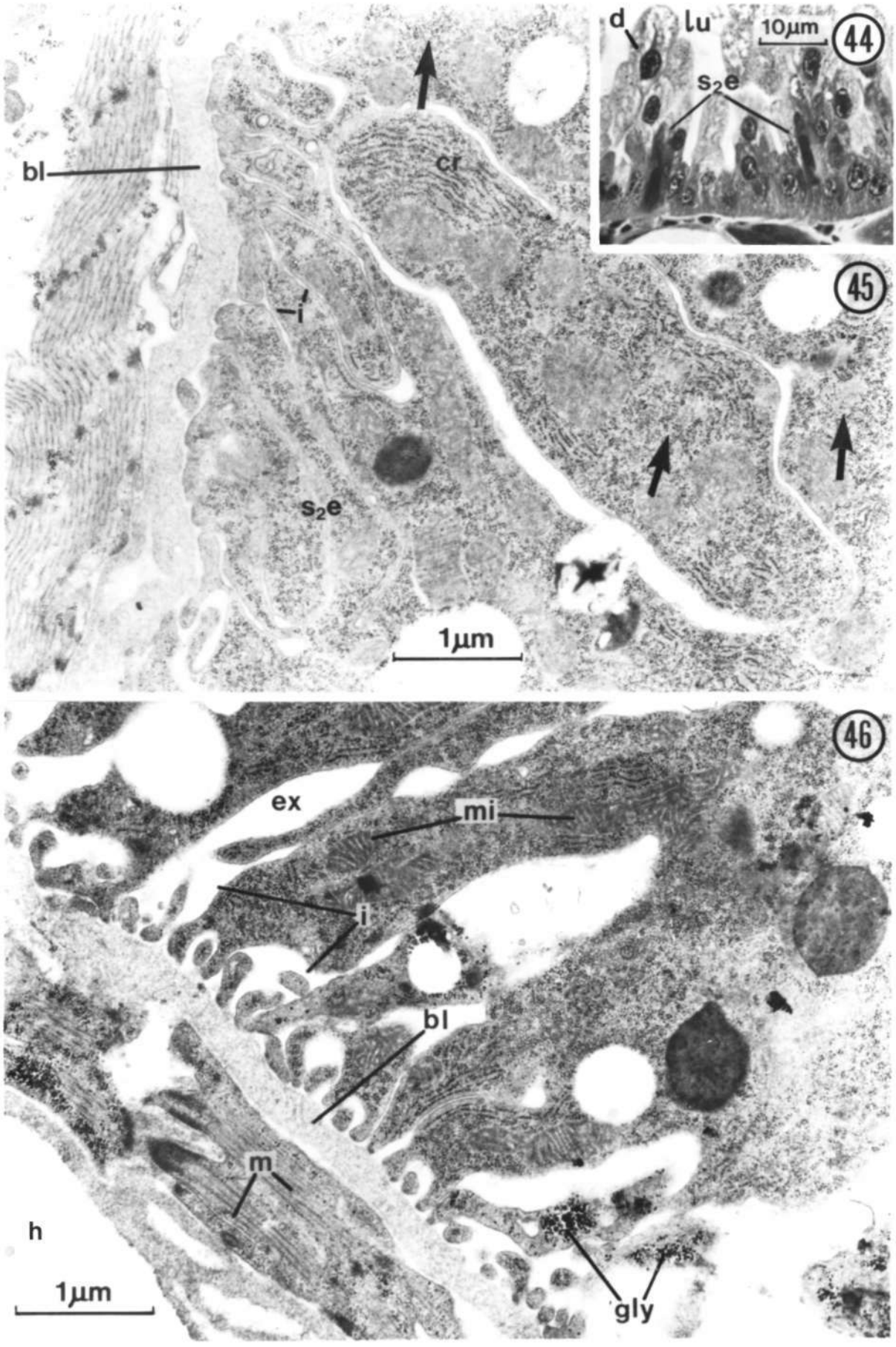
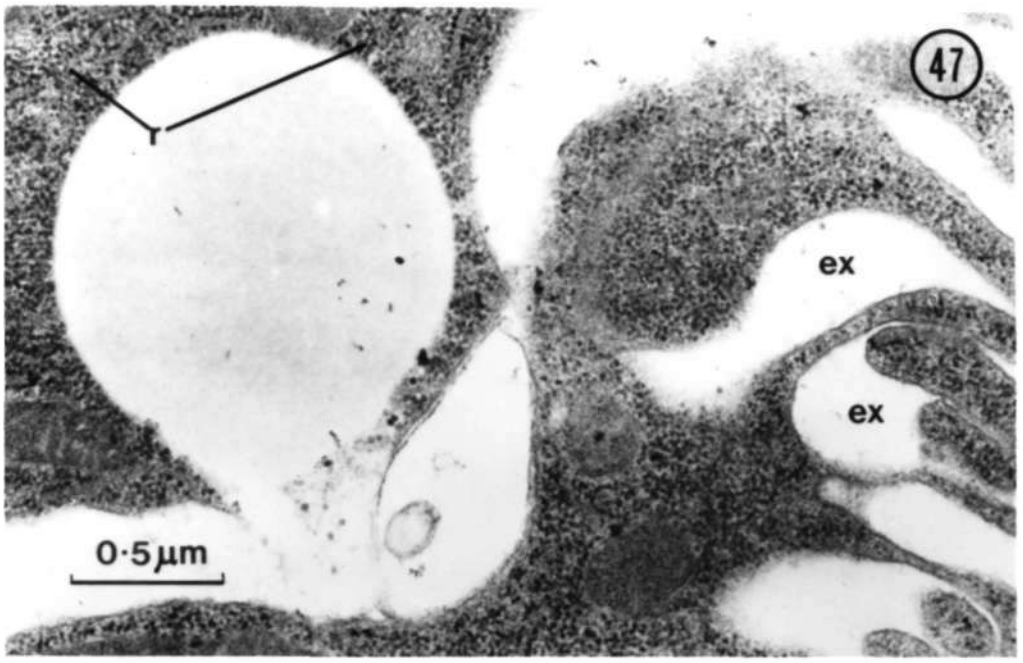
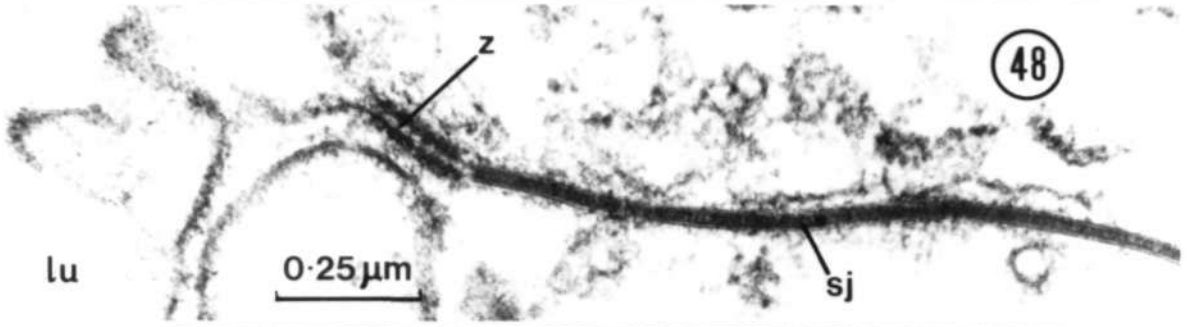

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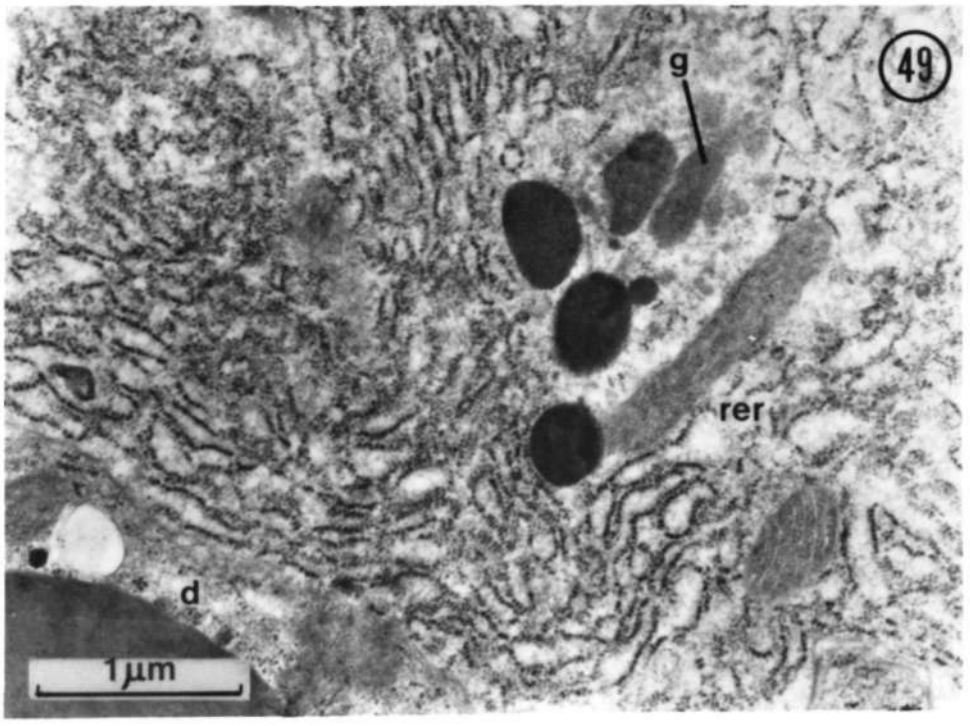
FIG. 47. T.E.M. of part of basophilic cell with distended extracellular space of the basal labyrinth, ex; free ribosomes, r. No en bloc.

FIG. 48. T.E.M. of junctional complex between gut cells; zonula adhaerentes, z; smooth septate junction, sj; lumen of gut, lu. en bloc.

FIG. 49. T.E.M. of basophilic cell after engorgement, with distended rough endoplasmic reticulum, r.e.r.; secretory granules, g; digest cell, d. No en bloc.


47
ex
ex
0·5µm


z
48
lu
0·25µm
sj


g
49
rer
d
1µm

remnants of the cells with their nuclei did not degenerate, but grew again and underwent some changes in internal structure during the period of rapid feeding (final day on the host). The organisation of the r.e.r. into whorls and parallel arrays had begun, but more noticeably, the basal infoldings of the plasma membrane extended deep into the cell cytoplasm, and the extracellular spaces were often distended (Figs. 43 & 47). There was some interdigitation of the lateral plasma membranes of the basophilic and digest cells,but this was nowhere extensive.

The lumen area of contact of the basophilic cells was relatively small and the plasma membranes had numerous, closely spaced and uniform sized microvilli, but no pinocytotic vesicles (Fig. 30). The junctional complex between gut cells close to the lumen appeared to be a zonula adhaerentes followed mainly by extensive smooth septate junctions (Figs. 45 & 48). The zonula adhaerentes had dense material on the cytoplasmic sides of the membranes and an intercellular gap of 2 to 4 nm.

After the tick dropped from the host, the basophilic cells enlarged to maintain contact with the expanding digest cells. The basal labyrinth contracted and the r.e.r. reached its maximum organisation into whorls or parallel arrays (Figs. 42 & 49). This is associated with the appearance of new granular material which is believed to be yolk proteins (Fig. 46) (Chapter 5).

6.5 DISCUSSION

In attempting to relate the water excretory mechanisms described in section 6.1 with the ultrastructure of the gut at the time of rapid feeding, it is clear that there is nothing in the gut comparable to the pressure filtration membrane of the coxal organ in *O. moubata* (Hecker *et*

al., 1969) or the mammalian kidney. However, on the available evidence, it cannot be excluded that for the long slow feeding period of female ixodid ticks, the passage of water across the gut is by either pressure filtration or diffusion from lumen to haemolymph, with water and ionic balance being regulated by the salivary glands. Diffusion of water through the gut cells to the haemolymph presumably occurs to some extent, given the osmotic difference between bovine blood 188 ± 22 mM/l NaCl and B. microplus haemolymph 231 ± 54 mM/l NaCl (Tatchell, 1969c); but pressure filtration would presumably have to occur between cells. That is the zonula adhaerentes and the smooth septate junction reported here must not form a barrier to water and small molecules, and evidence from other sources suggests that they do not (Lane & Skaer, 1980; Fawcett et al., 1981). One further problem occurs with a pressure filtration hypothesis, that is, there is no information on pressure differences across the tick gut. The internal pressure of O. moubata females rises to 40 Torr as feeding progresses (Kaufman et al., 1981) and similar results would be expected for the rapid feeding phase of B. microplus. The gut is surrounded by muscles which perform a vigorous peristalsis, and this could provide a sufficient local pressure difference across the gut for filtration.

During and after rapid final engorgement, diffusion of water across the gut may be too slow for adequate concentration of the blood meal, and there is evidence for an active rather than a passive process. In O. moubata, Kaufman et al.(1981) showed that movement of water across the gut was no greater with dilute blood meals where a greater osmotic gradient occurred, and that rate of fluid absorption was dependent on the Na^+ and Cl^- concentration of the diet. They proposed that water movement across the gut was coupled to active transport of NaCl. The gut of the ixodid

tick D. andersoni is selective in the passage of ions to the haemolymph, thus Na^+ and Cl^- pass but K^+ does not (Kaufman & Phillips, 1973) so passive diffusion or filtration is not indicated. Finally, Barker et al. (1977) showed that a gut preparation from Amblyomma maculatum maintained an electrical potential of 6.8 mV, gut negative to haemolymph, and in O. moubata the in vivo potential was 14-16 mV (Kaufman et al., 1981).

This and other evidence from the papers quoted indicates that a cell in the tick gut is capable of active transport of ions. In B. microplus, the only cell with the necessary ultrastructure appears to be the basophilic cell between its two phases of secretory activity. At this intermediate stage it has numerous mitochondria and an extensive basal labyrinth into which ions could be pumped to create an osmotic gradient. It is tempting to suggest that digest cells might also be involved. These cells have much greater area of contact with the lumen compared with the basophilic cells (even considering microvilli) and there is some increased lateral surface contact between the two cell types. As noted in Chapter 4 there was increased electron density of the blood meal in the digest cell endosomes indicating concentration of the blood in these vacuoles. It is proposed that basophilic cells actively create an osmotic gradient and water from the lumen and the digest cells follows into the haemolymph.

It may seem implausible that such complex transitions in form and function could take place in the basophilic cells. However, there is a parallel for this in the 'f' secretory cell of the salivary glands of R. appendiculatus (Fawcett et al., 1981a,b), a cell also found in B. microplus (Binnington, 1978). After a rapid secretory phase the cells transform, losing their organised cisternae of r.e.r. although retaining many free ribosomes. An extensive labyrinth is formed in association with

the abluminal interstitial cell (water cell), and numerous mitochondria are found in the filaments of cytoplasm. As already noted, they are considered to be actively involved in water excretion by the salivary glands (Fawcett et al., 1981b). The basophilic cell of the gut differs from the 'f' cell to some extent, its basal labyrinth is not so extensive, and it is never without some cisternae of r.e.r. This could be related to the later resumption of secretory activity.

In blood sucking insects there is evidence for active transport as a means of concentrating the meal, and the water transporting cells are rather similar in ultrastructure to the basophilic cells of B. microplus (Boehringer-Schweizer, 1977; Brown, 1980).

6.6 SUMMARY

The basophilic cell was examined in female ticks during the final, rapid phase of engorgement and beyond. Smaller numbers were located in the gut of nymphs.

Its ultrastructure, that is a basal labyrinth and numerous mitochondria, fitted it for a role in active water transport across the gut during rapid final engorgement.

During the post-engorgement phase, the basophilic cell reorganised its rough endoplasmic reticulum and resumed a secretory role.

CHAPTER 7

BABESIA BOVIS (BABES, 1889) DEVELOPMENT IN THE GUT OF B. MICROPLUS

7.1 LITERATURE REVIEW

Babesiosis is a disease of almost cosmopolitan distribution affecting many species of domestic mammals. Some Babesia parasites are shared by domestic and wild animals, while some are peculiar to wild animals, but the extent to which this disease occurs in wild animals remains largely undetermined. Bovine babesiosis has the widest distribution, being present in tropical and temperate areas and is found in all continents where cattle are reared. In other domestic mammals the disease appears to be of lesser importance, and its distribution is more limited (F.A.O. Animal Health Year Book, 1968). Lack of suitable tick vectors is probably the most important force in restricting the distribution of Babesia.

Bovine babesiosis was first recorded in Australia in 1880 by Gilruth (1912b), although it was probably introduced in 1872 when the sole vector of bovine babesiosis in this country, the tick B. microplus was introduced on cattle from Java (Seddon, 1952). Since then the vector has become widespread throughout most of Northern and Eastern Coastal Australia. The two species present are B. bigemina (Smith & Kilborne, 1893) and B. bovis (Starcovici,1893). B. bovis is the small pyriform Babesia occurring in the erythrocyte of cattle in Australia and is much more pathogenic than the larger parasite, B. bigemina, and is responsible for the majority of outbreaks of tick fever in Australia (Seddon, 1952).

The developmental cycle of Babesia in the vertebrate host may be entirely asexual. All development appears to be intra-erythrocytic, except for a brief period when newly divided parasites emerge from the

erythrocyte to infect fresh corpuscles. Krylov (1964) considered that an exo-erythrocytic cycle occurred but this opinion is quite contrary to the view held by most other authors. The intra-erythrocytic development appears to be a process of budding whereby the parasite divides equally into two and in some species into four "daughter" parasites (Fig. 50). These forms emerge from the host cell, usually causing it to rupture, and then infect other erythrocytes. This cycle seems to continue indefinitely, though mammals are seldom infected for more than two years (Levine, 1973) and with B. bovis it was often thought to be less than one year (Pierce, 1956) but Mahoney, Wright & Mirre (1973) showed positive parasitaemias for B. bovis 4 years after single transmission. Pre-immunity exists in Babesia infections. Hence mammals which recover from a clinical attack of babesiosis are immune to reinfection so long as a continuing low-grade infection persists. This low-grade infection is usually undetectable in thin blood smears and causes no obvious disease symptoms. The parasites are however available to the tick vector during feeding. Callow, McGregor, Parker & Dalgliesh (1974) showed that immunity will also persist after drug sterilisation of parasites for at least 6 months after treatment.

The transmission of Babesia by an arthropod vector was first discovered by Smith & Kilborne (1893). They reported in their classic monograph, the congenital transmission of the protozoan B. bigemina through the eggs of an arthropod, the cattle tick B. annulatus (Say, 1821) and also confirmed that the tick infected cattle through its bite. Since that date it has been shown for all species of Babesia, that ticks are the only known vectors. Much confusion still exists as to the nature of the developmental cycle in the tick and many early researchers were divided as to whether gametes occurred, and if so whether a sexual fusion of gametes was an integral part of the life cycle. Much of this confusion arose from trying to draw homologies between Babesia and Plasmodium.

The first detailed study of the life cycle of Babesia was by Koch(1905,1906) working with B. bigemina in Margaropus australis (= B. microplus);, R. evertsi and Hyalomma aegyptium. In the gut of the ticks he observed pear-shaped parasites which became amoeboid and had extended long, straight rays or thorn-like pseudopodia. These amoeboid forms associated in pairs and gave rise to organisms which possessed at first two nuclei which later fused to form a single nucleus. He recorded multiple division stages, but was unable to indicate how they were formed or their relationship in the developmental cycle. He called the rayed forms Strahlenkörper, and these developed in the gut lumen contents and gut cells of the replete female tick. Ultimately a final stage of Strahlenkörper, a spherical body was formed which turned into a multiple fission body in the cells of the epithelium of the gut. These fission bodies, when mature, released vermicules or merozoites which are clavate parasites later to be found in most organs especially the ova. Kleine (1906), reported the occurrence of similar stages during his cultivation experiments with canine blood, taken from dogs with a high parasitaemia of B. canis. Rather similar stages with spiky pseudopodia were also recorded by Dschunkowsky & Luhs (1910) but no information was given about their subsequent development. Crawley (1915) observed cigar-shaped bodies in smears from replete female B. annulatus, and from crushed preparations of eggs which they had oviposited. Although only a limited amount of material was available, he considered these forms to be identical with the clavate parasites described by Koch. In 1927, Rosenbusch described the development of B. bigemina in B. microplus in which he found numerous elongated forms in the gut caecae and haemolymph. Multiple division of these elongated clavate parasites occurred in the cells of the digestive and vascular organs. The salivary glands became infected, but transmission occurred only under defined conditions. Certain of these elongated

forms invaded the cells of the genital tube and without any further division were ultimately found in the ova. Multiplication commenced again only after the subsequent larvae fed on blood. Rosenbusch considered that the tick was the definitive host of this parasite, and that its transmission to cattle was not indispensable to its developmental cycle.

Dennis (1932) observed various stages in the development of B. bigemina in the gut and ova in replete females of B. annulatus and concluded that the initial development was the union of "isogametes" in the gut contents to form a motile "ookinete" which passed through the thin wall of the gut and penetrated the contiguous reproductive organs. The ova of the tick were invaded by the "ookinetes" which formed "sporonts". The sporonts gave rise to multinucleate "sporokinetes" which migrated throughout the tissues of the developing tick. Some of the "sporokinetes" came to occupy the salivary glands and finally underwent fragmentation to form minute "infective" sporozoites.

Regendanz (1936) disagreed totally with Dennis' work and concluded that the development of B. bigemina in the intestinal wall of B. microplus corresponded completely with that of B. canis in D. reticulatus as described by Regendanz & Reichenow (1933). After numerous binary fissions, the protozoa formed a motile vermiform stage which ultimately invaded the ovaries. Regendanz (1936) found no evidence of sexual forms, and he did not attempt to investigate the cycle in the larval and nymphal instars of the tick.

Muratov & Khessin (1959) studied the developmental cycle of B. bigemina in B. calcaratus = annulatus, and also described the formation of the club-shaped bodies (vermicules) observed by previous workers. These

club-shaped stages formed by binary fission or schizogony,penetrated into the epithelial cells of the intestine where multiple division, characterised by segmentation into amoeboid or round "agamonts" was observed. Some of these club-shaped stages entered the body cavity and ultimately penetrated all the organs of the female tick where further division occurred. These stages also penetrated the eggs of the tick where they continued reproduction by means of binary or multiple division. Amoeba-like agamonts were then formed which in turn developed into club-shaped forms. These increased in number during the process of incubation and were distributed throughout the organs of the developing larva. No sexual stages were demonstrated.

Riek (1964,1966) described the life cycles of both B. bigemina and B. bovis in the tick vector B. microplus. Reporting on B. bigemina he observed early development in the epithelial cells of the gut and after about 72 h mature "vermicules" were seen in the haemolymph and ova. A further process of multiple fission was also observed in the developing and engorged larvae. He observed that the final cycle occurred in the salivary glands of the nymph and forms infective for the vertebrate host were observed 8-10 days or more after the larvae were applied to the animals. He observed that multiplication was by multiple fission and although various forms that could be associated with a sexual cycle were observed he could not present irrefutable evidence for this. Potgieter & Els (1977) working with B. bigemina in larvae, nymphae and non-replete females of B. decoloratus observed that primary schizogony occurred as a continuous repetitive process in all 3 stages of the tick's cycle spent on the host. The primary schizonts and the large merozoites (= vermicules) were observed in the gut epithelium, haemocytes, muscles and peritracheal cells. Secondary schizogony they claimed led to the formation of small

merozoites (= infective forms) which occurred mainly in the salivary glands but was also observed in the cortex of the synganlion. Mature small merozoites they observed in nymphal and adult ticks only. Potgeiter et al. (1976) also described "merozoites" of B. bovis in the epithelial cells of the gut of B. microplus after seven days post incubation of the replete female but made no commitments as to sexual stages in the tick.

