STUDIES OF *ANAPLASMA MARGINALE* THEILER IN NYMPHAL *DERMACENTOR ANDERSONI* STILES

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NYMPHAL _DERMACENTOR ANDERSONI_ STILES

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CHAPTER I

INTRODUCTION

Bovine Anaplasmosis

Anaplasmosis is a major disease of cattle that may cause severe anemia and death in the bovine host. Black-tailed deer may have inapparent or carrier infections, and thus serve as important reservoirs for this disease.[1] The disease is prevalent worldwide, especially in tropical and subtropical areas,[2,3] and is a significant contributor to economic losses in the cattle industry. In the United States, anaplasmosis has been ranked as one of the most important diseases of cattle.[4] The causative agent, *Anaplasma marginale* Theiler, was first observed by Smith and Kilborne who thought the inclusion body in the red blood cells of cattle was a stage in the life cycle of *Babesia bigemina*.[5] Later, in 1910, this parasite was recognized as a separate disease-causing organism by Theiler.[6]

A. marginale is an obligate, intracellular parasite that is morphologically similar in bovine erythrocytes to other rickettsiae[7] and is presently classified in the Order Rickettsiales, Family Anaplasmataceae.[3] The genus *Anaplasma* contains four species, all infective for ruminants. *Anaplasma marginale* is the most pathogenic species for cattle, and *A. centrale* causes a mild disease in African cattle. *Anaplasma ovis* causes a mild infection in sheep and goats.[7] A new species, *A. mesaeterum*, has recently been reported in sheep.[8]

The parasite in bovine erythrocytes is characterized by the presence of inclusion or marginal bodies at the periphery of the host erythrocyte;[2] the name "marginale" refers to this location.[9] The erythrocytic inclusion vacuole contains one to several initial bodies.[10] It has been suggested that these initial bodies resemble reticulated forms, which are a developmental stage of the parasite seen in ticks; ticks serve as invertebrate host and vector for the parasite.[11] The organism contains both DNA and RNA and possesses a branching filamentous network in the cytoplasm.[2,10,12] Each initial body is surrounded by a double membranous organismic envelope consisting of a thick outer membrane and a thin inner cell membrane.[10,13-15] The inclusion membrane of the vacuole is thought to originate from the plasmalemma of the host erythrocyte.[16,17] Small electron-dense particles have also been observed in association with initial bodies.[10] Some organisms have tail-like inclusion appendages which are visible only after lysing infected erythrocytes.[10,18-23] These appendages appear in loop, comet, or dumbbell forms that display longitudinal and transverse periodicity.[10,18,21]

A hypothesis for the developmental cycle of _A. marginale_ within the bovine erythrocyte has been proposed.[24] The initial body, which has been thought to be the infective unit, enters the erythrocyte by invagination of the plasma membrane and forms a membrane-bound vacuole.[12] The initial body then multiplies by binary fission to produce more initial bodies which eventually leave the cell by exocytosis to infect other erythrocytes.[24] During exocytosis the erythrocyte is not lysed by the parasite.[25]

Ticks as Vectors of Disease

Disease transmission by ticks was first demonstrated in 1893 by

Smith and Kilborne in their studies of the cause of Texas cattle fever.[5] Since then, ticks have been recognized as both vectors and reservoirs of a variety of disease-causing organisms including rickettsiae, bacteria, spirochetes, viruses, and protozoa.[26,27] Ticks appear to be better adapted than insects for the development of rickettsiae and viruses because rickettsiae and tick-borne viruses do not multiply in bloodsucking Diptera, whereas many dipteran-borne viruses are capable of development in ticks.[28] Ticks also make good reservoirs of these disease agents because the organism can remain dormant in the tick for long periods and sometimes can be transmitted to their offspring via the egg for a number of generations.[29]

Tick-borne diseases affect human beings and many other animals and cause significant economic losses to the livestock industry throughout the world. The most important tick-transmitted blood parasites affecting cattle are Cowdria ruminantium (heartwater disease), Babesia bovis (babesiosis), Theileria parva (East Coast fever), and Anaplasma marginale (anaplasmosis).[30]