Petrov (1941) studied the development of B. divergens (= "B. bovis") in I. ricinus. He described a developmental cycle very similar to that reported by Dennis (1932) for B. bigemina. He stated that the "isogametes" fused in the tick's intestine to form an "ookinete" which passed through the intestinal wall and entered a developing ovum. Here the ookinete became globular and formed "sporoblasts" which in turn formed "sporozoites" which passed to the salivary glands. The larvae, nymphae, and adults, of the succeeding generation all transmitted the parasite. Polyansky & Kheissin (1959) found that B. divergens followed essentially the same pattern in I. ricinus as described by Muratov & Kheissin (1959) for B. bigemina in B. calcaratus (= annulatus). They stated that it reproduced by binary fission or schizogony in the tissues of the tick and in the eggs of infected females. They found no stages of sexual reproduction or sporogony.

Friedhoff & Bürscher (1976) rediscovered Koch's Strahlenkörper of B. bigemina and Friedhoff (1981) described a hypothetical life cycle in which he asserted that the Babesia stages that initiated development in the replete female were still unknown; but maintained that the peculiar irregular shaped forms (some with long rays, and some with spikes) which developed in the tick gut as had already been described by Koch (1905,1906) represented sexual stages. He retained the name

Strahlenkörper (= rayed forms or bodies) although syngamy still had not been demonstrated. Webber & Friedhoff (1977) described the presence of Strahlenkörper in the gut contents and gut epithelial cells. These parasites they believed developed from the erythrocytic phase of the parasite and had the characteristic spike-like projection and microtubules which they regarded as an identifying characteristic of parasites sexual stages. These forms are believed to be short-lived and formed kinetes within the gut epithelial tissues shortly after their appearance.

Wattendorff (1980) also recognised different types of Strahlenkörper, one of which he considered formed fission bodies that produced many spherical daughter cells, the precursors of kinetes or vermicules. Another type occurred in small numbers and persisted in the gut without apparent development. Sexual stages were also described for B. canis in the gut of D. reticulatus by Melhorn, Schein & Voight (1980).

Dalgliesh, Stewart & Rodwell (1981), using B. bovis parasites derived from stabilates of a laboratory strain prepared at intervals during several years of blood passaging in cattle, observed increased numbers of a particular developmental stage of the parasite in smears of gut contents. They considered them to be a type of Strahlenkörper. They associated the presence of a large number of these parasites with reduced infectivity of the strain for ticks.

Drolesky, Holman, Craig, Wagner & Mollenhauer (1983) reported the presence of sexual stages (parasites which possessed spike-like projections containing microtubules) of B. bovis in a culture medium and within cultured cells. These parasites they believed developed from the erythrocytic phase; as the parasites used for inoculating the cultures were derived from freshly collected bovine blood with a B. bovis

parasitaemia of 8.5% and was differentially lysed and inoculated onto previously prepared monolayer cultures of a B. microplus cell line. This in vitro conversion of erythrocytic B. bovis parasites to a sexual stage normally found in the tick gut they believed was prompted by culturing at 32°C in 3% carbon dioxide coupled with the interaction of B. microplus cells, as they observed that the cells had phagocytised Babesia parasites.

Only Rudzinska, Spielman, Riek, Lewengrub & Piesman (1979) distinguished clearly two stages of Babesia microti - a reproductive stage that produced merozoites and another stage that was in the minority. Its cytoplasm remained undifferentiated and no reproduction took place. This stage became larger than the first type and acquired an odd shape by folding, coiling or twisting together with a resulting reorganisation and differentiation. The authors assumed that these erythrocytic stages were Strahlenkörper (gametocytes). Rudzinska, Spielman, Lewengrub, Piesman & Karakashian (1982) further described for the first time the presence of a peritrophic membrane (P.M.) in the gut of feeding larvae, nymphae and adults of Ixodes dammini which is a vector of B. microti. This temporary structure divided the lumen of the gut into two concentric compartments, an internal endoperitrophic space (the lumen proper) and an external ectoperitrophic space, located between the P.M. and the epithelial cells. As a result the P.M. provided a mechanical barrier so that even small particles such as ribosomes derived from ingested reticulocytes were retained in the lumen proper and were never found in the ectoperitrophic compartment. They also described the passage into the ectoperitrophic space of B. microti which was aided by its highly specialised organelle, the arrow head. They further noted that only Babesia equipped with the arrow head structure were able to cross the P.M. Such stages have not been described in Babesia other than B. microti, nor has a P.M. been observed in other ticks.

Rudzinska, Spielman, Lewengrub, Trager & Piesman (1983a) showed that during the crossing of the P.M. the arrow head lost its density and once in the endoperitrophic space the arrow head touched the epithelial cell. It is not clear whether this contact was with the microvilli on the surface of these cells. However, they further observed that at the point of contact, the membrane of the host cell invaginated and encircled the parasite. The point of contact between the parasite's plasma membrane and the host membrane appeared to be at the site of the coiled structure. The coiled structure then, they believed, released enzymes which caused the host membrane to rupture and nearby host cell cytoplasm to degenerate. Eventually the parasite became surrounded by a halo of homogeneous material derived most probably from the host cytoplasm. The cell type invaded by the parasite was not identified.

The identity of the Strahlenkörper as a developmental stage of Babesia and Theileria can no longer be questioned; as Strahlenkörper have now been demonstrated in five species of Babesia (B. bigemina, B. ovis, B. canis, B. microti and B. bovis), and three Theileria (T. annulata, T. parva and T. ovis). Whether the sexual fusion of these Strahlenkörper, or bodies arising from the Strahlenkörper, is present in any or all members of the genera Babesia and Theileria remains to be seen.

7.2 INTRODUCTION

Babesia in tick tissues undergo dramatic changes during development from the erythrocytic stages ingested to the final forms found in the salivary glands of the larvae. The apparent difficulty in describing accurately the various developmental stages arose in part from our insufficient knowledge of the Babesia life cycle in the tick vector and more importantly our scant knowledge of the gut structure and function in ticks.

The terminology used to describe the stages is also confusing. Many forms in the tick gut have been described under different names. Nevertheless the old terminology of Koch (1905,1906) has been retained by most recent authors and in a recent review, Friedhoff (1981) described the life cycle for Babesia in mammals and suggested a terminology for the various stages of the parasite. His review was concerned only with bovine and ovine Babesia. It should be noted that the life cycles of "Nuttallia"-type Babesia of rodents, insectivores and probably cats and equines are different and in this regard resemble the Theileria life cycles (Friedhoff & Smith, 1981).

The Babesia stage that initiates the development in the ticks, is now known as Strahlenkörper (Friedhoff & Büscher, 1976; Friedhoff, 1977), and as a rule the engorging female tick is the only stage that can acquire infection from its mammalian host. Callow (1968) provided evidence that infection of B. microplus with B. bigemina usually occurred only during the last 24 hours that the tick spends on the host, that is, the time of the final rapid engorgement of the adult. Babesia from rodents are an exception to this rule. These "Nuttallia"-type Babesia are acquired by engorging larvae and transmitted by nymphs (Friedhoff & Smith, 1981).

Most studies on tick fever of cattle have focussed on the elucidation of the complex life cycle of B. bigemina, B. bovis and B. ovis in the tick vectors without correlating this with the physiological developmental activities in the tick gut. Researchers who studied the tick gut such as Williams (1905); Christophers (1906); Roesler (1934); Douglas (1943); Hughes (1954); Till (1961); Chinery (1964); Balashov (1972); Balashov & Raikhel (1976) did not study the Babesias.

In an excellent combination of these two fields of study Fawcett, Büscher & Doxsey (1981a,b,1982a,b), and Binnington, Young & Obenchain (1983) have followed the development of the salivary glands of R. appendiculatus and the selection by T. parva of type 'e' cells of Acinus III for development within the salivary glands. Moltman, Mehlhorn, Schein, Voigt & Friedhoff (1983) have also followed the development of the salivary glands of three ixodid tick species (H. anatolicum anatolicum; H. a. excavatum; R. turanicus) and the formation of B. equi sporozoites in them. They observed that the development of the sporozoites was identical in all three vectors.

In this work a start has been made on a similar approach to Babesia development in the gut of B. microplus. The earlier chapters on the gut of B. microplus both at light and electron microscope levels, showed that during the feeding of nymphs and adults the gut caecae consisted of five cell types. The stem cell, which differentiated into any one of the other cell types, two subtypes of digest cells (dhl,dhv), two secretory cells (s_1, s_2) and a basophilic cell (b). By the time the engorged tick dropped from the host to digest the blood meal and lay eggs, only two cell types remained in the gut, the digest cells (dhl,dhv) and the basophilic cells (b) (Chapters 3, 5 & 6). The digest cells are involved with intracellular digestion of the blood meal, while the basophilic cells have a variety of functions which probably includes the secretion (exocytosis) of vitellogenin (Chapter 5).

The above background provided a basis for answering the following questions on the B. bovis life cycle in the tick.

1. Are Strahlenkörper really part of the life cycle in B. bovis infection in cattle?

2. In which of the two cell types mentioned above do the parasites develop? Is there any evidence for selectivity of a particular cell type for development?
3. What is the morphology of the fission body in the gut epithelial cell?
4. How do vermicules (kinetes) released from the fission body get to the haemolymph?

Most of the earlier work on the life cycles relied on gut smears which were allowed to dry and were then fixed and stained. This procedure is prone to errors as Babesia stages thought to be in the lumen, could have been squashed from the gut cells. Drying also could distort shapes and forms and dry fixing rather than wet fixing could produce the same false results. Better techniques are now available which enhance resolution at the light microscope level (for example semi-thin sections of plastic embedded material), and the fact that many gaps still exist in our knowledge of the Babesia life cycle in the adult female tick, suggests the need for a reappraisal.

7.3 MATERIALS AND METHODS

7.3.1 *In vitro* Feeding

The in vitro feeding system used in these experiments was a modification of the one used by Chabaud (1950a), Gregson (1957) and Joyner & Purnell (1968). Details of the modification are described by Willadsen, Kemp & McKenna (in press). Briefly, a glass capillary tube was drawn from a Pasteur pipette, such that the diameter of the tube decreased to a fine tip that was oval in cross-section, and fitted over the tick's mouthparts. The females were inverted and stuck to a glass slide with double-sided tape. The hypostome and chelicerae were fitted inside the

tube, and the palps were splayed along the outside of the tube and glued in position with a drop of cyanoacrylate adhesive (Selleys Supaglue, Australia). The tube was filled with 0.5 ml of heparinised bovine blood (preservative free heparin, Evans Co., 10 units/ml of blood) and the ticks plus tube were placed on a thermostatically controlled hot plate at 35°C overnight (giving approximately 17 hours of feeding).

Female ticks which were to be fed *in vitro* were collected from a donor calf at noon, and left at room temperature for 2 h to shed the cement cone from the mouthparts. The ticks were approximately 6 mm in length and were due to engorge by the next day (Wharton & Utech, 1969). The feeding was started at 2.00 p.m. and the experimental feeding period of each tick timed from when it was placed on the hot plate. Ticks were weighed at the start and after engorgement the next day before being incubated at 28± 1°C and > 90% R.H. Four *in vitro* experiments were carried out using the virulent tick-transmissible Samford strain of *B. bovis* at blood infection rates of 1%, 3%, 5% and 10% parasitaemia.

7.3.2 *In vivo* Feeding

An *in vivo* experiment was conducted in order to confirm the results obtained *in vitro*. A 10 month old calf was splenectomised and infected with the same strain of *B. bovis*. Several batches of larvae were infested on the calf resulting in engorged females being available as the parasitaemia rose to about 1%. The engorged ticks were incubated at 28±1°c and > 90% R.H.

7.3.3 Histology

A total of 34 ticks from the *in vitro* experiments were fixed after 3, 6 and 17 h of feeding. The remainder of the ticks were incubated and

fixed after 24, 48, 72 h post engorgement, and after 96 h when vermicules (kinetes) were found in the haemolymph.

Ticks from *in vivo* experiments corresponded with 17 h of feeding *in vitro*. Three freshly engorged ticks were fixed on collection and the remainder incubated and three ticks were fixed each day at the same intervals as above (see Table 8).

Ova from ticks in both experiments were disected out in phosphate buffered saline pH 7.2 at 168 h post engorgement and fixed.

Ticks for examination by light microscopy were fixed in Carnoy's fluid and Karnovsky's fixatives. Carnoy's was used because gluteraldehyde/paraformaldehyde fixation when used for demonstration of parasites in sections precluded the "Romanowsky" staining of nuclei which has traditionally been used to differentiate piroplasm from other cellular inclusions in smears and paraffin sections (Shortt & Cooper, 1948; Binnington *et al.*, 1983). The ticks were then processed as described in Chapter 2 and embedded in methacrylate. 1.0 µm sections were stained using Giemsa (Hogg's modified technique, as contained in Gurr Biological Staining Methods, 1979), and the pH adjusted to 6.8 in order to demonstrate clearly the *Babesia* parasites.

For electron microscopy small pieces of gut were fixed in Karnovsky's fixative and processed as described in Chapter 2. Before making ultrathin sections of infected gut, semi-thin survey sections, mounted on slides were stained with 0.2% Martinotti's toluidine blue (Morzaria *et al.*, 1976). This technique was useful in locating infected regions of the tick gut before ultrathin sections were made and stained (Chapter 2).

7.4 RESULTS

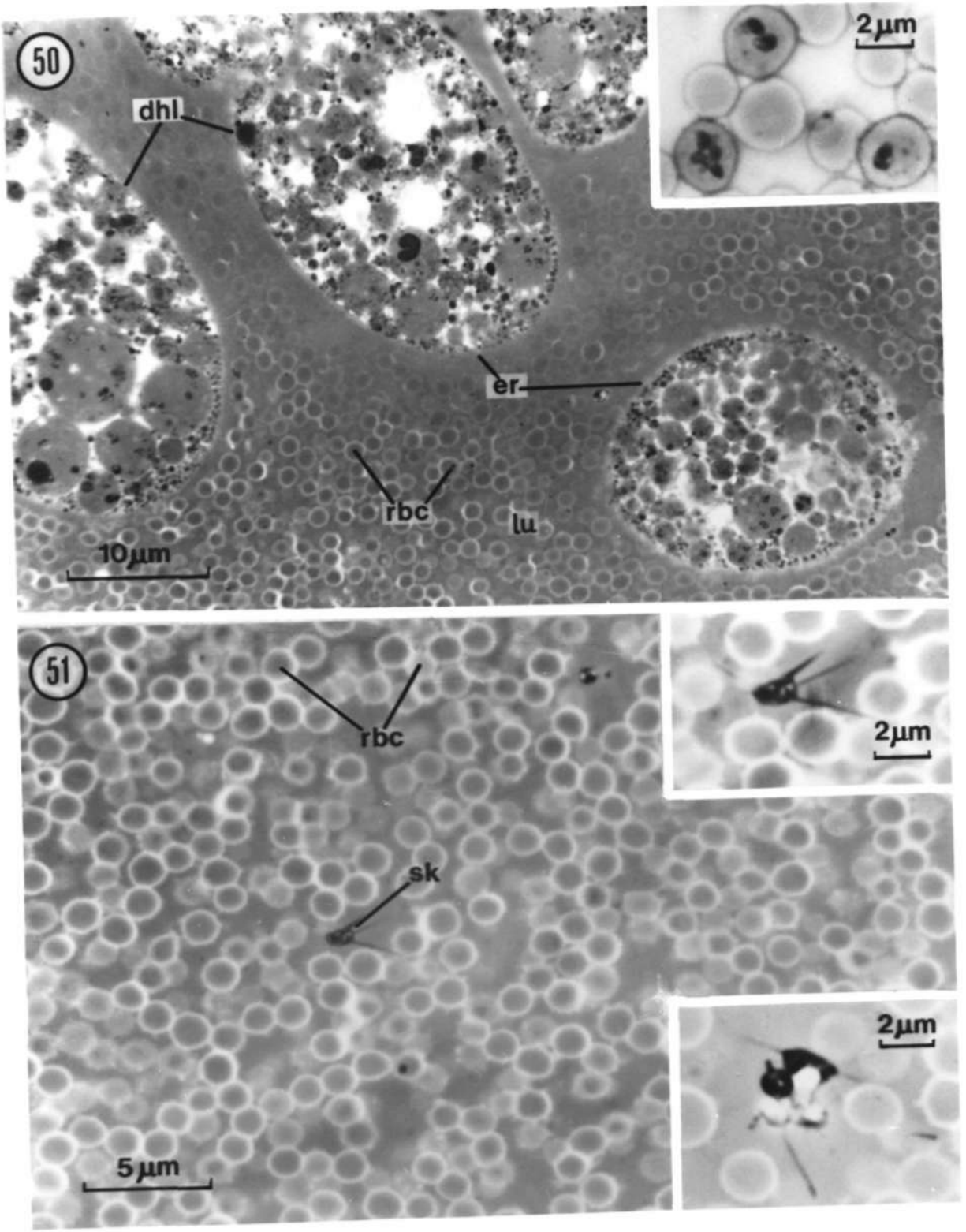
By the time the female ticks from the *in vitro* experiments had fed overnight (17 h) they had increased in weight on average by about 148 mg, or 5-6 times their original weights (Tables 4, 5, 6, 7 & 8).

Light microscope examination of the sections from experiments I-IV showed that increasing the parasitaemia did not increase the infection rate in the gut cells. A total of 55 ticks were examined, five at each stage. Most of the erythrocytic stages (merozoites) normally seen in blood films could be found in the intact red cells in the gut lumen of the tick after three hours of feeding. By six hours of *in vitro* feeding, lysis of red blood cells occurred in all regions close to the digest cells resulting in a band of homogeneous material which separated the digest cells from the intact red cells in the centre of the lumen (Fig. 50). Most of the particulate materials which included the *Babesia* parasites and host leukocytes had been taken into the dhl cells by phagocytosis and these cells bulged far into the lumen. The non-particulate materials, haemoglobin and other non-cellular components of the diet were being incorporated into dhv cells by pinocytosis.

By 17 h *in vitro*, a few *Babesia* merozoites appeared to have transformed into Strahlenkörper. The numbers and their lengths varied. They had long rays or spikes and were similar to those present in the first phase of development as described by Friedhoff (1981). Some were found free in the lumen and appeared larger than those in the red cells. Freshly collected ticks from the *in vivo* experiment showed a similar picture (Fig. 51). The diameter of the Strahlenkörper averaged 2.10 ± 0.01 µm and the longest spike was 3.54 ± 0.5 µm. For comparison, red cells in the lumen had a diameter of 3.53 µm. The morphological sequence

FIG. 50. Engorged female with 3rd generation digest cell subtype I, dhl; lumen, lu; intact host erythrocyte, rbc; region of lysed erythrocytes, er. (Methacrylate section, Giemsa). Inset shows merozoites of Babesia in smear of red blood cells.

FIG. 51. Gut lumen of engorged female with intact host erythrocytes, rbc; Strahlenkörper, sk. Insets show higher magnification of Strahlenkörper with long rays or spikes. (Methacrylate section, Giemsa).


50
dhl
er
rbc
lu
10µm
2µm
51
rbc
sk
5µm
2µm
2µm

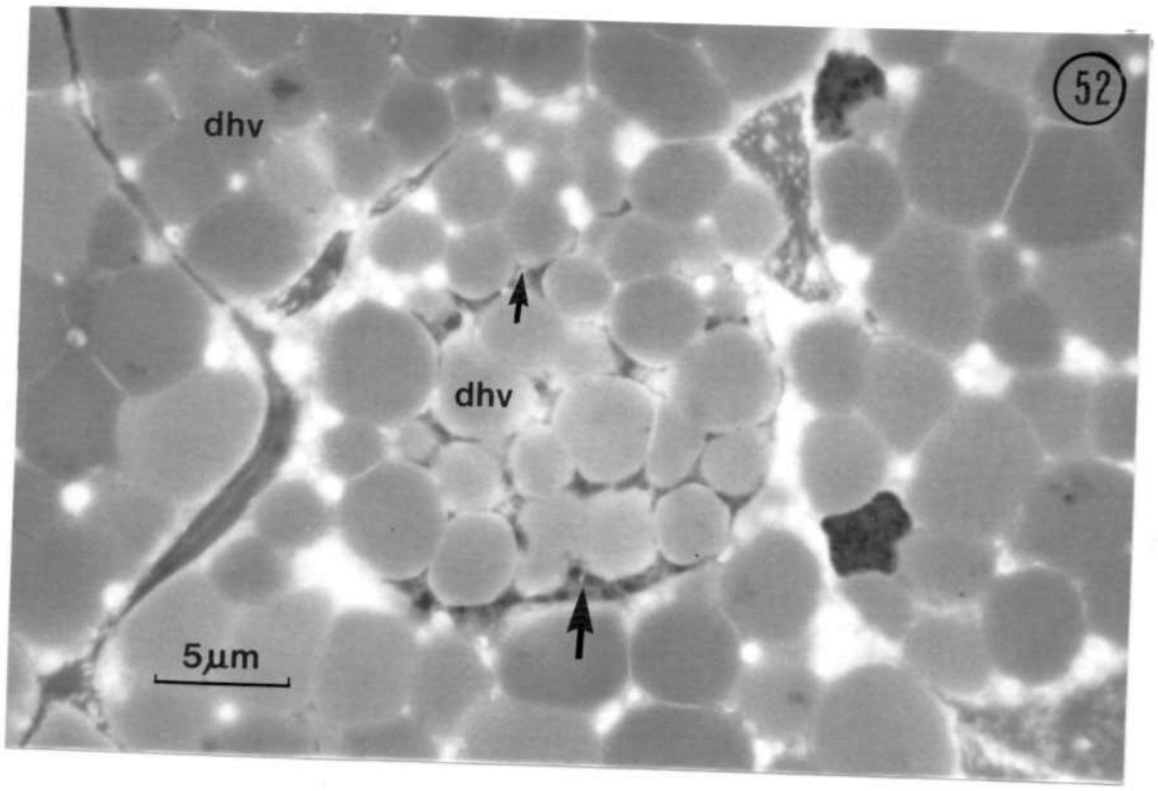
in the development of the Strahlenkörper could not be clarified in this study. Strahlenkörper of varying shapes and forms were present in the gut lumen and red blood cells up to 2 days post incubation. However, there was an increasing number of the rounded forms without very prominent rays or spikes by day 2. These parasites appeared amoeboid and had dense chromatin at the borders of the nuclei. This stage of the parasite appeared to correspond with the fifth phase described by Friedhoff (1981). The width of the body was 3.73 ± 0.11 µm and a few still had long spikes which were 5.26 ± 0.52 µm in length. A new stage was very noticeable by day 3 in the dhv cells, lying in the cell stroma apparently squeezed between the enlarging food vacuoles. Multiplication of this stage in the dhv cells was very marked, giving rise to many star-shaped parasites with a central dense nucleus (Fig. 52). In fact, the cytoplasmic shape appeared to be determined by the available space between the food vacuoles. The sizes of the parasites at this stage also varied considerably. The largest was 7.10 µm in length by 3.99 µm in width, the smallest was 3.88 µm in length by 2.96 µm in width and all were touching by their extended rays.

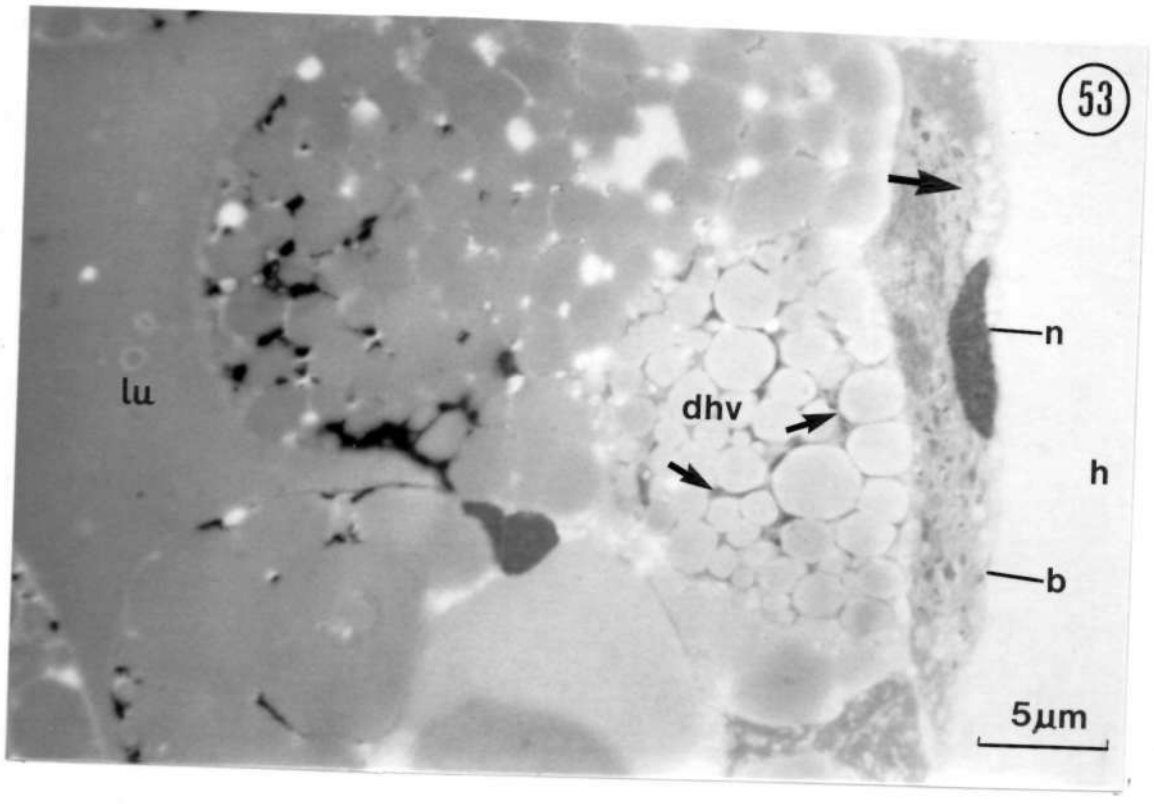
The individuals in these conglomerates of four, ten or more parasites were larger than the Strahlenkörper and were not fission bodies. By day 4 these parasites had invaded the adjacent basophilic cells en masse and there were no Babesia left in the digest cells (Fig. 53). These stages gave rise to the immature multiple fission bodies (Fig. 54) and then mature multiple fission bodies (Fig. 55) in the basophilic cells. Development in the gut was completed when these transformed into kinetes (vermicules) which are motile stages and when mature are commonly found in the haemolymph by day 5 and later.

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FIG. 52. Digest cell bodies in infected 3rd generation digest cell subtype II, dhv; large arrow indicates star-shaped parasites, between the endosomes; small arrow indicates dark red parasite nuclei; uninfected digest cell subtype II, dhv. (Methacrylate section, Giemsa).

FIG. 53. Digest cell bodies in 3rd generation digest cell, dhv; and invading basophilic cell, b; small arrows indicate parasite in digest cell; large arrow indicates parasites in basophilic cell, nucleus of basophilic cell, n; haemolymph space, h; lumen, lu. (Methacrylate Section, Giemsa).


52
dhv
dhv
5µm


53
lu
dhv
n
h
b
5µm

Hypertrophy of the infected basophilic cell nuclei was observed. A comparison of infected versus non-infected basophilic cells showed that the nuclei increased in diameter from 8.24 ± 0.31 µm to 13.09 ± 0.90 µm, suggesting that the parasite stimulated the cells to increased synthetic activity. Parasitised cells were less basophilic than non-parasitised cells, and showed signs of degeneration as the fission bodies matured.

By day 6, basophilic cell infection was no longer detectable as vermicules had all invaded the haemolymph. By day 7, vermicules had invaded the developing ova and other internal organs of the tick. The vermicules rounded up after penetrating the ova and multiplied within the cytoplasm of the ova in between the yolk granules (Fig. 56). Few ova were infected (less than 5%) but infected ova were heavily parasitised resulting in local degeneration of the ovum.

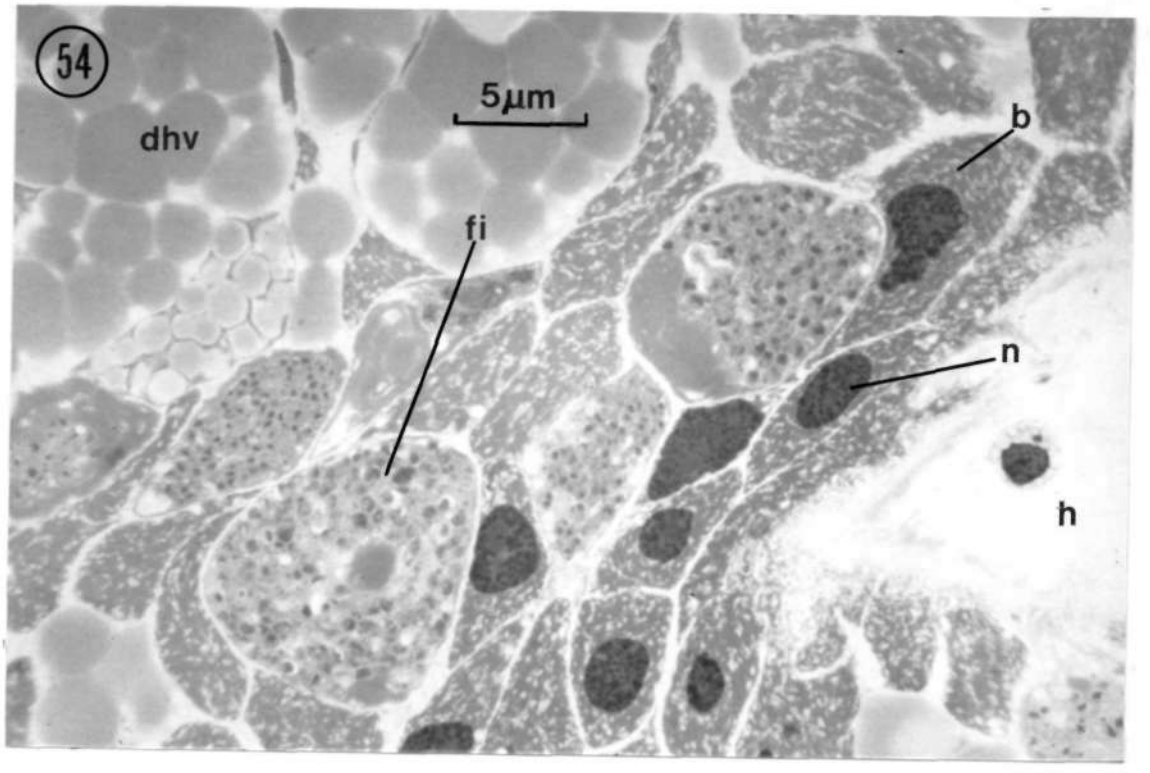
Hypertrophy of the nucleus in the infected cells of the developing ovum was not observed and the parasite divided in a specific pattern different from the gut stages. The events as they occurred in the ovum appeared to be exactly the same as those reported in detail for *B. ovis* in *R. bursa* (Moltmann, Mehlhorn & Friedhoff, 1982) and *B. major* in *Haemaphysalis punctata* (Morzaria *et al.*, 1976) and therefore were not investigated any further.

7.5 ULTRASTRUCTURE OF *B. BOVIS* IN GUT BASOPHILIC CELLS

Only the fission bodies which resulted from the invasion of basophilic cells by the star-shaped parasites, four days post-incubation at 28°C were examined during the course of this study.

FIG. 54. Immature fission bodies, fi, in infected basophilic cell; uninfected basophilic cell, b; nuclei of basophilic cell, n; digest cell, dhv; haemolymph space, h. (Methacrylate section, Giemsa).

FIG. 55. Mature multiple fission bodies, fm, in a basophilic cell; uninfected digest cell, dhv; vermicule, v. (Methacrylate section, Giemsa).


54
5µm
dhv
b
fi
n
h

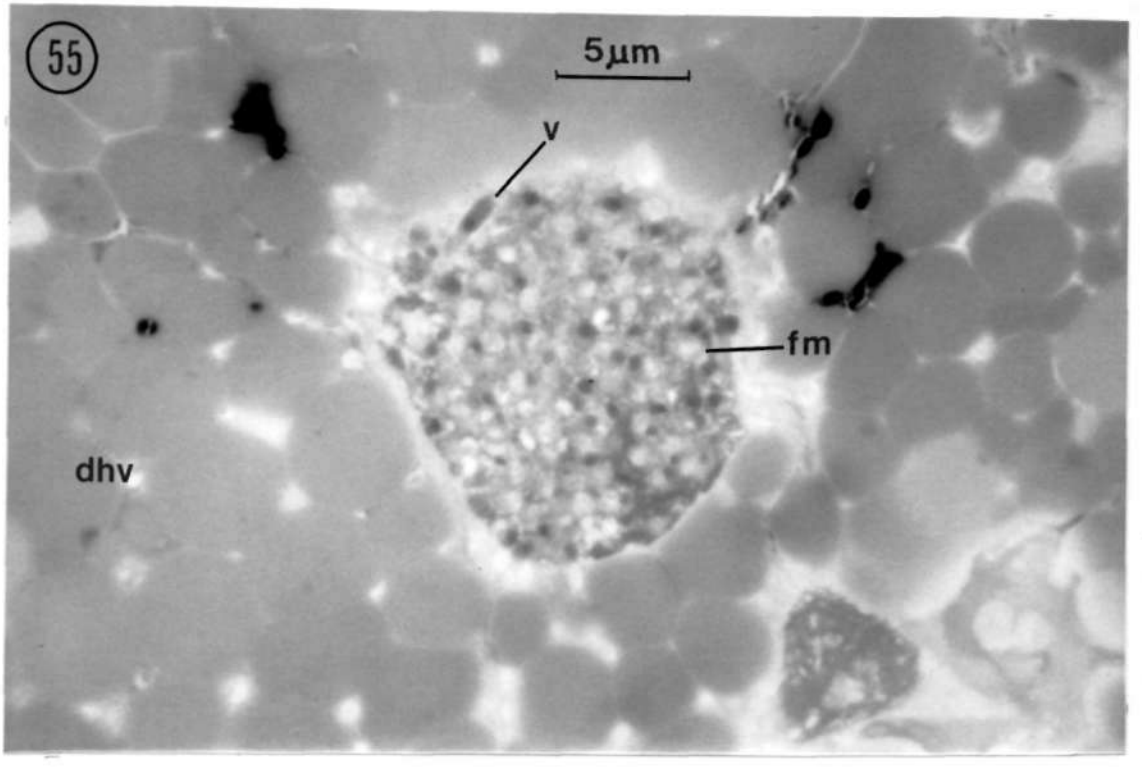
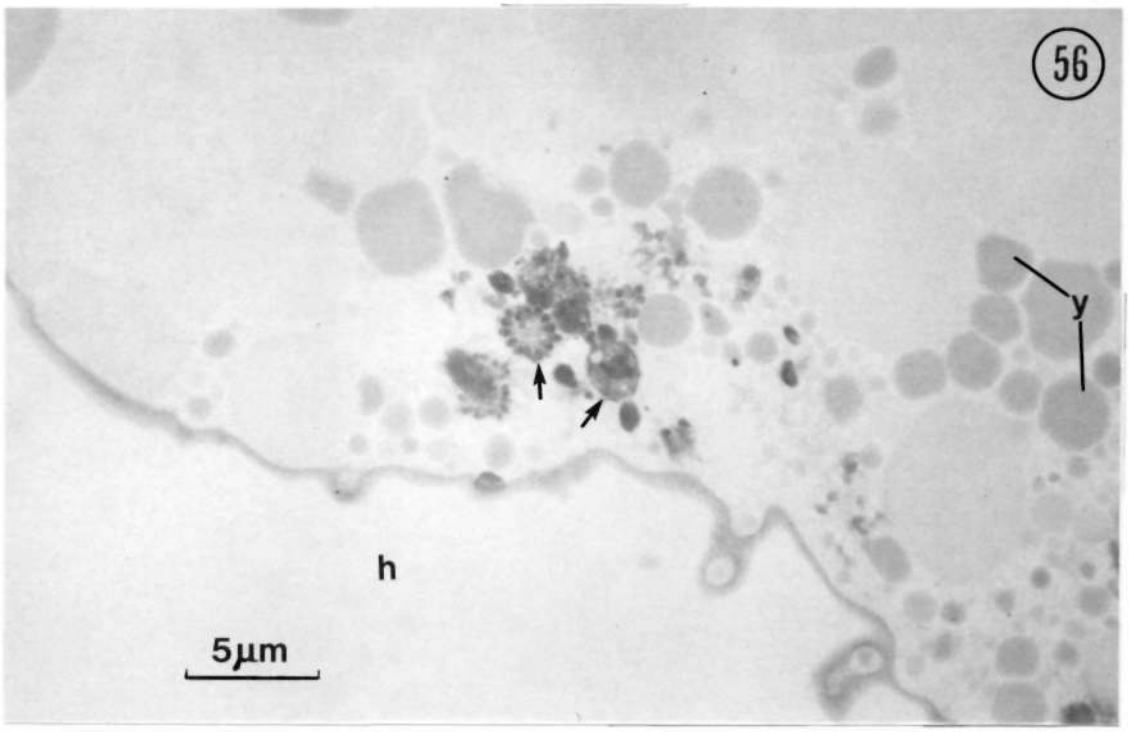

55
5µm
v
fm
dhv

FIG. 56. *B. bovis* parasites in ovum indicated by arrow; yolk granules, y; haemolymph space, h. (Methacrylate section, Giemsa).


56
y
h
5µm

Of a total of 12 ticks examined, some 84 gut cells were infected with fission bodies, and all were basophilic cells. Two main stages of the parasite were found, the immature fission body in which only the limiting plasma membranes were clearly distinguishable (Fig. 57) and the mature fission body which had well defined membranes and organelles (Fig. 58). Both stages appeared to be developing in a small pool of amorphous material within the cells which had extensive whorls of rough endoplasmic reticulum. The maturing vermicules were broad anteriorly and tapered gradually from the nuclear region to the posterior end (Fig. 58) and had a slightly protruding anterior pole (Fig. 59).

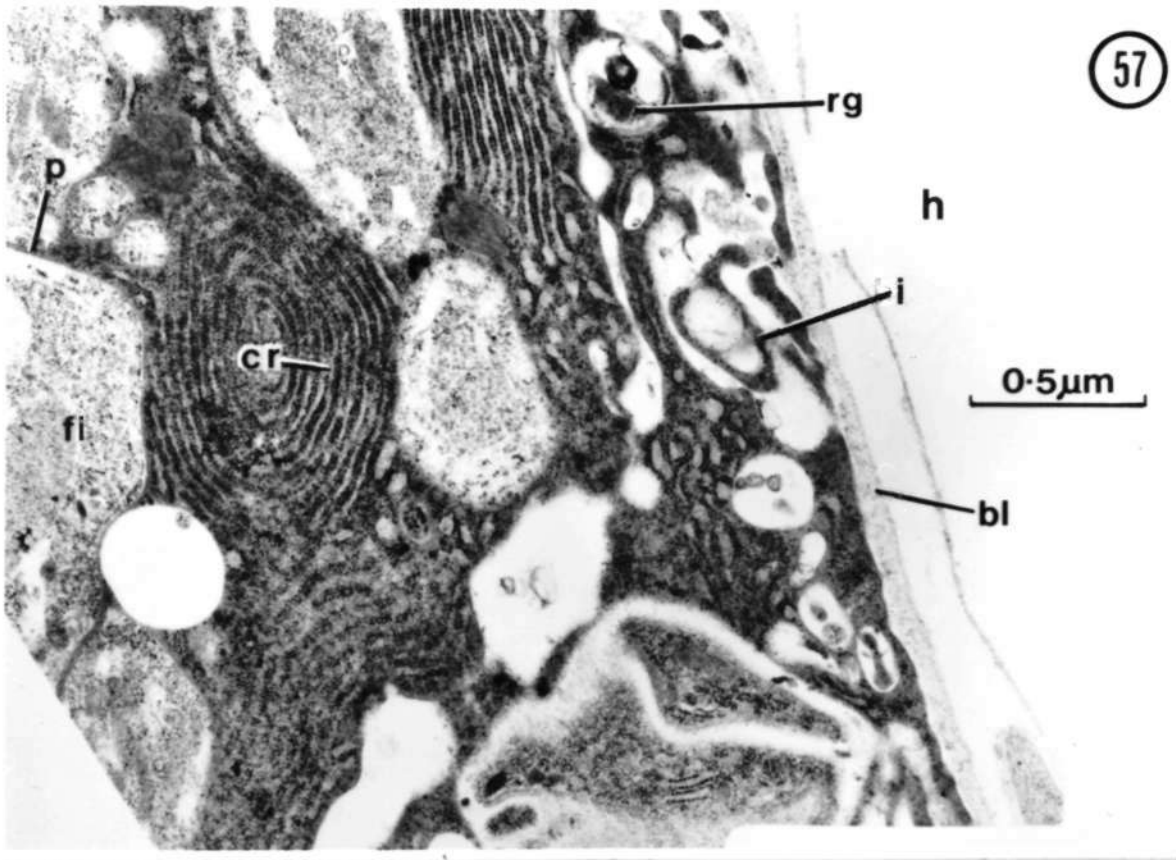
The structure and function of the Babesia organelles has been reviewed by Friedhoff & Scholtyseck (1968), Sholtyseck & Melhorn (1970), Friedhoff (1974), Morzaria et al. (1976), Potgieter & Els (1976), Potgieter et al. (1976), Potgieter & Els (1977), Mehlhorn, Schein & Voigt (1980) and Moltmann et al. (1982). Only a brief comparison is made here with earlier descriptions.

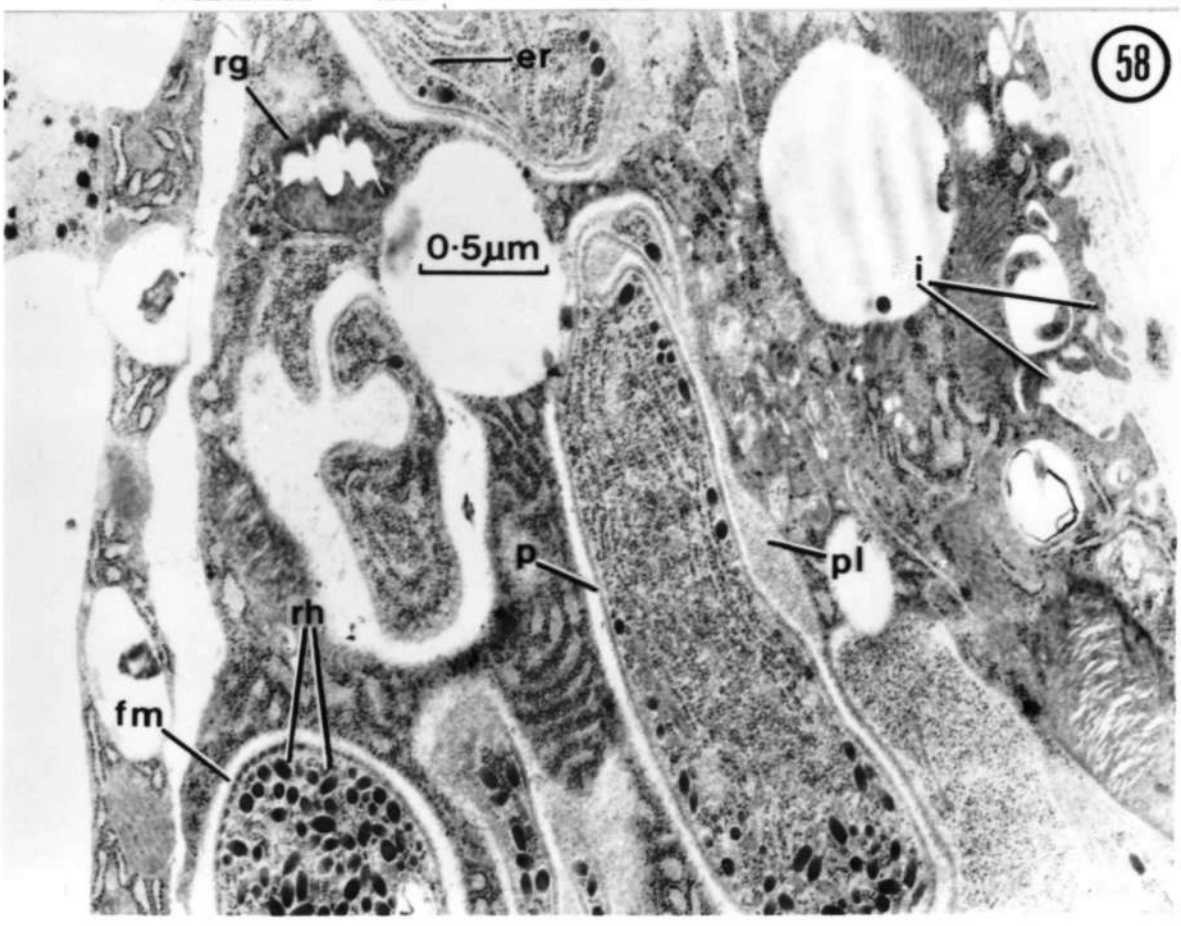
Pellicle of the parasite. There were three membranes in the pellicle of the mature vermicule (Fig. 60). The outer membrane was a thin single layer, followed by a wider, poorly staining middle membrane, and the inner membrane. In places, the inner membrane appeared to be double layered with the inner most parts intimately associated with the underlying microfibrillar layer.

Osmiophilic layer. This was particularly visible in sections of the mid-region of the developing vermicule, where this layer consisted of numerous, regularly spaced, longitudinal microfibrils lying between microtubules. Near the tapering anterior end, these microfibrils converged

FIG. 57. T.E.M. of immature fission bodies in the gut basophilic cell of B. microplus females on the 3rd day after engorgement. Immature fission bodies, fi; poorly defined plasma membrane of fission body, p; cisternae of rough endoplasmic reticulum, cr; remnant secretory granule, rg; basal lamina, bl; basal labyrinth, i; haemolymph space,h. No en bloc.

FIG. 58. T.E.M. of mature fission bodies in the gut basophilic cell of B. microplus females on the 4th day after engorgement. Mature fission body, fm; plasma membrane of mature fission body, p; remnant secretory granule, rg; basal labyrinth, i; rhoptries of mature fission body, rh; rough endoplasmic reticulum of fission body, er; pool of amorphous material surrounding fission body, pl. No en bloc.


57
rg
p
h
i
cr
0·5µm
fi
bl


58
rg
er
0·5µm
i
p
pl
rh
fm

as a number of at least 15 visible relatively thickened osmiophilic "ribs" (Fig. 61).

Pellicular microtubules. Longitudinally arranged microtubules were observed underneath the inner membrane of the pellicle, and in close association with the osmiophilic layer. At the anterior pole, numerous microtubules were observed radiating backwards; but how far they extended down the body wall of the vermicule was not determined (Fig. 62).

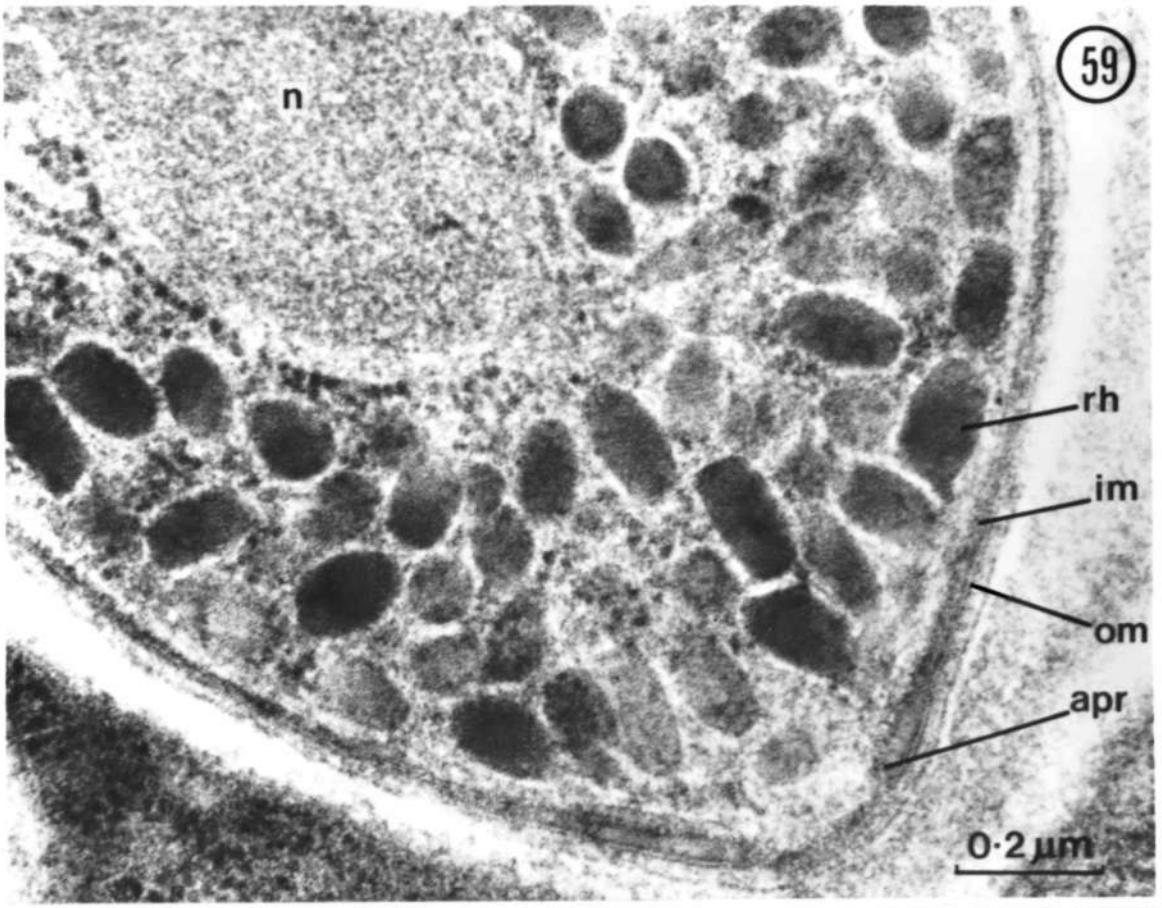
Polar ring. The polar ring could only be identified with certainty in the anterior region; where the microtubules appeared to be radiating backwards. No individual microfibrils or ribs could be distinguished within or as part of the actual polar ring (Fig. 62).

Nucleus. The nucleus appeared as a granular structure with no conspicuous nuclear membranes. Most reports recorded double membranes which were not observed in this study. Also no nucleoli were seen (Fig. 59).

Rhoptries. Mature vermicules contained these homogeneous, electron dense, osmiophilic structures, located as a rule at the anterior end of the parasite between the nucleus and the polar ring (Figs. 59 & 62). Occasionally similar structures were seen beside the nucleus and at the posterior end of the parasite. They were round to oblong or pear shaped structures from which duct-like structures are thought to extend forward and terminated at the anterior end of the parasite. Micronemes which have been described as small osmiophilic organelles could not be distinguished from rhoptries.

FIG. 59. T.E.M. of the anterior pole of a vermicule; outer membrane, om; inner membrane, im; anterior polar ring, apr; rhoptries, rh; nucleus, n. No en bloc.

FIG. 60. T.E.M. of the pellicle of vermicules; outer membrane, om; middle membrane, mm; inner membrane, im; rhoptries, rh; rough endoplasmic reticulum, rer. No en bloc.


59
n
rh
im
om
apr
0·2 µm

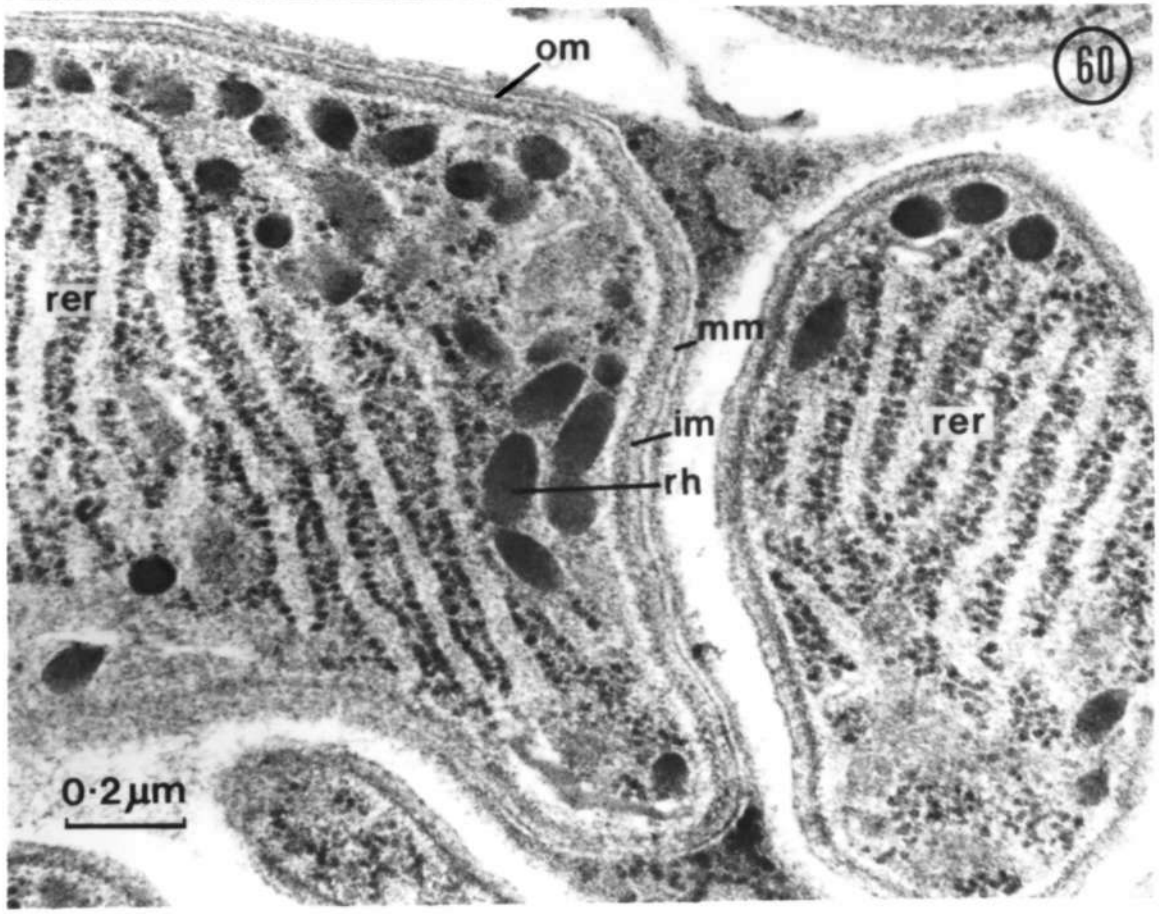
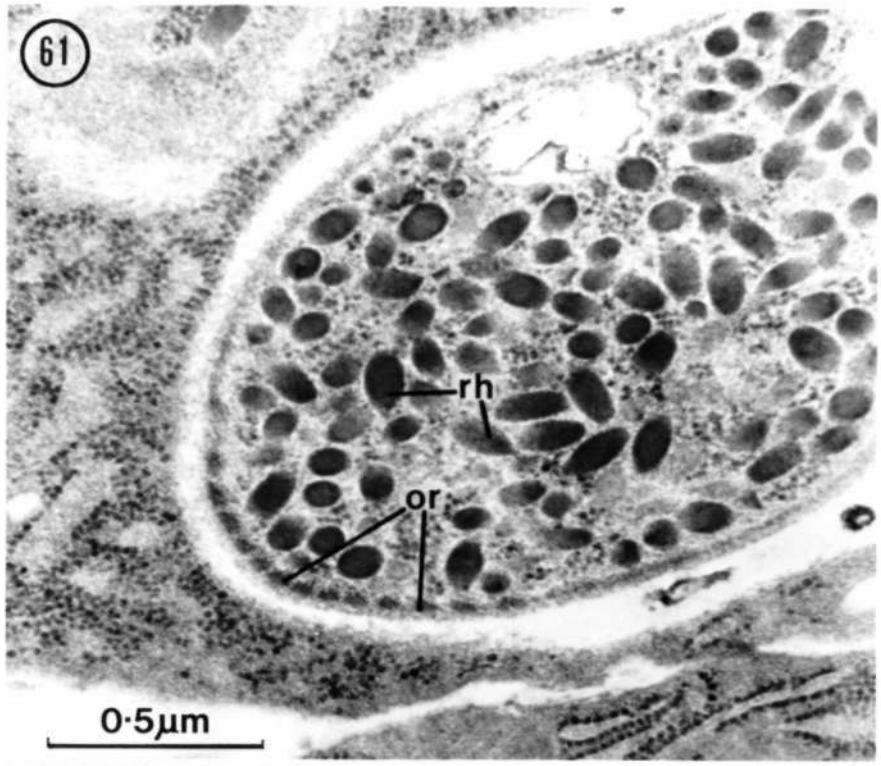
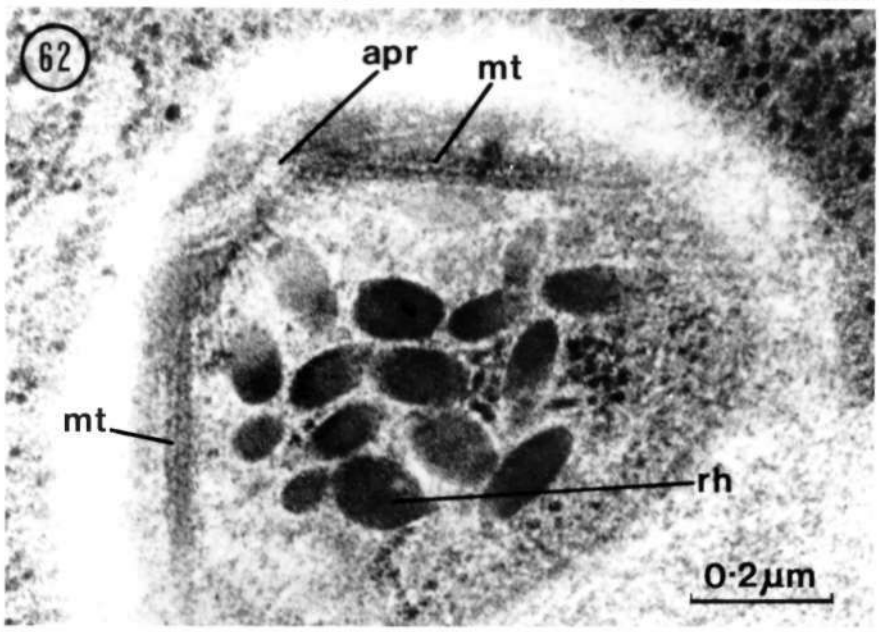

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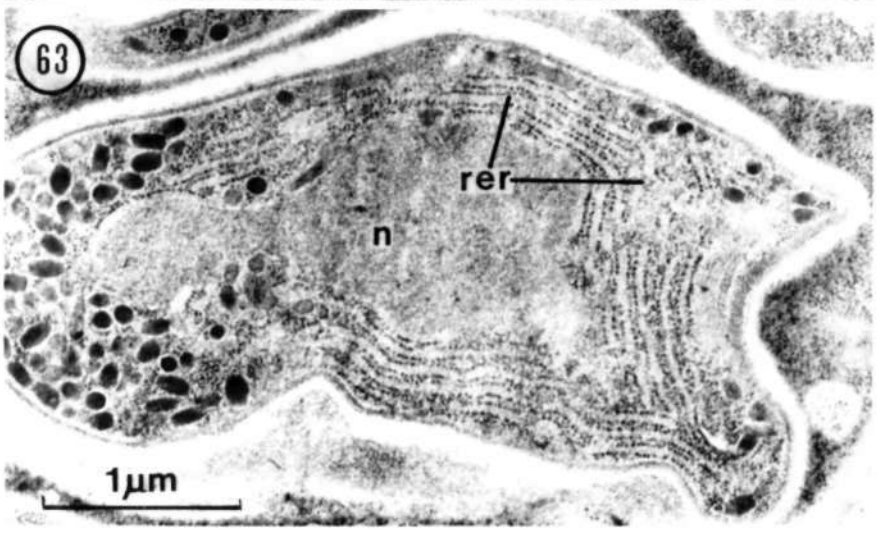
FIG. 61. T.E.M. of vermicule showing the thickened osmiophilic "ribs", or; rhoptries, rh. No en bloc.

FIG. 62. T.E.M. of anterior pole of vermicule; anterior polar ring, apr; microtubules, mt; rhoptries, rh. No en bloc.

FIG. 63. T.E.M. of vermicule with concentric rings of rough endoplasmic reticulum, rer; around the nucleus, n. No en bloc.


61
rh
or
0·5µm


62
apr
mt
mt
rh
0·2µm


63
rer
n
1µm

Endoplasmic reticulum (ER). The endoplasmic reticulum was well developed and was found in concentric rings round the nucleus (Fig. 63). In young maturing vermicules the er was lamellar in structure, and associated with osmiophilic dark bodies, the ribosomes (Fig. 60). As the vermicule matured further, the canaliculi of the r.e.r. which were granular became represented by vesicles or cisternae of various sizes.

Other organelles. A Golgi complex, typical protozoan mitochondria, food vacuoles, micropores, nucleoli, tubular structures and posterior polar ring could not be identified.

TABLE 4. IN VITRO FEEDING - EXPERIMENT I (1% PARASITAEMIA)

Tick number	Weight of tick before feeding (grams)	Weight of blood meal (grams)	Time when tick was processed (hours after feeding began)
1	0.0317	0.0269	1
2	0.0331	0.0474	3
3	0.0288	0.0424	3
4	0.0287	0.0946	17*
5	0.0360	0.0857	17
6	0.0269	0.0911	17
7	0.0283	0.1079	24
8	0.0342	0.1147	47
9	0.0338	0.1277	96
10	0.0235	0.1779	96
11	0.0233	0.1245	168

* Ticks were removed from in vitro feeding at 17 h.

TABLE 5. **IN VITRO** FEEDING - EXPERIMENT II (3% PARASITAEMIA)

Tick number	Weight of ticks before feeding (grams)	Weight of blood meal (grams)	Time when tick was processed (hours after feeding began)
1	0.0280	0.0702	10
2	0.0290	0.0991	17*
3	0.0366	0.1305	24
4	0.0363	0.1702	24
5	0.0447	0.1435	48
6	0.0329	0.1415	72
7	0.0354	0.1494	96
8	0.0322	0.1517	96
9	0.0340	0.1012	168
10	0.0286	0.1443	168

TABLE 6. **IN VITRO** FEEDING - EXPERIMENT III (5% PARASITAEMIA)

Tick number	Weight of ticks before feeding (grams)	Weight of blood meal (grams)	Time when tick was processed (hours after feeding began)
1	0.0380	0.0568	3 h 15 mins
2	0.0234	0.1240	24*
3	0.0359	0.1624	24
4	0.0376	0.1839	48
5	0.0337	0.1460	72
6	0.0354	0.1496	72
7	0.0300	0.1287	96
8	0.0403	0.1702	96
9	0.0429	0.1434	168
10	0.0222	0.1727	168

* Time when ticks were removed from *in vitro* feeding.

TABLE 7. IN VITRO FEEDING - EXPERIMENT IV* (10% PARASITAEMIA)

Tick number	Weight of tick before feeding (grams)	Weight of blood meal (grams)	Time when tick was processed (hours after feeding began)
1	0.0383	0.0537	3
2	0.0344	0.0765	17**
3	0.0364	0.0754	17
4	0.0330	0.1294	24
5	0.0351	0.1532	48

* This experiment was done to repeat specific stages indicated.

** Time when ticks were removed from in vitro feeding.

TABLE 8. IN VIVO FEEDING - EXPERIMENT V (1% PARASITAEMIA)

Batch No.	Mean Weight of engorged ticks at incubation (grams) (100 ticks/batch collected daily)	Time when ticks were processed (days after engorgement)
1	0.2610	0 1
2	0.3440	2 3
3	0.2906	4 5
4	0.2670	6 7

7.6 DISCUSSION

In the past, the nomenclature of various Babesia stages in the tick has been unnecessarily confusing and to avoid this the nomenclature of Friedhoff (1981) has been used here. The term motile kinete (vermicule) has been used to describe the mature products of the multiple fission which occurs in the basophilic cells of the gut.

Feeding ticks *in vitro* made it easier to study the early morphological changes of the parasite in the tick gut because it became possible to fix ticks at specific time intervals during the final rapid phase of feeding. The feeding was successful and most engorged females had good weight gains comparable to ticks fed *in vivo*.

Gametes of *B. bovis* could not be identified at the light or E.M. level. Gamete fusion was also not observed. Friedhoff (1981) reported that sexual fusion of gametes has not been seen in ovine and bovine *Babesia*, though Rudzinska *et al.* (1982,1983a,b) have distinguished male and female gametes and also demonstrated gamete fusion of *B. microti* in *I. dammini*. They further showed that penetration of the ookinete into the gut epithelial cells was an active process possibly through the use of enzymes present in the coiled structure within the parasite.

Although the issue of gamete fusion has not been resolved in this study of *B. bovis*, the evidence available suggests that the Strahlenkörper present in larger numbers by day 2 initiated the development in the dhv cells. All the earlier stages that were taken in by phagocytosis into the dhl cells appeared to be digested and only a few parasites left in the lumen managed to penetrate the right digest cell (dhv). This penetrating stage of Strahlenkörper is similar to the fifth phase described by Friedhoff (1981) which gave rise to multiple fission bodies in the epithelial cells of the gut (Friedhoff, 1969). In this study it has been shown that although these Strahlenkörper penetrated the dhv cells, they did not form fission bodies, but they did divide within these cells, giving rise to a large number of star-shaped stages. This is the first report in literature on the presence of this intermediate stage of *Babesia* parasites in the gut cells of *B. microplus*. It is quite distinct from the Strahlenkörper and will be referred to as the digest cell body (dcb).

The food vacuoles in the dhv cells increased in size by the accretion of small vacuoles (Chapters 3 & 4). The fact that these dcb stages were not incorporated into the food vacuoles, meant that they were in no danger of being digested, and it is suggested that the shape of these intermediate stages is determined by the available space between the food vacuoles in these dhv cells. Also the presence of the dcb stages outside the food vacuoles suggested that entry into the cell was an active process and did not take place passively with the food. This agrees with recent findings by Rudzinska et al. (1983b) that possibly enzymes from the coiled structure digested away the host cell membrane before internalization of the parasite into the gut epithelial cells. Also Wright, Goodger & Mahoney (1981) found that parasites with esterase (proteolytic enzyme) were tick transmissible, but parasites without this protease were non-tick transmissible suggesting that this enzyme had some role in establishing infection in the tick vector. Electron microscopy is required to resolve these problems.

Another interesting point is the development of the basophilic cell. This cell appeared to develop from the secretory cell type 2 present only in the non-replete female (Chapters 3, 5 & 6). The basal portion left after the completion of the first phase of secretion expanded again, and extensive rows of r.e.r. reorganized and developed in the cytoplasm. The cells began their second phase of activity when they synthesised new material believed to be large amounts of egg protein or vitellogenin which is presumably secreted into the extra-cellular spaces and thence into the haemolymph and eggs. It is possible from the timing of these events that the evolution of the Babesia-Boophilus relationship has favoured the development of the ability of the parasite to recognise and penetrate this tick gut cell which has the machinery for active

synthesis. Fission bodies were all concentrated within the basophilic cells, and hypertrophy of the host cell nucleus confirmed the close association of this parasite and the cells. The parasites may have stimulated the host nuclei into greater activity leading to synthesis of materials of use to the Babesia. A similar close association of parasites and host cells was observed with T. parva in the salivary gland cells of R. appendiculatus (Fawcett et al., 1982b).

Selection of the basophilic cell for development would also make it easier for the liberation of mature vermicules into the haemolymph. In addition, the extensive basal infoldings of the basophilic cell with a large surface area in contact with the haemolymph, would provide an easy and direct access for the vermicules into the haemolymph.

These studies also give a possible explanation for the apparent tropism or preferential attack of the developing ova by the motile kinetes (vermicules) circulating in the haemolymph. Parasites have been demonstrated in various internal organs of the tick and although at this stage of development the ova in the ovaries constituted a very large proportion of the body mass, there is abundant opportunity for vermicules to be led astray. However, vitellogenin must pass whole into the ova, and the Babesia vermicules could recognise receptors for these proteins on the surface of the ovarian cells. No studies on the behaviour of the vermicules in the haemolymph of ticks are available and this is required for further interpretation.

7.7 SUMMARY

B. bovis infected ticks were obtained by in vivo and in vitro feeding on infected blood.

The Strahlenkörper (possibly sexual stages) were detected in the gut lumen, and the studies showed that they are part of the life cycle of *B. bovis*. Gametes and fertilization could not be demonstrated.

One new stage of development was identified. This was observed after 24-48 h of incubation in the digest cell subtype II (dhv), where the parasites multiplied and developed between the large endosomes. This stage was quite distinct from the Strahlenkörper and was named digest cell body (dcb). The *en masse* invasion of the basophilic cells by the dcb was also shown.

Fission bodies developed not in digest cells but in the basophilic cells which have been suggested as the site of synthesis of egg proteins.

The basophilic cells presumably provided the required nutrients for *Babesia* development and it is proposed that this high selectivity of the basophilic cells is linked with the subsequent selection of the ovary and eggs for development.

The ultrastructural study confirmed the presence of the *Babesia* parasites in the basophilic cells.

CHAPTER 8

EFFECTS OF VACCINATING CATTLE AGAINST THE TICK B. MICROPLUS: REJECTION OF TICKS AND HISTOLOGY OF DAMAGED TISSUES

8.1 LITERATURE REVIEW

Ticks are the most important ectoparasites of livestock throughout the world (Hoogstraal, 1973). In Australia, losses in cattle production due to *B. microplus* ticks were estimated some years ago to cost $100 million annually (Anon,1975). The losses are caused by host anaemia and anorexia (Seebeck, Springell & O'Kelly, 1971), tick fever deaths, hide damage and the costs of tick control and tick fever vaccines. *B. microplus* is the most important tick of cattle in Australia although *Haemaphysalis longicornis* and *I. holocyclus* occasionally cause deaths due to heavy infestations or to paralysis respectively (Dicker, 1978; Ross, 1924,1935; Andrew, Bonin & Williams 1946).

Tick control is essential, and ever since arsenic was first used as an external dressing, reliance has been placed on chemicals for control. Unfortunately, ticks have developed resistance to these acaricides, which has increased the costs and threatened to limit this approach to control (Roulston, Wharton, Nolan, Kerr, Wilson, Thompson & Schotz, 1981). Acaricide resistance has been a serious problem in control of *B. microplus* against which the full armoury of organophosphates and other acaricides has had to be employed (Beesley,1982).

As an alternative measure, biological control has been attempted using pasture spelling (Wilkinson, 1955, 1956, 1957), and though this has been shown to be successful is difficult to implement. Eradication of ticks is another alternative, but on a large scale it has been

unsuccessful (Mackerras, Waterhouse, Maiden & Edgar, 1961). One practical demonstration of eradication of ticks, however, was shown at Magnetic Island, north Queensland (Johnston, Wharton & Calaby, 1968). This method is impractical and too expensive under the conditions of cattle rearing in most of Queensland.

There is also growing recognition that host resistance provides a valuable basis for parasite control (Tatchell, 1981), although some emotive opposition from the chemical industry is still present in Africa (Anon, 1982). The trend will be beneficial only if the role of host resistance is kept in perspective (Sutherst, 1983). In Australia the use of tick resistant cattle with the ability to reduce tick survival has been widely accepted, particularly as there is only one tick species of major economic importance (Nolan, 1981).

The concept basically envisages the management of tick populations by utilising host resistance as a major mortality factor in the tick's life cycle, and the restoration in a relatively sophisticated cattle industry of the equilibrium between host and parasite which evolved under natural conditions. The use of tick resistant cattle in Australia has been advocated for many years (Johnston & Bancroft, 1918; Kelley, 1943; Anon, 1955; Wilkinson, 1962; Kennedy & Chirchir, 1971; Wharton, Utech & Sutherst,1973; Wharton, 1975,1976; Wharton & Norris, 1980), and resistant cattle have been found useful for tick eradication in "marginal" tick areas. Their use has not yet been widely advocated or accepted for the alleviation of multi-host tick problems, but the results of investigations with Haemaphysalis by Dicker & Sutherst (1981) and Amblyomma by Garris, Stacey, Hair & McNew (1979) have demonstrated that the principle is applicable to other species.

One problem with the use of tick resistant cattle is that testing for resistance relies on the tedious process of counting ticks on animals exposed to ticks artificially or in the field. Certainly a test, which would provide a rapid visual assessment of tick resistance of individual animals would considerably enhance progress in the use of tick resistant cattle, but such a test is unlikely to be available soon. The most likely answer to this problem in the long term will probably come from immunological research (Nolan, 1981).

Willadsen, Williams, Roberts & Kerr (1978) isolated an antigen from B. microplus which produced an immediate type hypersensitivity reaction at the injection site, and the intensity of the reaction correlated with the degree of resistance of the animals. However, the precision required in antigen selection and injection suggested that further refinement would be required before a field test could be applied. Also disappointing was the fact that although another antigen was specific in indirect haemagglutination tests, there was a negative correlation between resistance and antibody titres. This negative correlation may not be strong enough to be useful in selecting for tick resistant cattle.

Undue emphasis on selection for resistance is likely to be at the expense of other productive characteristics, for example, Zebu (B. indicus) type cattle tend to be more difficult to handle, also there are some fertility problems. In the dairy industry, there are problems of maintaining high milk production when Sahiwal (B. indicus) has been crossed with European (B. taurus) cattle to increase tick resistance (Anon,1977). Recently, Utech & Wharton (1982) reported lower milk production from dairy cattle selected only for tick resistance over several generations, which indicated a need to test the correlation

formally. Selection of B. indicus x B. taurus cattle for tick resistance per se resulted in the selection of animals with more Zebu characteristics such as greater adaptability to stress and reduced potential for growth (Frisch, 1976). Nevertheless, with time and careful management, these problems can be overcome but it would be useful to extend the gene pool of the cattle in some circumstances.

Resistance of cattle to ticks has been reviewed by Willadsen (1980). Consideration of resistance naturally leads to the question of artificially raising resistance levels in animals of moderate resistance, or to the induction of resistance in susceptible animals. This would increase the available gene pool of cattle in tick infested areas. Various authors have suggested that vaccinating cattle against ticks could contribute to tick control, and Allen & Humphreys (1979) showed that fecundity of D. andersoni was lower after ticks had fed on vaccinated cattle. The yield of the cattle-tick B. microplus was reduced by 70% on vaccinated animals compared to cattle which acquired resistance by tick infestation (Johnston et al., 1983). Vaccination thus shows some promise as a new alternative method of tick control which could be of value in an intergrated approach to tick management and as a result deserves further investigation.

8.2 INTRODUCTION

A review of the literature since 1979 has shown that in some tick-host relationships it had been possible to reduce the burden of ticks on vaccinated animals (Allen & Humphreys, 1979; McGowan, Homer, O'Dell, McNew & Baker, 1980; Ackerman, Floyd & Sonenshine, 1980; Johnston et al., 1983). While some results have not been spectacular, there was a significant reduction in both tick burdens and/or fecundity and some

susceptible cattle were made 100% resistant to B. microplus (Johnston et al., 1984, submitted for publication). The source of the antigens so far used for vaccination has been crude extracts of tick organs or of whole ticks, and the implications in some of the papers has been that a "novel" approach has been made in the immunisation against ticks. However, there was no good evidence for this, and the antigens used might simply be the same as those injected by the tick in its saliva. If this were the case, no significant advantage would be expected from vaccination compared to repeated tick infestation.

Since CSIRO had a project on vaccination of cattle against ticks, a joint approach was undertaken between Dr. D.H. Kemp of CSIRO and myself in order to compare the rejection of ticks from vaccinated cattle, with that from cattle with acquired resistance. The results of the joint work have been submitted for publication. Further work was needed to identify precisely the sites of host induced damage to ticks, the histology of the gut and other tissues, and this was carried out by me. The importance of the whole study was that if vaccination was to be successful, it must enhance the level of acquired resistance or add a genuinely "novel" form of resistance to the existing one.

Following on from the basic histological study of B. microplus gut (Chapter 3), it was possible to observe the histopathological changes in the gut of ticks which had fed on vaccinated animals compared with those which had fed on control cattle.

8.3 MATERIALS AND METHODS

B. taurus (Hereford) heifers about 12 months of age, were vaccinated as described by Johnston et al. (1984, submitted for publication), using antigens from B. microplus females collected on the day before the ticks

completed engorgement. Briefly, the ticks were washed, homogenised in phosphate buffered saline 0.012M, pH 7.2 (PBS) using a Virtus blender and the empty cuticles filtered off through sterile glass wool. The filtrate was centrifuged at 30,000 g for 30 min, the supernatant removed and suitable aliquots were emulsified with Freund's Complete Adjuvant (FCA). The pellet was resuspended in PBS, sonicated in a 180 watt Rapidis Sonicator for 30 seconds and aliquots were also emulsified in FCA. The supernatant and pelleted material were injected at different sites. The first two intramuscular injections were given 30 days apart and a final subcutaneous injection without Freunds was given 21 days later. Three animals (Nos. 1,2,3) received antigens and three (Nos. 4,5,6) had 0.15M Saline with FCA.

The cattle had no prior exposure to B. microplus and were kept in tick-free conditions during immunisation. The animals were challenged with adult B. microplus after their third vaccination. Adult ticks were obtained from engorged nymphs which moulted in an incubator at 35 ± 0.5°C, 70-80% relative humidity and were placed in aluminium rings (2.5 cm in diameter) glued to the clipped skin of the animal's back near the dorsal midline. Between 20 to 36 females and 15 to 30 males were put in each ring. Adult ticks were also fed in vitro on blood from one of the vaccinated and one of the control animals. They fed through a thin baudruche membrane as described by Waladde, Kemp & Rice (1979). Briefly, male and female ticks were placed on the membrane and then enclosed by a nylon gauze. The membrane, together with its chamber and cup were taped onto a hot plate and warmed at 35 ± 0.5°C for 6 h which allowed the ticks time to secure attachment (Kemp, Stone & Binnington, 1982). The hot plate and feeding chambers were then inverted to allow the host cells to sediment to the ticks' mouthparts which had penetrated the membrane soon after attachment.

From the in vivo experiment, ticks were picked off after 1, 2 and 6 days and sampled also on day 8, one day after engorgement. Ticks from the in vitro experiment were sampled only up to 42 h post infestation. Two ticks from vaccinated and two from control animals were sampled at each interval, giving a total of 32 ticks with which to study the histopathology of the damaged gut. The ticks were processed for paraffin and methacrylate embedding, and were sectioned and stained as described in Chapter 2.

8.4 RESULTS

8.4.1 Effects of Vaccination on Ticks In Vivo and In Vitro

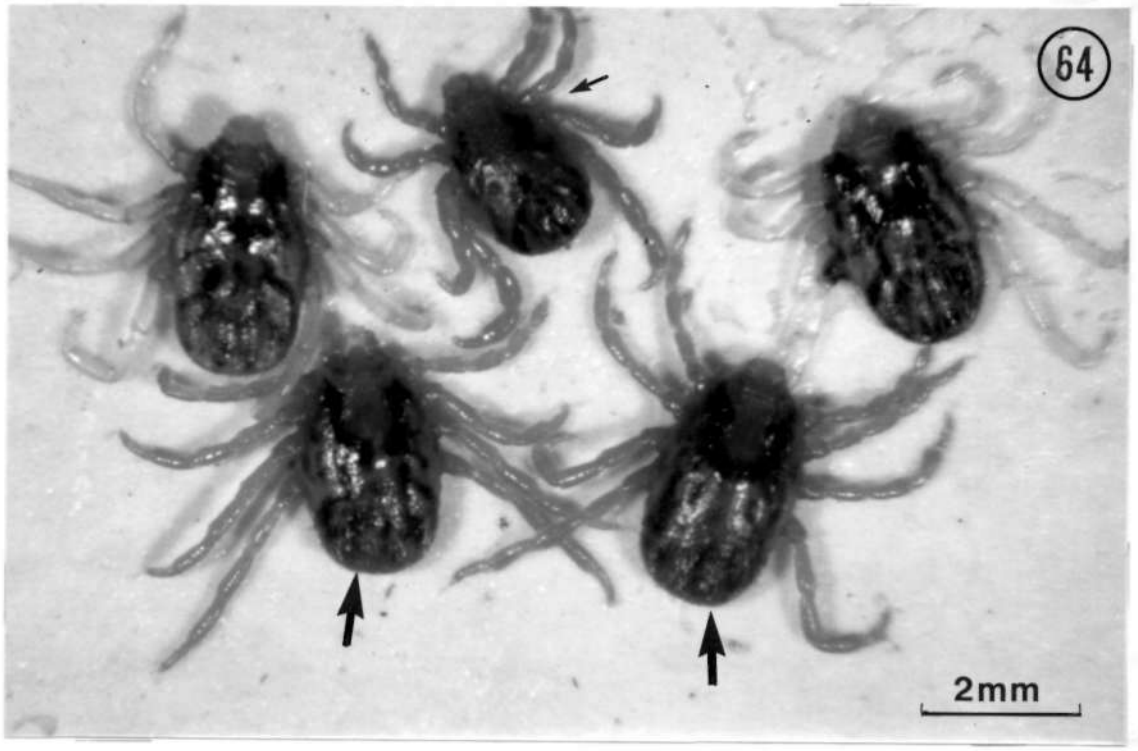
The effects of vaccination on B. microplus females in vivo are summarised in Table 9. Two animals showed continued loss of ticks throughout feeding so that 23 and 53% had died in the rings by day 8. On one vaccinated animal, there was no greater loss of ticks than on the controls. Many surviving females on all three vaccinated animals were abnormal, as some had a ruptured gut with leakage of bovine erythrocytes or haemoglobin into the tick's haemolymph (Fig. 64). In others, the ticks were bloated due to distended gut caecae. Up to 60% of the females had a damaged gut and many of them died on the host.

Some of the effects of vaccination were reproduced after feeding ticks in vitro for 16 up to 42 h. Gut damage occurred in ticks feeding on blood from vaccinated animals but there was no greater percentage of dead ticks, as compared to the controls.

8.4.2 Histology of Ticks from Vaccinated and Control Animals

Ticks with damaged gut were observed after only 24 h on vaccinated cattle and also after 24 h of feeding in vitro on blood from a vaccinated

FIG. 64. Young feeding female B. microplus ticks 24 h on a vaccinated host (large arrows); male tick after 24 h on a vaccinated host (small arrow) and two normal female ticks from a control host.


64
2mm

animal (No.3). The only conspicuous difference between these two groups of ticks was the greater proportion of ticks on the host which had distended caecae. The gut caecae of these ticks were filled with intact red cells, host leukocytes and sloughed off gut cells (Fig. 65). Some gut caecae were normal, but others had their linings reduced to thin strips with the cells completely destroyed and only the basal lamina and muscle cells remained. Other ticks from the vaccinated animals were not distended, but had turned red, due to rupture of the gut caecae with leakage of bovine erythrocytes or haemoglobin into the tick haemolymph. In contrast, the ticks from the control animals appeared normal and their gut caecae were lined with stem cells and digest cells. The lumina contained homogeneous tissue fluid and the digest cells showed normal accumulation of lipid and food vacuoles (Fig. 66). It was noted that salivary glands adjacent to damaged gut appeared unaffected by vaccination (Fig. 67).

By 48 h, the surviving damaged females had accumulated more leukocytes and red cells in their gut lumen and there was greater destruction of the digest cells (Figs. 67 & 68). More red ticks, that is ticks with ruptured caecae, were observed and acumulation of debris from host cells had increased in the lumen. Some caecae appeared relatively normal but the presence of host cells in the haemolymph showed that the gut had been ruptured elsewhere (Fig. 69). Ticks from the control animals had developed normally. Stem cells were in division and many digest cells were beginning to extend into the lumen as digestion progressed (Fig. 70).

The ticks which had survived to day 6 were delayed in their development. Fig. 71 shows the tissues of a female tick 7 mm in length which was collected on the day before final engorgement. Secretory cell type 2 (s_2m) were present and the colloidal mass were being liberated by many of these cells, but the gut had ruptured and the haemolymph was packed with host

FIG. 65. Gut caecum of female tick after 24 h on a vaccinated host; digest cells sloughed off into in lumen, d; host erythrocytes, rbc; salivary gland acinus, sg; basal lamina, bl. (Methacrylate Section, H & TB-P).

FIG. 66. Gut caecum of female tick after 24 h on control host; complete lining of stem cells and digest cells, d; lumen, lu. (Methacrylate section, H & TB-P).

FIG. 67. Gut caecum of female tick after 48 h on vaccinated host. Caecum with relatively normal lumen, lu_1; same caecum with distended lumen, lu_2; cell debris and leukocytes from host, db; normal salivary gland, sg. (Methacrylate section, H & TB-P).

FIG. 68. Gut caecum of female tick after 48 h on vaccinated host; caecum with distended lumen and almost completely destroyed gut cells, lu_2; cell debris and leukocytes from host, db. (Methacrylate section, H & TB-P).

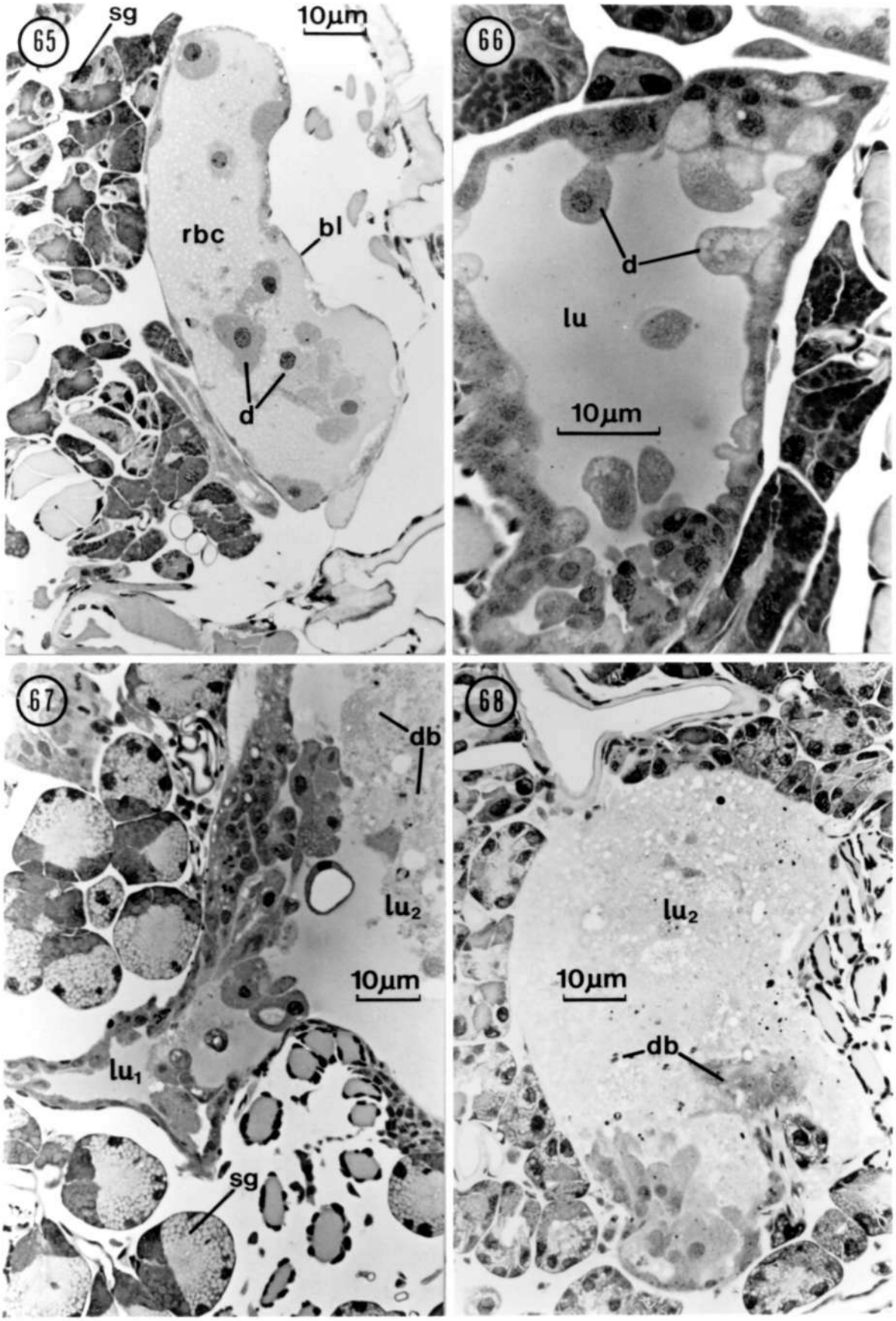
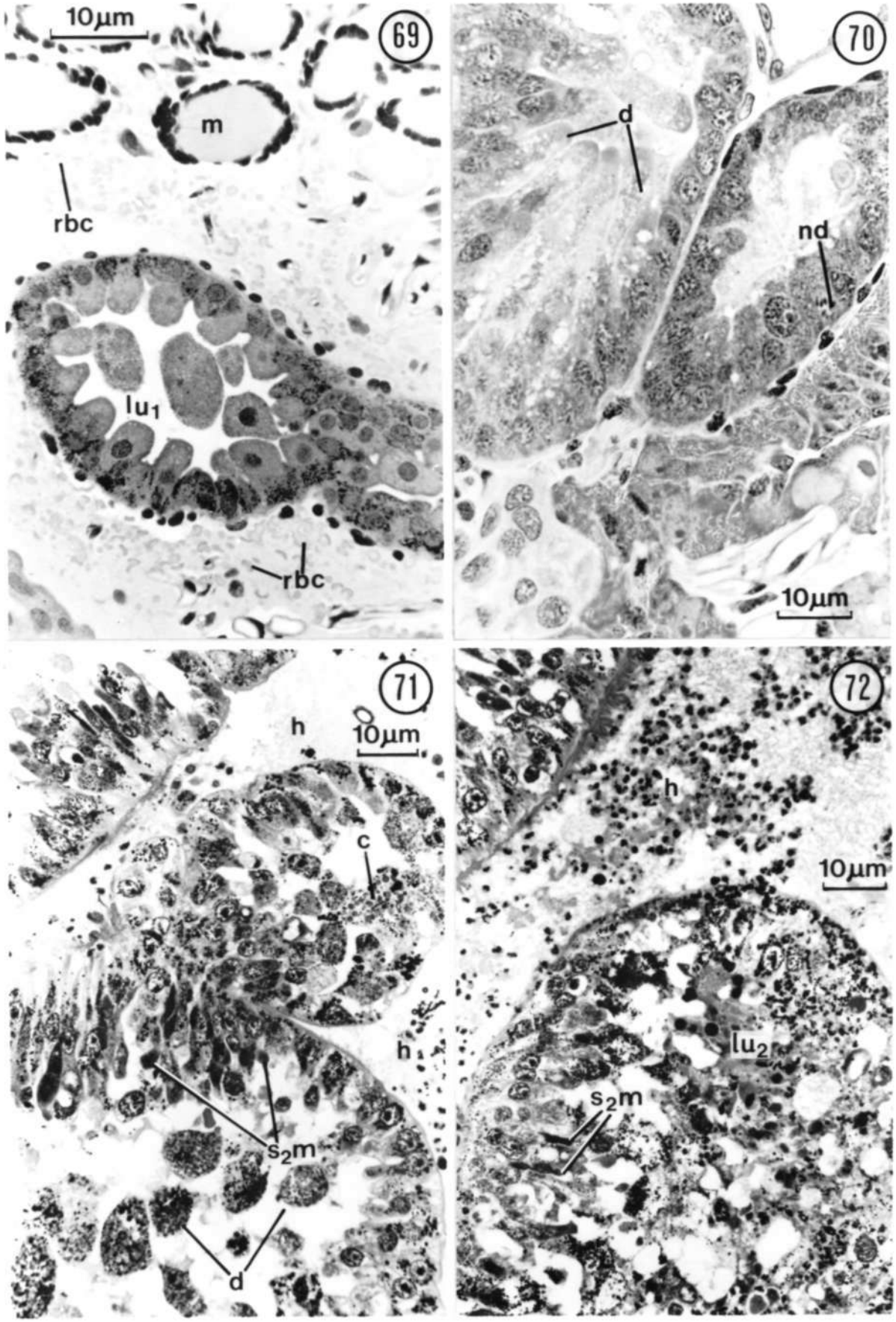

65
sg
10µm
rbc
bl
d
66
d
lu
10µm
67
db
lu2
10µm
lu1
sg
68
lu2
10µm
db

FIG. 69. Gut caecum of female tick after 48 h on vaccinated host; caecum with relatively normal lumen and digest cells, lu_1; host erythrocytes in tick haemolymph, rbc; tick muscle, m. (Methacrylate section, H & TB-P).

FIG. 70. Gut caecum of female tick after 48 h on control host; complete lining of stem cells and digest cells, d; nuclear division,nd. (Methacrylate section, H & TB-P).

FIG. 71. Gut caecum of female tick after 6 days on vaccinated host; secretory cell type 2, s_2m; "colloid mass", c; tick haemolymph space with host cells, h; digest cells, d. (Paraffin section, H & E).

FIG. 72. Gut caecum of female tick after 6 days on vaccinated host; secretory cell type 2, s_2m; tick haemolymph space with host erythrocytes, leukocytes and lysed tissue products, h; lumen with tissue debris, lu_2. (Paraffin section, H & E).


10μm
69
m
rbc
lu1
rbc
70
d
nd
10μm
71
h
10μm
c
h
s2m
d
72
h
10μm
lu2
s2m

red cells, leukocytes and lysed tissue products (Fig. 72). The activity of the 3rd generation digest cells (subtype I, dhl) was apparently inhibited and the digest cells subtype II (dhv) and the basophilic cell (b) could not be identified. On the other hand, ticks of the same age from the control animals had developed normally. Their secretory cells type 1 & 2 had completed their function, their digest cells (dhl) and (dhv) were active and the basophilic cells were maturing (Fig. 73). Once the gut had been damaged and host leukocytes had escaped, other tissues were attacked, for example the muscles and malphigian tubules (Figs. 74 & 75). Leukocytes could be seen penetrating the sarcolemma and in between degenerating muscle fibres.

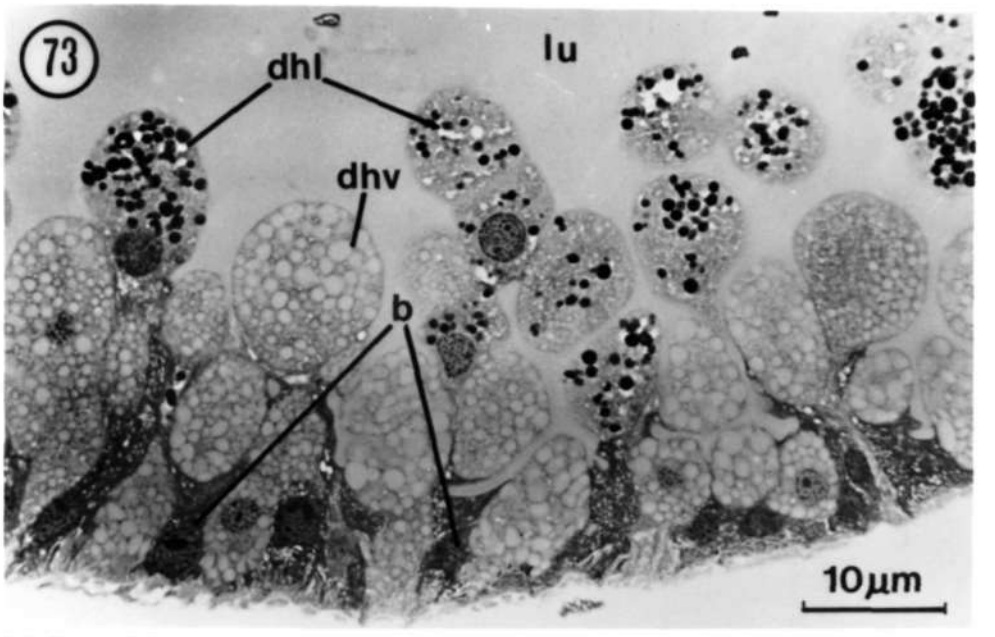
Ticks which had engorged and detached from the vaccinated hosts on day 7 were sampled on day 8, and the various types of damage which were found in engorged red ticks are as follows. Damage to the basophilic cells only is shown in Fig. 76. The basophilic cell plasma membranes appeared to have been separated from the cell, and the cell cytoplasm showed signs of vacuolation, leading to possible degeneration of the cell. Fig. 77 shows damage to the subtype II digest cells (dhv) and the basophilic cells. Specifically the outer plasma membranes lining the luminal surface of the cells appeared to lyse and some of the contents of the cells were released into the lumen. The basophilic cells were also damaged in this caecum whereas other caecae had damaged digest cells, but the basophilic cells appeared normal (Fig. 78).

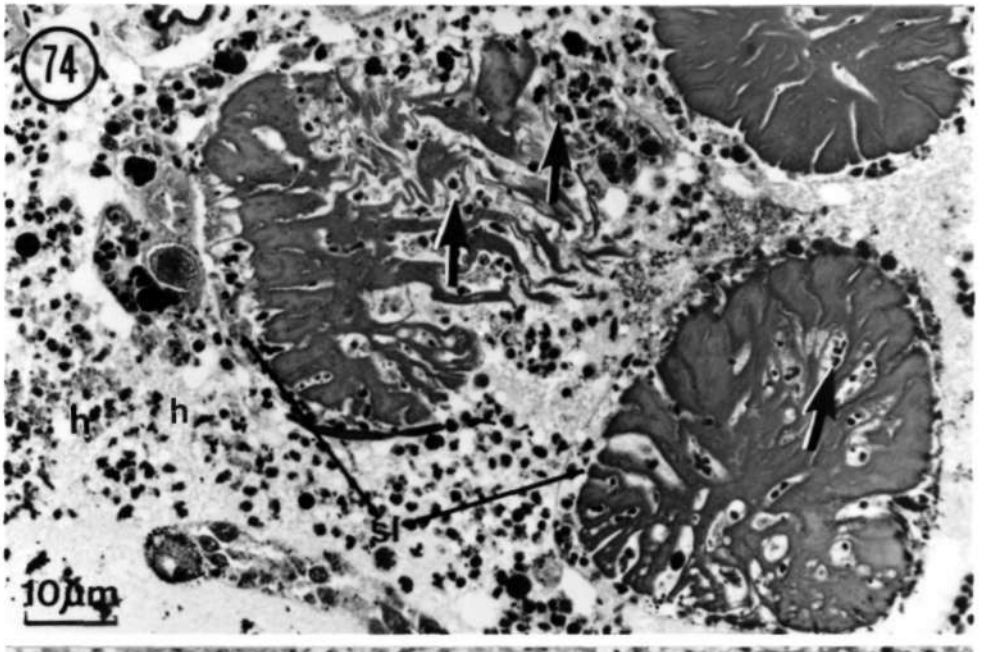
Males presented a very striking picture. As early as 24 h post-infestation, dead males were observed. Some of the surviving ones showed a damaged gut as in the females with increased intake of leukocytes. Leukocytes destruction of the accessory reproductive gland, particularly the median lobe (Fig. 79) was observed after the gut had ruptured.

FIG. 73. Gut caecum of female tick after 6 days on control host; digest cell subtype I dhl; digest cell subtype II, dhv; basophilic cell, b; lumen, lu. (Methacrylate section, H & TB-P).

FIG. 74. Muscles in female tick after 6 days on vaccinated host. Arrows show host leukocytes penetrating between degenerating muscle fibres; damaged sarcolemma, sl; tick haemolymph space with host leukocytes, h. (Paraffin Section, H & E).

FIG. 75. Malphigian tubules in female tick after 6 days on vaccinated host; relatively normal malphigian tubule, mp_1; damaged malphigian tubule, mp_2; nerve axons from brain, na. (Methacrylate section, H & TB-P).


73
lu
dhl
dhv
b
10µm


74
h
h
sl
10µm

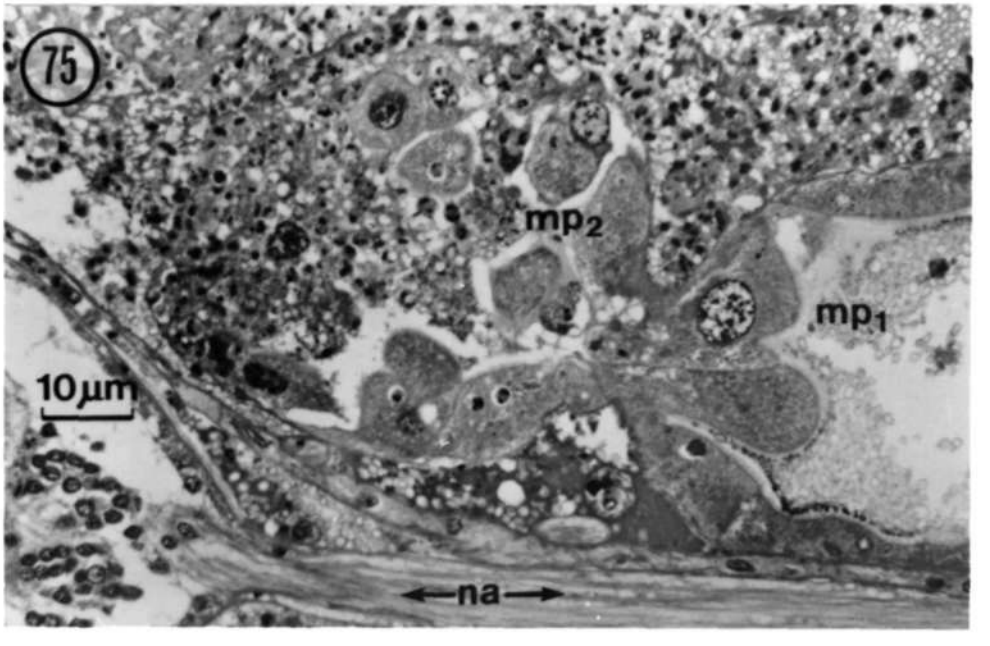
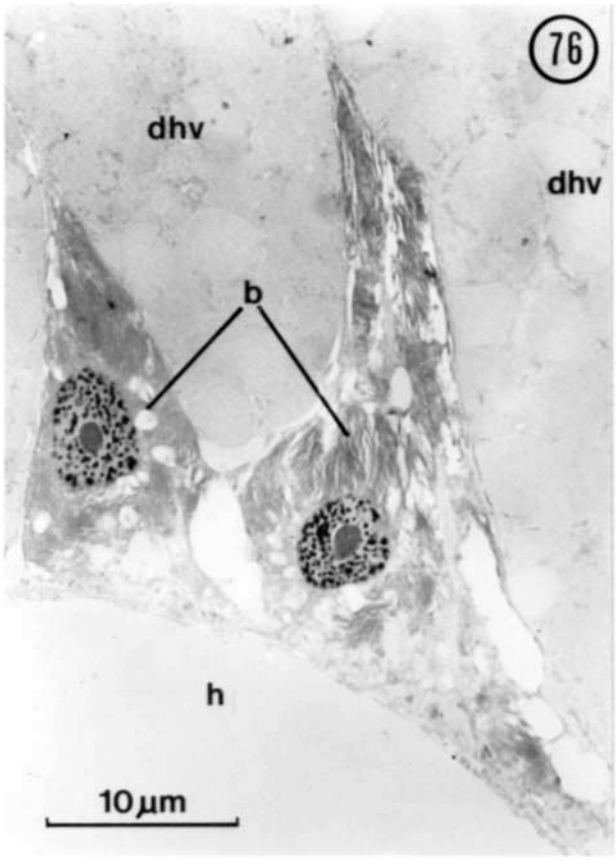

75
mp2
mp1
10µm
na

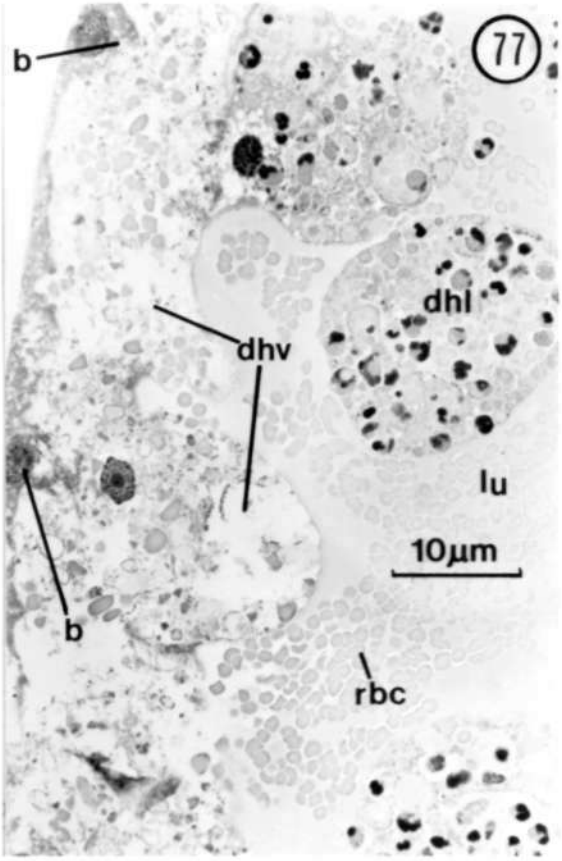
FIG. 76. Gut caecum of female engorged (day 8) on vaccinated host; basophilic cell with vacuolation of cytoplasm, b; digest cell, dhv; haemolymph space, h. (Methacrylate section, H & TB-P).

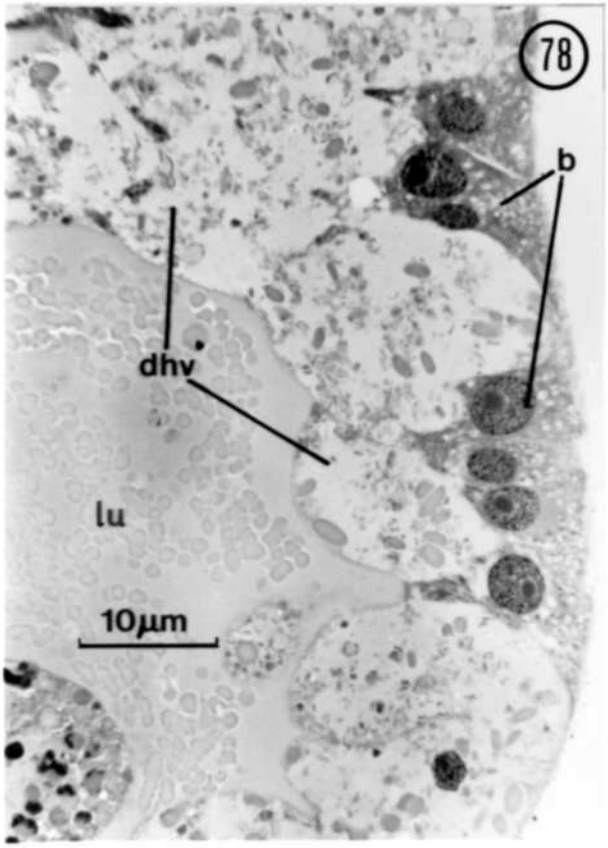
FIG. 77. Gut caecum of female engorged (day 8) on vaccinated host; damaged digest cells subtype II, dhv; damaged basophilic cells, b; lumen, lu; digest cell subtype I, dhl; host erythrocytes, rbc. (Methacrylate section, H & TB-P).

FIG. 78. Gut caecum of female engorged (day 8) on vaccinated host; damaged digest cells subtype II, dhv; intact basophilic cells, b; lumen, lu. (Methacrylate section, H & TB-P).

FIG. 79. Median lobe of accessory gland in reproductive organ of male, fed for 24 h on vaccinated host; tissue damaged by host leukocytes, dt; normal tissue, nt. (Methacrylate section, H & TB-P).


76
dhv
dhv
b
h
10µm


77
b
dhl
dhv
lu
10µm
b
rbc


78
b
dhv
lu
10µm

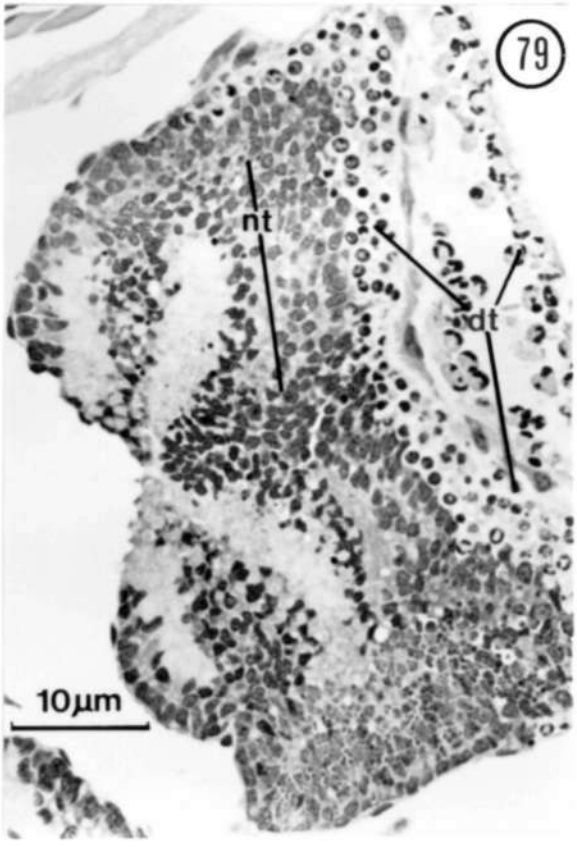

79
nt
dt
10µm

TABLE 9. EFFECTS OF VACCINATION ON **B. MICROPLUS** FEMALES **IN VIVO**. THE PERCENTAGE OF DEAD TICKS AND THE PERCENTAGE WITH DAMAGED GUT.*

	Cumulative % dead or gut damaged**					
	Vaccinated			Controls		
% Dead on days after infestation	Animal 1	2	3	4	5	6
Day 1	12	8	13	14	9	10
Day 2	12	8	13	15	12	11
Day 3	13	12	21	15	13	11
Day 4	13	13	30	15	15	11
Day 6	13	15	42	15	15	11
Day 7	13	15	51	15	15	11
Day 8	14	23	53	15	15	11
% gut damaged on days after infestation						
Day 1	**2**	**16**	**4**	**0**	**0**	**0**
Day 2	**3**	**22**	32	0	0	0
Day 3	**4**	**23**	33	0	0	0
Day 4	**5**	**37**	38	0	0	0
Day 6	**9**	**37**	38	0	0	0
Day 7	10	57	42	0	0	0
Day 8	11	***60	***45	0	0	0

* Taken from Kemp *et al.* (1984) submitted for publication.

** 3-6 rings with 80-177 females for each figure. Gut damaged ticks had ruptured gut with escape of host erythrocytes or grossly distended gut.

*** Lower than expected damage because dead ticks could not be assessed for gut damage. Most damaged females did not engorge. Those which did, failed to oviposit.

8.5 DISCUSSION

The results in this chapter showed that rejection of ticks as a result of vaccination was different from resistance to ticks acquired over a prolonged period of exposure to tick feeding. In acquired resistance to B. microplus, it is well known that ticks are rejected mainly at the establishment of each instar and particularly at the larval stage (Roberts, 1968; Wagland, 1979). An immediate type hypersensitivity reaction to salivary antigens with serous exudation and cellular infiltration is positively correlated with rejection (Schleger, Lincoln, McKenna, Kemp & Roberts, 1976; Willadsen, Williams, Roberts & Kerr, 1978).

On vaccinated cattle there was no greater loss of establishing females compared with the controls, but there was a loss of females throughout feeding on two out of three vaccinates. There was no serous exudation at the attachment sites or obvious signs of hypersensitivity reaction to the adults. This is contrary to the effects of acquired resistance, suggesting that vaccination had stimulated a different immunological response, leading to a different rejection mechanism. This was confirmed by the high percentage of gut damaged females on vaccinated cattle compared with the controls.

The histopathological changes observed in 32 female ticks from vaccinated cattle further supports this contention. The large numbers of ticks with distended gut caecae on the vaccinated hosts were particularly interesting. These ticks differed from engorged serum ticks or "haemolymph ticks" from anaemic animals which had large haemolymph volumes and small contracted caecae. The distended ticks had relatively normal haemolymph volumes, but many caecae were stretched and in these parts of the caecae no stem, digest or developing secretory cell type 2 (s_2e, s_2m)

were found on the basal lamina, as most were either destroyed or sloughed off prematurely into the lumen. Some gut muscle cells were also damaged.

Damage to the gut resulted in leakage from the caecae and in many cases the haemolymph was packed with gut lumen contents. The attack on muscle cells and Malphigian tubules within the tick haemocoel was observed in a few of the damaged ticks examined, and appeared to be mediated by host leukocytes. There was no detectable muscle or Malphigian tubule damage when the haemolymph was free of these cells.

Another significant finding was that in some females the early secretory cell type 2 (s_2e) and the mature secretory cell type 2 (s_2m) in the female gut were both attacked. Secretory activities were delayed, and this delayed the development of the basophilic cells (b). Females which had fully engorged also showed damage to these same cell types. This attack on the s_2e, s_2m & b cells could be expected, because histology of the 5-6 mm females used for vaccination showed that they contained the s_2m cells at their peak of development and secretion (Chapter 3). In male ticks, the reproductive tract was the only tissue other than gut which was being destroyed and again the host leukocytes were involved.

During slow feeding when many ticks became damaged on vaccinated cattle, the predominant cells in the gut were digest cells. Since these cells are frequently ruptured, it is suggested that the most important antigens related to vaccination are located on the lumen side of the plasma membranes in these cells. Host antibodies and cells would have ready access to the surface of these cells during feeding. The microvilli of gut cells in ixodid ticks possesses a fibrillar layer consisting of

glycocalyx, which is a mucopolysaccharide (Raikhel, 1978) and this is known to be an important part of the plasma membranes of intestinal cells involved in absorption. Damage to these components could impair the function of these cells or in this case cause leakage of the cells. Reactions against other tissues which are antigenic (muscle, Malphigian tubule etc) could then occur and this may also be important in the rejection of ticks.

It has been suggested by Kemp et al. (submitted for publication) that host antibodies and complement are required for damage to gut cells. The histological study of the gut indicates that there may also be a contribution from host cells. Male ticks had ingested numerous host leukocytes early in feeding, as opposed to males on control animals. Female ticks on vaccinated cattle had more leukocytes in the gut and leukocyte associated damage suggested that these cells play some role in the success of vaccination and this would differ from the complement dependent gut damage.

As stated earlier, the damage to ticks recorded here occurred in ticks infested soon after vaccination. It is not yet known what host reactions are important for the high levels of resistance which are maintained for several months after vaccination (Johnston et al., 1983). However, damaged ticks were still observed in these later infestations so the vaccination effects must still be operating to some extent.

The natural host defences against arthropod parasites relies on an acquired resistance to antigens usually present in the salivary secretions of the parasite. Reliance on these antigens limits the potential scope of the immunity, but this can apparently be circumvented by injecting non-

salivary antigenic material into the host (as was the case in the vaccination experiments reported here) to stimulate the production of antibodies which will be ingested by the parasite with detrimental effects (Nogge & Giannetti, 1980). This novel idea had already been shown to have promise against blood sucking insects (Khan, Connell & Darcel, 1960; Alger & Cabrera, 1972; Schlein & Lewis, 1976) and it is only recently that similar encouraging results have been published on host vaccination against ticks (Allen & Humphreys,1979; Ackerman et al., 1980; McGowan et al., 1980; Johnston et al., 1983). The results presented here is further indication that vaccination produces greater deleterious effects on the tick than does natural immunity due to tick exposure (salivary antigens).

8.6 SUMMARY

Rejection of B. microplus ticks from vaccinated cattle was shown to be different from rejection which resulted from acquired resistance to infestation by ticks.

The main difference was the damage to gut cells which in light microscope sections were shown to be completely destroyed in some ticks. In others, the outer plasma membranes lining the luminal surface of the cells appeared to lyse and some of the contents of the cells were released into the lumen.

Significant damage was also found in muscle, Malphigian tubules and cells of the male reproductive organs, when host leukocytes and erythrocytes escaped into the haemolymph.

In some vaccinated cattle a high proportion (>60%) of female ticks were prevented from feeding by reactions which damaged gut and other tissues.

CHAPTER 9

VACCINATION OF RABBITS WITH R. SANGUINEUS ANTIGENS AND A SEROLOGICAL COMPARISON WITH B. MICROPLUS ANTIGENS. A LABORATORY MODEL

9.1 LITERATURE REVIEW

Trager (1939a) presented the first experimental evidence on the development of acquired resistance to a blood sucking arthropod. His work was based on the study of the American dog tick, Dermacentor variabilis and he found that guinea pigs and rabbits which were infested once with larval ticks showed an increased degree of resistance to tick challenge. The mechanism of resistance was suggested to be (i) blocking of the blood flow to the sites of attachment of subsequent batches of larvae, nymphs and adults, (ii) hypersensitivity. Brumpt & Chabaud (1947) and Chabaud (1950b), after investigating several host-tick systems, concluded that only some of the individual hosts acquired resistance. Riek (1962) showed that there was a wide spectrum in resistance of cattle to the cattle tick B. microplus. He also described a state of hypersensitivity in some animals.

Feldman-Muhsam (1964) endorsed the findings of Brumpt & Chabaud (1947) and Chabaud (1950) and stated that from 25-90% R. sanguineus larvae placed on three rats were recovered in the engorged state. These wide variations she put down to the many variable factors influencing the success of feeding viz. batch variation, age and viability of ticks, age and sex of hosts, together with the temperature and humidity of the environment. Roberts (1968) showed that there was an acquired resistance in cattle against B. microplus. He concluded that the resistance exhibited was the result of an immune response by the host. Several other workers have reported variable successes and these have been reviewed by Nelson, Bell, Clifford & Keirans (1977).

The mechanisms of acquired resistance to ticks are varied and sometimes uncertain. An immunological basis for resistance has been postulated by numerous investigators with papers on the subject by Roberts (1968); Boese (1974); Wikel, Graham & Allen (1978), and Willadsen (1980). It has been suggested that it is complement-mediated (Berenberg, Ward & Sonenshine, 1972), prostaglandin mediated (Higgs, Vane, Hart, Potter & Wilson, 1976), or other immune-mediated inflammatory rections which rendered the bite site unfavourable for the feeding process (Riek, 1962) or grooming is stimulated thus, reducing infestation loads (Bennett, 1969; McGowan, 1974). Protection has been successfully transferred with immune sera by some researchers (Trager, 1939b; Riek, 1958; Roberts & Kerr, 1976; Brossard, 1977; Brossard & Girardin, 1979), but not by others (Pogorelyi, 1966; Bagnall & Rothwell, 1974; Wikel & Allen, 1976). Some other workers have claimed successful transfer of resistance only with immune lymphocytes or a combination of lymphocytes and serum factors (Askenase, 1977). It is apparent that a number of different immunological reactions are acting against ticks and which is of greatest importance for tick rejection depends on the particular host and parasite.

The induction of anti-tick immunity by vaccination using tick antigen preparations has been examined by relatively few authors. Resistance to tick larvae by guinea pigs which were injected intracutaneously with larval extracts from the same species of ticks was first observed by Trager (1939b). Riek (1958) reported that egg extracts of Haemaphysalis bispinosa (≡ longicornis) protected laboratory animals against larval attachment. Several groups of investigators have also demonstrated that salivary gland extracts of D. andersoni or R. sanguineus could be used to vaccinate guinea pigs against tick infestation (Garin & Grabarev, 1972; Wikel, 1977).

Allen & Humphreys (1979) were the first to report an apparently novel immunological method of tick control in guinea pigs and cattle. They showed that feeding and reproductive performances of D. andersoni infesting immunised guinea pigs and calves were significantly reduced. This obvious success resulted from the use of antigenic extracts of partially fed ticks, but not unfed ticks, and this may well reflect the antigenic changes which occurred in the tissues of the feeding female ixodid tick (Balashov, 1972; Chapter 3 & 4).

Ackerman, Floyd & Sonenshine (1980), demonstrated delayed attachment, reduced engorgement weights, lengthened pre-oviposition periods, and reduced egg production in adult D. variabilis females infesting rat hosts immunised with extracts derived from whole ticks or tick midguts. Similar findings were observed by McGowan et al. (1980) in their experiments with Amblyomma maculatum. In addition, they observed the more rapid formation of the feeding lesion (2 h) in immunised rabbits following attachment of adult ticks. This is suggestive of a type 1 immediate hypersensitive reaction, presumably mediated by a homocytotropic antibody (IgE). Boese (1974) had reported the development of such an antibody in approximately 75% of rabbits repeatedly infested with nymphal Haemaphysalis leporispalustris ticks. McGowan, Barker, Homer, McNew & Holscher (1981) reported limited success in vaccination of cattle against A. americanum.

Most of the resistance levels induced in vaccinated hosts were not high, but recently, the yield of the cattle-tick B. microplus was reduced by 70% on vaccinated animals compared with controls (Johnston et al., 1983).

9.2 INTRODUCTION

The literature cited in this chapter and in Chapter 8 suggests that immunisation against ticks has, in recent years, become an important topic for study.

With the novel effect of vaccination using crude extracts of whole ticks or tick guts now documented and the need to test small amounts of purified extracts, the use of cattle for vaccination trials is very expensive. While it is desirable to use cattle and their specific host tick, B. microplus, it is often impossible to have enough animals, and a suitable laboratory model is needed. Allen & Humphreys (1979), for example, used guinea pigs first as a guide to their vaccination trials with D. andersoni. A laboratory model has some obvious advantages.

(i) Statistically adequate numbers of experimental and control animals.

(ii) Use of both isogenic and allogenic strains of animals.

(iii) Ease of testing many antigens or combinations of antigens from purification procedures or from genetic engineering of tick proteins.

(iv) Economic feasibility.

Because of their size, rabbits are an obvious choice for a laboratory animal model for work with ticks.

It would have been ideal to rear and study the cattle tick B. microplus on rabbits but earlier workers found that the cattle-tick would not feed successfully on rabbits or other laboratory animals (Loomis, 1971). It became necessary to select a tick species that could feed normally on rabbits.

R. sanguineus ticks were chosen because they fed normally on rabbits and a laboratory maintained strain was available at the Animal Research Institute, Yeerongpilly, Brisbane.

In this experiment rabbits were immunised against R. sanguineus using an extract of semi-engorged (3.5 mm) adult female ticks. The aim was to test the suitability of the laboratory model for tick:host vaccination studies.

9.3 MATERIALS AND METHODS

9.3.1 Rabbits

Eight male, 4-4 $\frac{1}{2}$ months old, closed strain Dutch bred rabbits were used for this experiment. They were maintained at 25 ± 1°C and were fed rabbit and guinea pig pellets (Feed Supplies, Brisbane, Australia).

9.3.2 Ticks

Nymphs of the brown dog tick, R. sanguineus were obtained from Mr. Peter Green of the Department of Primary Industries, Yeerongpilly to start a culture in the laboratory. The ticks had originally been collected at Oakhurst, west of Maryborough, Queensland in 1971 (Green, 1972). All fed ticks and the eggs were maintained at 26 ± 1°C and 90-100% relative humidity (RH). However, the larvae and adults which emerged before the rabbits were ready for infestation, were kept at 18 ± 1°C and 80-90% RH.

Before these ticks were used for infestation, they were kept at room temperature for 2 h, and the active ones were sampled, counted and used for infestation.

9.3.3 Antigen Preparation

The crude antigen (CA) preparation was obtained by homogenising 100 semi-engorged adult females (3.5 ± 0.5 mm in length) in a Virtis blender containing 3 ml cold (4°C) 0.012M phosphate buffered saline 0.15 M NaCl, pH 7.2 (PBS) containing penicillin and streptomycin (100 units of each per ml). The ticks were homogenised for 2 min and the disruption of the tissues was completed using a pre-chilled mortar and pestle. The resulting crude preparation was filtered through sterile glass wool by suction and the filtrate was made up to 10 ml with PBS.

The CA was sonicated at 4°C for 30 sec in a 180 watt Rapidis Sonicator (Ultra Sonics Ltd., Shipley, Yorks, England) using a 9 mm probe at a power setting of 12 and tuned to maximum amplification.

Four millilitres of the CA was used to vaccinate the rabbits that day and 4 ml was frozen in vapour phase liquid nitrogen (-100°C) and later used for a second injection. The balance of 2 ml was taken and spun at 45,000 g for 1 h. The supernatant was pipetted off and stored at 4°C to be used for protein concentration determination, gel diffusion tests and immunoelectrophoresis. Protein concentration of the supernatant was measured by the Biuret method (Layne, 1957).

9.3.4 Bleeding

Prior to each injection and before tick infestation, the rabbits were bled from the marginal vein of the ear. Sera were separated by clotting, and were stored at -20°C in 2 ml aliquots.

9.3.5 Vaccination

The four controls and four vaccinates were given two injections four weeks apart. Each vaccinate received 1 ml CA emulsified with 2 ml of Freund's complete adjuvant (FCA). The controls each received 1 ml PBS with 2 ml FCA. The first injection was given subcutaneously at four separate sites, at a dose of 0.75 ml per site. The second injection, four weeks later was given intramuscularly, in the inner thigh muscles at four different sites.

9.3.6 Tick Infestation and Collection

Larvae and adults were put on the rabbits four weeks after the second vaccination. Approximately 100 larvae were counted while being chilled on a cold plate, and were used to infest each of the three controls and three vaccinates. Ten males and ten females were infested each on one control and one vaccinate. The larvae and adults were placed on the ears of the rabbits to which a cloth bag had been attached, and they were restricted from grooming by the use of Elizabethan collars similar to those described by Bailey (1960).

The following parameters were monitored to determine the degree of host resistance.

(i) Time for attachment of adults (larvae were not monitored as they were barely visible).

(ii) Period from infestation of the host to detachment.

(iii) Engorgement weights of larvae and females.

(iv) Moulting time from engorged larvae to nymphs.

(v) Presence or absence of damaged ticks.

(vi) Weight of egg masses laid.

Four days post-infestation, the rabbits were transferred from large cages to smaller ones held over water to collect engorged stages. The ticks were collected using a soft brush and were dried on tissue paper before being transferred to a plastic container which held a strip of filter paper. The ticks were then stored briefly at 18 ± 1°C and greater than 90% RH before being weighed. Groups of larvae were weighed using a Mettler H20T balance accurate to 0.01mg; and individual adults were weighed with a Mettler H10T balance. Randomly selected larvae from both control and vaccinated animals were weighed individually using a Mettler UM7 (Ultra Microbalance).

9.3.7 Gel Diffusion and Immunoelectrophoresis

Ouchterlony & Nilsson (1978) immuno-diffusion and immuno-electrophoresis methods were used to evaluate the precipitating antibodies in sera of immunised rabbits

For gel diffusion 4 ml of 0.75% Oxoid Ion agar No. 2 (Oxoid Ltd., London) in PBS pH 7.4 was poured onto 2" x 2" glass slides and allowed to set. Wells of 5 mm diameter were made in the gel with a distance between wells of 5.0 mm and 40 µl of the CA or the different sera filled the wells. The slides were incubated at 4°C for 48 h in a humidified chamber, for the precipitation lines to develop.

The CA was also fractionated by electrophoresis in 1.5% Ion agar No. 2 (Oxoid Ltd., London) and sodium barbitone buffer (0.025 M, pH 8.6), according to the Shandon Electrophoresis apparatus manual - Technical Bulletin SAE-05-106 (Shandon Scientific Co. Ltd., London). The agar was prepared on microscope slides using the Shandon Southern Immunodiffusion kit. The wells were enlarged to a diameter of 2.0 mm. Five microlitres of CA were pipetted into the wells, and run for 2 h at a constant 200 V and 20 mA from negative to positive. The central troughs were then cut out and 150 µl of appropriate antisera were added and allowed to react for 48 h at 4°C in a humidified chamber.

In both experiments, the immunoprecipitin patterns were recorded after 48 h and the slides were washed for one week in saline with 0.04% sodium azide the solution being changed every two days. The slides were later washed for 1 h in distilled water and dried at 37°C overnight before being stained with 0.1% Napthalene (Amido) Black 10B (George T. Gurr Ltd., London). The slides were destained in 5% glacial acetic acid in distilled water, rinsed in distilled water, dried and photographed.

9.3.8 Sera for Gel Diffusion and Electrophoresis

9.3.8.1 Rabbit antibodies to *B. microplus* haemolymph.

This was prepared by pooling haemolymph from female ticks and storing it at -20°C. Then 0.5 ml of haemolymph plus 0.5 ml FCA was emulsified and injected intramuscularly into the hind legs of a rabit (No. 32). Four weeks later the same volumes were inoculated intradermally. After another four weeks the same volumes were administered subcutaqneously.

The rabbit was bled two weeks after the last injection and serum separated by clotting. This serum will be referred to as R.32.

9.3.8.2 Rabbit antibodies to **B. microplus** gut cells

Female ticks were collected the day before engorgement and the gut caecae were disected out, washed in PBS (containing penicillin and streptomycin) and homogenised for 2 min at 4°C in a Virtis blender. The mixture was then sonicated in 4 x 10 ml aliquots in a 180 watt Rapidis Sonicator for 30 sec using maximum power and a 9 mm probe at 4°C. After pooling the material it was centrifuged at 30,000 g for 1 h at 4°C and the supernatant was used for inoculating a rabbit (No. 56). Two ml of the supernatant plus 3 ml of FCA were emulsified and injected intramuscularly, 1.5 ml to each hind leg, and another 2 ml subcutaneously on the back in 10 separate sites. Four weeks later 1 ml supernatant plus 2 ml FCA were injected intramuscularly in the hind legs (1.5 ml/site/leg). Two weeks later an intravenous injection of 0.2 ml of supernatant was given.

The rabbit was bled two weeks later and serum separated by clotting. This serum will be referred to as R.56.

9.3.8.3 Bovine antibodies to **B. microplus** whole tick extract

A Hereford calf (No. 659) was vaccinated with an extract from female B. microplus ticks collected the day before engorgement. Whole females were homogenised and 4 ml of this CA plus 8 ml of FCA was emulsified and injected intramuscularly. Four weeks later the same volume was given subcutaneously. Four weeks after the second injection, 4 ml of the crude antigen alone was given subcutaneously. The animal was bled three weeks later and serum was separated by clotting the bovine blood. This serum will be referred to as K.659.

9.3.8.4 Rabbit antibodies to **R. sanguineus**

Blood was taken from the rabbit which was used for feeding nymphs and adults R. sanguineus to produce the CA. The infestation was repeated once more and a total of 700 nymphs and 180 partially fed adults were recovered from the rabbit. The time interval between the two feeding cycles was 30 days. As many fewer ticks were recovered during the second infestation, the development of an acquired immunity was suspected and the rabbit was bled. The serum was collected as before and will be referred to as RIP.

All sera were stored in 2 ml aliquots at -20°C until needed.

9.4 RESULTS

9.4.1 Protein Concentration

The protein concentration of the supernatant of the CA was 7.6 mg/ml.

9.4.2 Immuno-precipitation Analysis

Reaction patterns from Ouchterlony immunodiffusion slides are shown in Figs. 80, 81, 82 & 83. No antibodies to the R. sanguineus CA were detectable in the sera from the control rabbits (Fig. 80A-1); and no lines detectable in the vaccinated rabbits after the first injection (Fig. 80A-2). Fig. 80A-3 shows multiple precipitation lines formed against the CA by R.56 serum (rabbit vaccinated with B. microplus gut cells), and R.32 serum (rabbit vaccinated with B. microplus haemolymph), but no lines with RIP serum (rabbit infested with R. sanguineus ticks only). Fig. 80B-1 refers to control rabbits after their two injections and shows no lines against the CA, whereas Fig. 80B-2 which refers to the four sera of the vaccinated rabbits after the two injections, shows precipitation lines against the CA indicating that vaccination induced a response in the rabbits. Fig. 80B-3 shows precipitation lines with R.56 and R.32 reacting

FIG. 80 A-1. Gel diffusion slide for control rabbit sera 1-4, four weeks after the first inoculations. Centre well contained the R. sanguineus C.A.

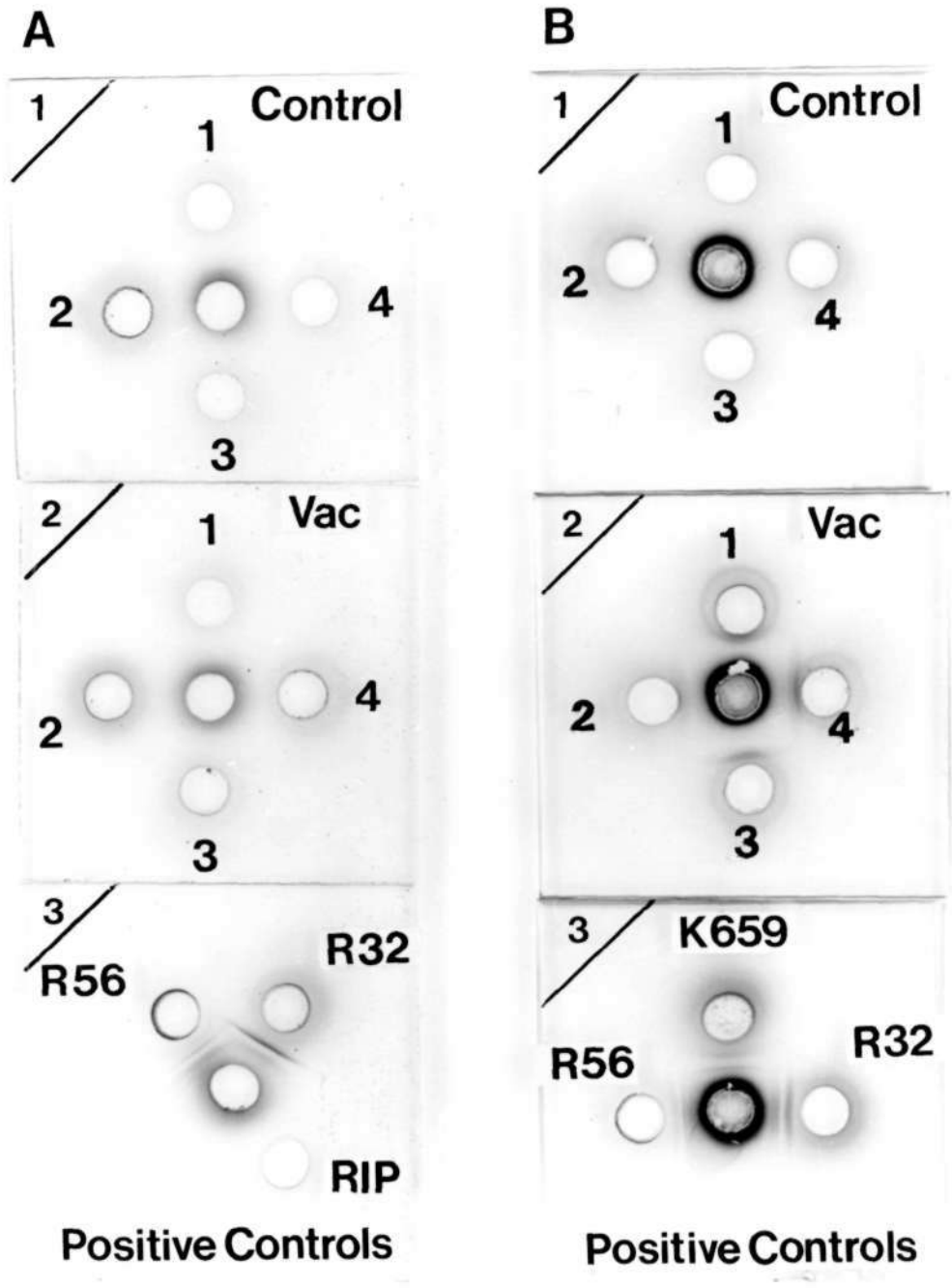
FIG. 80 A-2. Gel diffusion slide for vaccinated rabbit sera R1-R4, four weeks after the first inoculations. Centre well contained R. sanguineus CA.

FIG. 80 A-3. Gel diffusion slide for positive control rabbit sera, R56; R32 and RIP. Centre well contained R. sanguineus CA. Note precipitin lines with R56 and R32 sera.

FIG. 80 B-1. Gel diffusion slide for control rabbit sera (1-4) four weeks after second inoculations. Centre well contained R. sanguineus CA.

FIG. 80 B-2. Gel diffusion slide for vaccinated rabbits R1-R4, four weeks after second inoculations. Centre well contained R. sanguineus CA. Note precipitin lines with sera 3 and 4.

FIG. 80 B-3. Gel diffusion slide for positive control sera from R.56; R.32, and bovine K.659. Note precipitin lines with all sera.


A
1
Control
1
2
4
3
2
Vac
1
2
4
3
3
R56
R32
RIP
Positive Controls
B
1
Control
1
2
4
3
2
Vac
1
2
4
3
3
K659
R56
R32
Positive Controls

against R. sanguineus CA. Since no response was obtained from RIP (Fig. 80A-3), serum from K.659 (bovine vaccinated with B. microplus whole tick extract) was substituted, and precipitation lines were observed when this serum (K.659) was reacted against the CA of R. sanguineus (Fig. 80B-3).

These results with sera from rabbits and cattle vaccinated with R. sanguineus and B. microplus CA's gave the expected homologous reactions but there were also some cross reactions. These raised two questions. Are there common antigens in ixodid ticks of different genera? Do the host reactions to tick antigens confer any protection against ticks? Attempts were made firstly to fractionate the CA (Fig. 81). Using this method, poor separation of proteins was obtained. However, one conspicuous band had migrated furthest towards the anode and was common to all the rabbits vaccinated (Fig. 81a,b,c & d) and bovine (K659) (Fig. 81-e). This line was observed in R.32 (Fig. 81f) but not R.56 (Fig. 81g), suggesting that the antigen was in haemolymph but not gut. Fig. 81h shows precipitation lines when B. microplus CA was reacted with the homologous antiserum of bovine origin. The common line was also present when K.659 serum (bovine vaccinated with B. microplus CA) was reacted against the fractionated (CA) of R. sanguineus (Fig. 81e).

If this antigen was indeed shared between R. sanguineus and B. microplus then it should be possible to demonstrate lines of identity when these various antisera were cross reacted against the CA of both tick species. The result of a third experiment are given in Fig. 82. Fig. 82a shows the reaction of R. sanguineus CA producing lines of identity with all four rabbit vaccinates (R_1-R_4) and R.32 (B. microplus haemolymph) and K.659 (B. microplus whole tick extract). Various combinations of R_{1-4} were mixed with K.659, R.32 and R.56 (B. microplus gut) such that each one

FIG. 81 a-h. Fractionation of CA by immunoelectrophoresis.

a. wells contained R. sanguineus CA. Central trough contained serum from vaccinated rabbit R1.

b. wells contained R. sanguineus CA. Central trough contained serum from vaccinated rabbit R2.

c. wells contained R. sanguineus CA. Central trough contained serum from vaccinated rabbit R3.

d. wells contained R. sanguineus CA. Central trough contained serum from vaccinated rabbit R4.

e. wells contained R. sanguineus CA. Central trough contained serum with bovine antibodies to B. microplus whole tick extract (K659).

f. wells contained R. sanguineus CA. Central trough contained rabbit serum with antibodies to B. microplus haemolymph (R32).

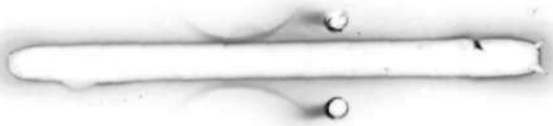
g. wells contained R. sanguineus CA. Central trough contained rabbit serum with antibodies to B. microplus gut cells (R.56). Note absence of precipitin lines.

h. wells contained R. sanguineus CA. Central trough contained serum from vaccinated bovine (K.659).

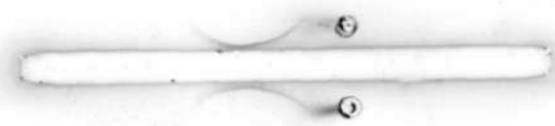
a R1



b R2



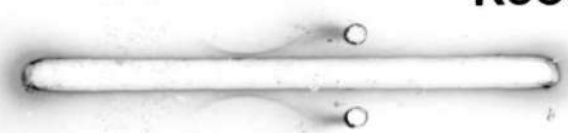
c R3



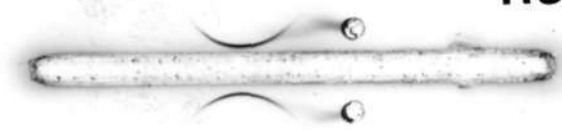
d R4



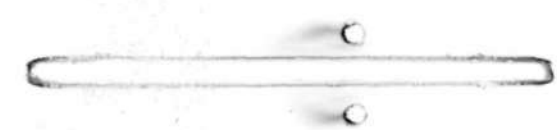
e K659



f R32



g R56



h K659

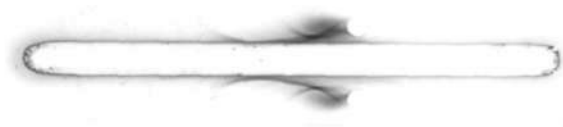


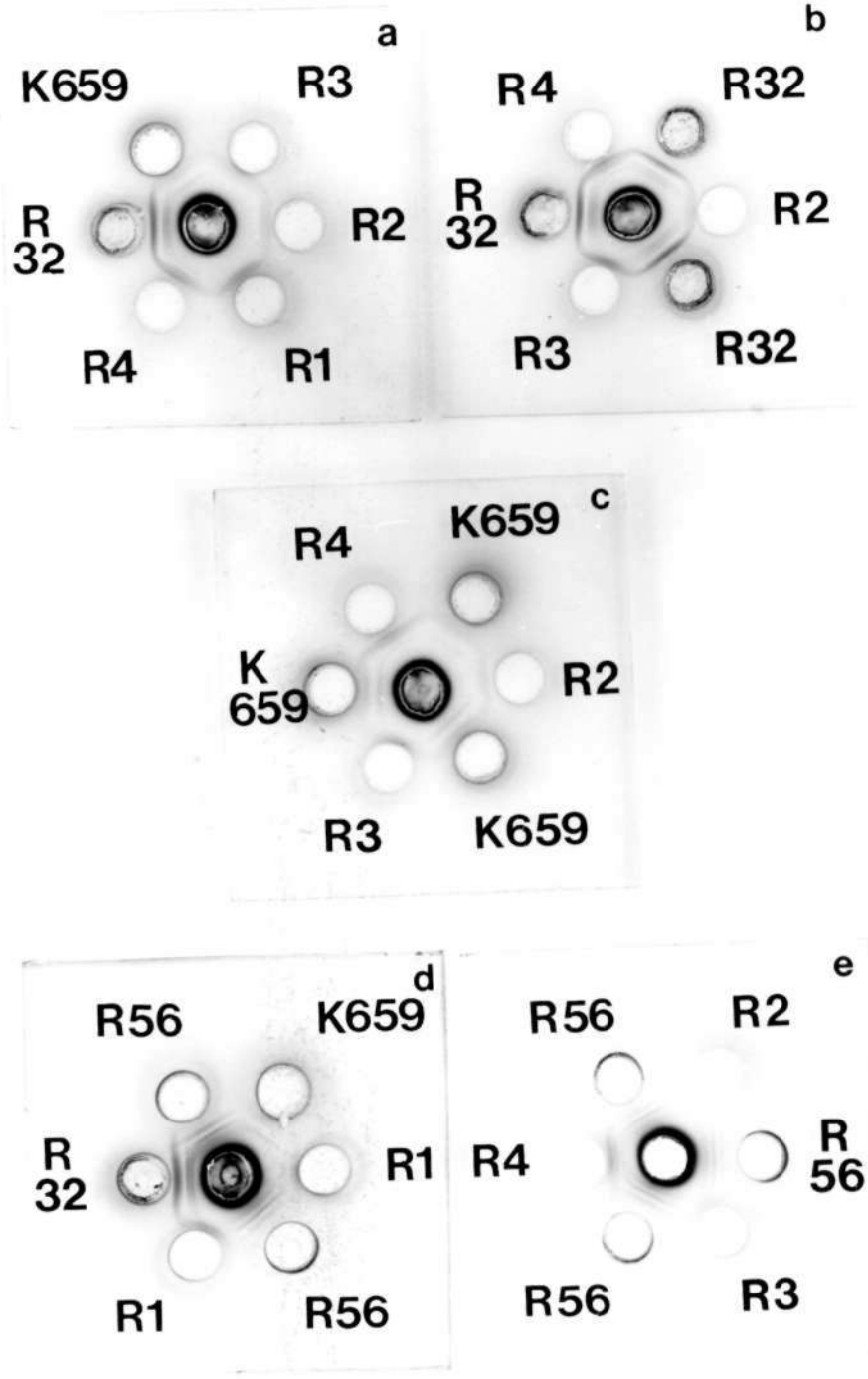
FIG. 82-a. Gel diffusion slide for vaccinated rabbit sera R1-R4; R.32 and bovine K.659. Centre well contained R. sanguineus CA. Note precipitin lines of identity.

FIG. 82-b. Gel diffusion slide for vaccinated rabbits sera R2, R3, R4, and R.32. Centre well contained R. sanguineus CA. Note precipitin lines of identity.

FIG. 82-c. Gel diffusion slide for vaccinated rabbit sera R2, R3, R4 and bovine K659. Centre well contained R. sanguineus CA. Note precipitin lines of identity.

FIG. 82-d. Gel diffusion slide for vaccinated rabbits sera R1, R32, R56 and K549. Centre well contained R. sanguineus CA. Note precipitin lines on non-identity.

FIG. 82-e. Gel diffusion slide for vaccinated rabbits R2, R3, R4 and R56. Centre well contained R. sanguineus CA. Note precipitin lines of partial identity.


a
K659
R3
R
32
R2
R4
R1
b
R4
R32
R
32
R2
R3
R32
c
R4
K659
K
659
R2
R3
K659
d
R56
K659
R
32
R1
R1
R56
e
R56
R2
R4
R
56
R56
R3

of R_1-4 could cross react with the positive controls as shown in Figs 82b, c, d and e. R.56 (B. microplus gut) consistently failed to produce heavy precipitation lines. At least three other less heavy lines were present against R.32 (B. microplus haemolymph), two of which showed identity with R_1-4 (Figs. 82a,b & d).

Fig. 83a^1-e^1 is identical to Fig. 82 a-e, except that the centre wells contain B. microplus CA. The haemolymph proteins again showed two heavy lines which were shared with R. sanguineus vaccinated rabbits R_1-4 (Fig. 83 b^1, c^1, d^1 and e^1). At least one conspicuous cross over (spur) line indicating non-identity or only partial identity was observed (Figs. 82 d & e; Figs. 83 d^1 & e^1).

Immunodiffusion tests with prevaccination sera of R.32, R.56 and K.659 against the R. sanguineus CA, showed no precipitation lines; also B. microplus CA, when reacted against prevaccination sera of R_1-4 showed no lines.

9.4.3 Effects of Immunisation on Tick Attachment and Duration of Feeding

All the adult ticks on the control rabbit were attached by 2 days post infestation and increasing in sizes. There was little or no signs of reaction on the rabbit. On the other hand, within 12 hours of infesting the vaccinated rabbit with adults, signs of itching and restlessness became apparent but this subsided quickly, but by day 2 only two female ticks had settled down completely. On the 3rd day the rest of the adult ticks became attached but did not appear to be feeding well when examined on day 5. A large pox-like swelling became visible under the mouthparts of the attached ticks (Fig. 84). Two females (each 3.5 mm in length) from the vaccinated and control rabbits were sampled on day 5 and processed for

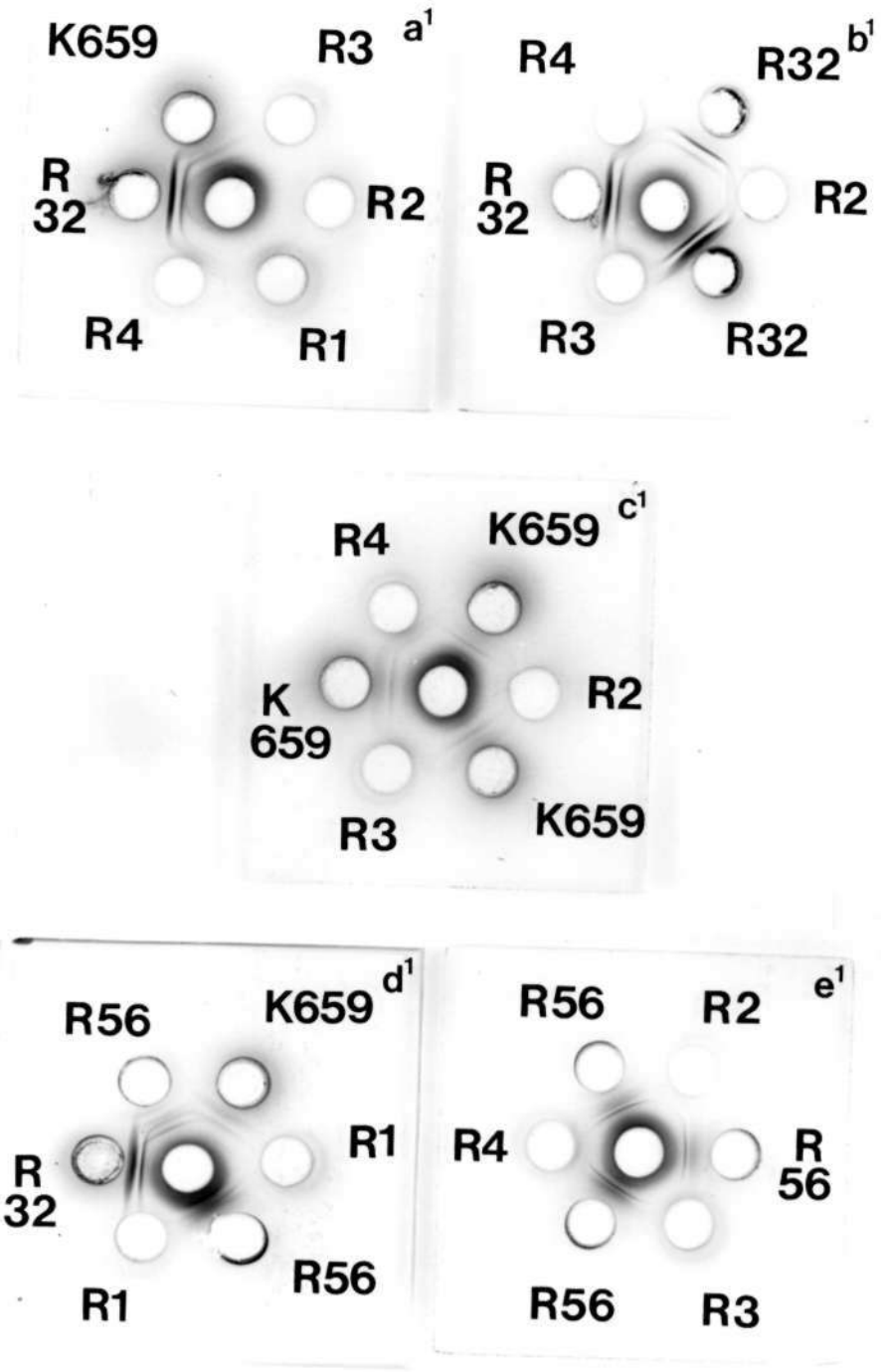
FIG. 83-a[1]. Gel diffusion slide for vaccinated rabbit sera R1-R4; R32 and bovine K.659. Centre well contained B. microplus CA. Note precipitin lines of identity.

FIG. 83-b[1]. Gel diffusion slide for vaccinated rabbit sera R2, R3, R4 and R32. Centre well contained B. microplus CA. Note precipitin lines of partial identity.

FIG. 83-c[1]. Gel diffusion slide for vaccinated rabbit sera R2, R3, R4 and K659. Centre well contained B. microplus CA. Note precipitin lines of partial identity.

FIG. 83-d[1]. Gel diffusion slide for vaccinated rabbit sera R1, R32, R56 and K659. Centre well contained B. microplus CA. Note precipitin lines of non-identity.

FIG. 83-e[1]. Gel diffusion slide for vaccinated rabbit sera R2, R3, R4 and R56. Centre well contained B. microplus CA. Note precipitin lines of partial identity.


K659
R3
a1
R
32
R2
R4
R1
R4
R32
b1
R
32
R2
R3
R32
R4
K659
c1
K
659
R2
R3
K659
R56
K659
d1
R
32
R1
R1
R56
R56
R2
e1
R4
R
56
R56
R3

methacrylate embedding as already described in Chapter 2. This size was approximately the size of ticks collected and processed for CA preparation. Histology of the gut in these ticks is shown later in Figs. 85, 86 & 87.

Females on the control rabbit took 7-9 days to complete engorgement. Six fully engorged females were collected by day 7 and two not so fully engorged (7.00 mm in length) by day 9 (two were taken earlier for histology)(Table 12). These two (7 mm females) weighed less than the engorged females but still laid a large number of eggs. No engorged females dropped from the vaccinated rabbit. Only two females fed to the semi-engorged state (5 mm in length) (Fig. 84). It was observed that several adult males feeding on the vaccinated rabbit died prematurely. The two females that reached the semi-engorged state became discoloured (pale brown to ash) and finally died in situ before dropping.

The response to larvae was different. Engorged larvae started dropping from the control rabbits by day 4 and finished by day 7. There was up to 24 h delay before engorged larvae started dropping from the vaccinated rabbits and they had finished by day 9. There was no visible reaction on the ears of both the vaccinated and control rabbits. The larvae recovered were weighed (Table 10) and although there appeared to be a difference between the group mean weights of the larvae from the control rabbits compared to the larvae from the vaccinated rabbits, this was not statistically significant ($t = 0.67$) (Table 11). The larvae from the vaccinated rabbits were also bright red as opposed to the dark red to brown colour of the larvae from the control rabbits. Calculation of moulting time (up to 50% moult) gave a maximum delay (R_3 & R_4) of only 36h.

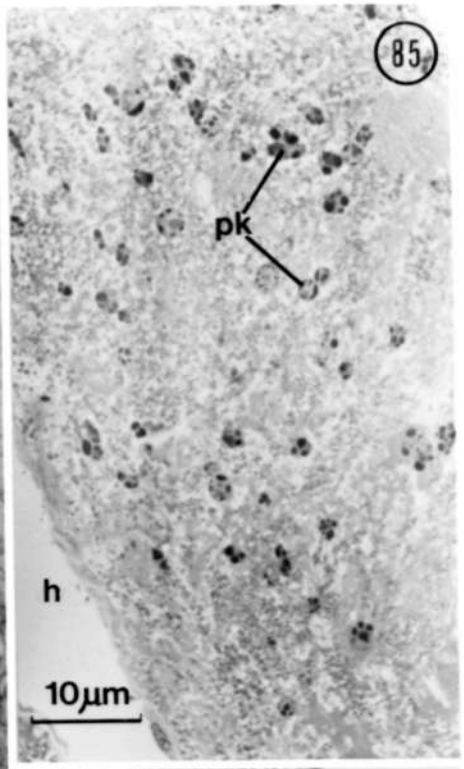
FIG. 84. Ear of vaccinated rabbit with pox-like swellings at the attachment sites of R. sanguineus females (arrowed).

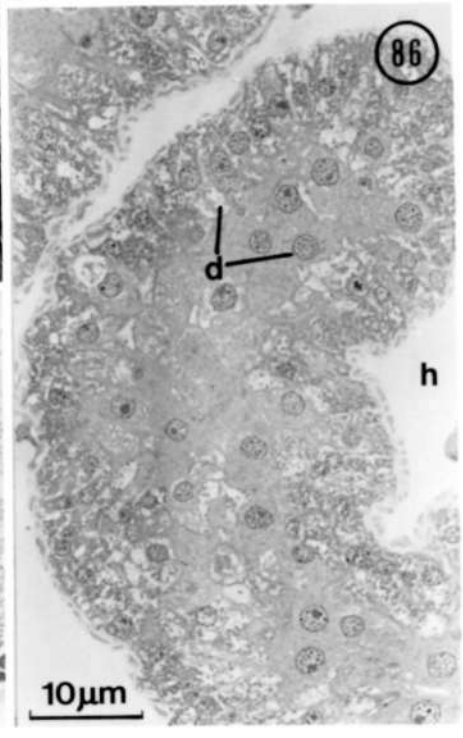
FIG. 85. Gut caecum of female R. sanguineus after 5 days on vaccinated rabbit; pyknotic nuclei of damaged digest cells, pk; haemolymph space,h. (Methacrylate section, H & TB-P).

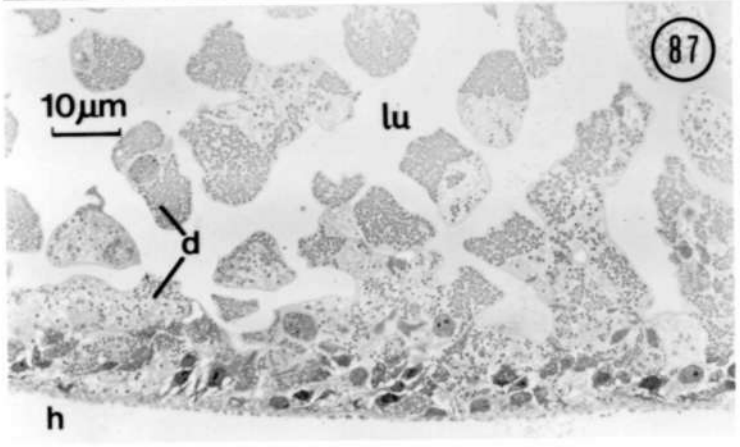
FIG. 86. Gut caecum of female R. sanguineus after 5 days on vaccinated rabbit; relatively normal digest cells, d; haemolymph space, h.

FIG. 87. Gut caecum of female R. sanguineus after 5 days on control rabbit; normal digest cells, d; lumen, lu; haemolymph space h.


84


85
pk
h
10µm


86
d
h
10µm


87
10µm
lu
d
h

There was no correlation between the intensity of the immunoprecipitation lines and the response of the rabbits to the challenge with the larvae. Also vaccinate 1 produced less heavy lines but was apparently strongly resistant as it killed most of the challenge adult ticks.

As no engorged adults were obtained from vaccinate 1, a comparison of the weights of engorged females could not be done (Table 12), and the effects of immunisation on egg production could not be investigated. However, the females processed for methacrylate embedding showed gut damage in the ticks from the vaccinated rabbit. This damage was not as extensive as already reported for B. microplus (Chapter 8) but gut damage was evident from the disrupted gut cells lining the basal lamina and a large number of the digest cells showing pyknotic nuclei (Figs. 85 & 86). Ticks from the control rabbit were normal (Fig. 87).

9.5 DISCUSSION

The immunogen used in this vaccination was a crude extract (CA) of partially fed, R. sanguineus females, and thus contained antigens from the gut, haemolymph and other internal organs. Many of these antigens may give rise to weak immunological reactions in the host or non-protective ones.

Trager (1939a,b), Boese (1969,1974) and McGowan et al. (1980) have reported that ticks fed on animals immunised with tick antigens or subjected to previous tick infestation often failed to engorge and suffered a reduction in engorgement weights or egg mass weights. Other studies indicated that inflammatory cells infiltrated the bite lesion, presumably stimulated by chemo-attractants (Berenberg, Ward & Sonnenshine, 1972); and others suggested that antigen/antibody complexes specific for

salivary antigens occurred at the bite site (Gregson, 1970; Kirkland, 1971; Allen, Khalil & Wikel, 1979). Rapid collagen deposition, constriction of blood vessels and proliferation of epidermis have been suggested as a possible mechanism for "walling off" the ticks mouthparts, thereby reducing the size and quality of the blood meal consumed (reviewed by Tatchell and Moorhouse, 1968; Tatchell, 1969; Arthur, 1970; Nelson et al., 1977). Still others have demonstrated strong allergic reactions against tick antigens (Riek, 1958,1962; Boese, 1974; Willadsen et al., 1978; McGowan et al., 1980).

The rabbit experiment with R. sanguineus has also demonstrated that more than one reaction is important in the vaccination model. First there was the intense host reaction to adults producing a pox-like lesion, presumably infiltrated by inflammatory cells. Second serological analysis demonstrated that immunisation of these rabbits induced specific antibody formation and when the ticks fed, their gut cells became damaged. The precipitation lines on vaccinate 1 was not as heavy as others suggesting a low concentration of specific antibody. The fact that less heavy immunoprecipitation lines were observed on this vaccinate 1 (which killed most of the attached ticks) is not unusual. Willadsen et al. (1978) measured antibody to a purified tick antigen by indirect haemagglutination, but its concentration did not correlate with the degree of immunity of the animal, and infact, higher titres tended to be found in cattle of low immunity. The most protective antibodies cannot always be demonstrated by immunoprecipitation.

Attempts to demonstrate immunoprecipitation lines with serum from the rabbit repeatedly challenged with R. sanguineus ticks (RIP) failed, which suggested that vaccination using tick antigens generated a different response than the challenge from repeated tick infestations.

One explanation for the successful vaccination against adult R. sanguineus is that ingested antibodies, probably specific for digestive tract antigens, resulted in alterations in the gut epithelium (Fig. 85). Other tissues such as salivary glands, seemed unaffected. This is similar to the results obtained in vaccination studies using B. microplus on cattle (Chapter 8). It was reasonable to expect that the early skin reactions were against salivary antigens injected into the host during feeding, since the vaccination CA could also contain salivary gland proteins. Alternatively antigens may be shared between different tick organs because in other host-tick reactions, immunofluorescence staining with sera from rabbits infested with both H. anatolicum and R. sanguineus showed antigens in the gut system as well as the salivary glands (Kohler, Hoffmann, Hochner & Weiland, 1967).

The importance of the antigens from gut and other tissues was reflected in the apparent success of immunisation of guinea pigs and calves using partially fed but not unfed adults of D. andersoni (Allen & Hymphreys, 1979). They suggested that feeding stimulated developmental and presumably antigenic changes in the gut and reproductive tissues of the feeding tick. The histology of the gut in B. microplus (Chapter 3) has shown this to be true, as many new cells and new cell types such as secretory cells develop as the tick feeds.

The number of antigens involved in the immune reaction in any one host-parasite system is unknown. In this experiment four immuno-precipitation lines were clearly demonstrated when the CA of R. sanguineus was reacted against homologous antisera. Heavy precipitation lines were also produced by R.32 (B. microplus haemolymph) and these showed identity with R_{1-4} lines. It would appear from this discussion that at least three

mechanisms could operate against blood sucking arthropods on vaccinated hosts:

(1) Gut damage

(2) Salivary gland antigen neutralisation and lesion formation

(3) Increased access of antibodies to target antigens within the parasite as a result of gut leakage.

A significant finding in this experiment is the presence of shared antigens between *B. microplus* and *R. sanguineus* (Figs. 82a-e). Confirmation of shared antigens is shown in Fig. 83b^{1}-e^{1} since *B. microplus* CA was placed in all the centre wells. The significance of these shared antigens is unknown. It can be suggested that if the shared antigens were protective, vaccination against, say *B. microplus*, could provide protection against *R. sanguineus* and *vice versa*. This implication for vaccination against ticks warrants further study.

Adult males feeding on vaccinate 1 died prematurely. Similar results were observed in the *B. microplus*:cattle system where ticks were placed on rings on both vaccinated and control cattle and feeding behaviour and deaths recorded (Kemp *et al.*, submitted for publication). Histological sections of these ticks showed that the gut of these males had whole blood with a large concentration of leukocytes after 24 h of feeding on the host but the females had a more homogeneous mixture devoid of identifiable red and white blood cells. This subject has been dealt with in detail in Chapter 8. Similar findings regarding dead males had been reported for *A. maculatum* by McGowan *et al.* (1980) but ticks with damaged gut were not recorded. It is possible that the deaths of these male ticks prior to mating could have been responsible for the failure of female ticks to become replete and produce eggs.

The results presented in this experiment showed that rabbits were a valuable model for studying tick:host interactions as they were capable of mounting effective immune responses following vaccination. Rabbits are also cheaper than cattle and more can be used for each experiment. The studies also showed that at least one antigen:antibody mechanism was similar to the B. microplus; cattle vaccinations, that is the mechanism leading to gut damage.

TABLE 10. TOTAL LARVAE COLLECTED AND THE GROUP WEIGHTS.

	Control 1	Control 3	Control 4	Vac 2	Vac 3	Vac 4
Total fed larvae collected from 100 infested	54	59	84	65	39	28
Group mean weight in µg	386	454	418	409	371	307

TABLE 11 INDIVIDUAL WEIGHTS OF LARVAE IN µgs

	Controls	Vaccinates
Mean weight of individually weighed larvae in µg	437.12	420.555
Standard deviation of individually weighed larvae	41.446	26.711
"t" test; 38 df	t = 0.67 no significant difference	

TABLE 12. INDIVIDUAL WEIGHTS IN MGS OF ENGORGED FEMALES FROM CONTROL RABBIT NO. 2.

224.2

256.0

261.0

233.0

235.5

251.9

(Table 12 Contd.)

Semi-engorged females from control 2 weight (mg) ≈ 7.0 mm in length.

111.0

132.0

9.6 SUMMARY

Rabbits were vaccinated against R. sanguineus to test the suitability of the laboratory model for tick:host vaccination studies. The crude antigen (CA) was a preparation from partially fed female ticks. The protein concentration of the supernatant was 7.6 mg/ml.

Gel diffusion experiments showed four precipitin lines in reactions between CA and vaccinated sera. Some of the antigens were apparently shared between B. microplus and R. sanguineus.

Vaccination of rabbits against R. sanguineus had only a minor effect on the feeding duration of larvae, but adults were unable to feed and grow on the vaccinated animal.

Histology showed that gut cells of female ticks were damaged as in B. microplus studies.

The laboratory model was therefore shown to give comparable results to B. microplus experiments on cattle and at much less expense.

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