Ticks have several characteristics that enable them to be good disease transmitters. They remain infectious with disease organisms over long periods of time due to their ability to overwinter and withstand prolonged starvation.[28] Ticks, especially argasids and three-host ixodids, frequently feed on more than one vertebrate host.[26,27] Disease organisms can be passed from one instar to the next in the arthropod vector, known as transstadial transmission, thus making it possible for both immature and adult stages to transmit disease. Transovarial transmission, the transfer of disease organisms within the arthropod vector to its offspring via the egg, is also important.[26,28] Finally, some disease

organisms can undergo cyclical changes as well as multiplication within the arthropod vector, a process known as cyclic propagation.[26]

Ticks become infected with pathogens by feeding on the blood of an infected host.[29] Concentration of infectious organisms in the host's blood and an extended feeding time enhance the ability of ticks to acquire infection.[28,29,31] The cutting mouthparts, or chelicerae, are used to penetrate the host's skin, and the heavily spined hypostome serves to anchor the tick while it feeds. A cementing substance is also secreted to aid in attachment. Ixodid ticks are slow feeders and remain attached for several days, thus allowing for long exposure to pathogenic organisms. Blood and tissue fluids are ingested and stored in the diverticulae of the midgut until uptake by digestive cells occurs. The feeding process is also aided by salivary secretions which inhibit blood coagulation in the feeding lesion and aid in the destruction of surrounding tissues.[32]

The peritrophic membrane, which is a membrane that forms in the midgut region of many bloodsucking arthropods to surround ingested food, is only known to be present in one species of tick.[33] Because most ticks lack a peritrophic membrane, parasites are able to penetrate the gut cells readily.[28] Extensive pinocytotic activity, which is thought to occur during digestion, allows penetration into the gut cells where multiplication of the pathogen frequently takes place. Some pathogens may migrate to the hemocoel where they multiply in the hemolymph, a process demonstrated for rickettsiae, piroplasms, and spirochetes.[28] Hemocytes are thought to aid in the dissemination of parasites to the salivary glands, ovaries, and various other organs.[29,31]

During the life cycle of ticks organs that harbor microorganisms, such as the midgut and Malpighian tubules, are gradually replaced, thus aiding in the survival of pathogens.[28,29] Transstadial transmission is enhanced in this way because the process of metamorphosis primarily affects ectodermally derived tissues.[29] Salivary glands completely degenerate during molting, so that pathogens must reinvade this organ when it develops again.[28,29]

Pathogens can be transmitted both mechanically and biologically by ticks. Mechanical transmission occurs when the vector transports parasites from host to host usually by mouthparts that have become contaminated with blood.[34] Argasids and many male ixodid ticks are good mechanical transmitters of disease because they are capable of feeding on numerous hosts.[29,35] Biological transmission occurs when the tick serves a necessary function in the life cycle of the pathogen.[34]

Transstadial and transovarial transmission, which are considered to be types of biological transmission, are the most common routes for passage of disease agents from one tick stage to another.[35] These modes of transmission, while common in ticks, are rare in bloodsucking insects.[28] Rickettsia rickettsii and other spotted fever group rickettsiae can be passed transovarially to 100% of the offspring if ticks feed during the peak of infection.[36-38] Both transstadial and transovarial transmission can be important for the maintenance of certain rickettsiae and other pathogens in nature.[31,37,39] Sexual transmission by infected male ticks to noninfected females occurs occasionally with viruses[40] and rickettsiae.[41,42] Most infectious agents transmitted by ticks cause disease in the vertebrate host and appear to be nonpathogenic to ticks.[39] Symbiotic rickettsiae have also been reported in many species of ticks and are

passed transovarially from one generation of ticks to another. They have not been associated with any known disease in vertebrates.[28]

The mechanism of infection of vertebrates with organisms transmitted from ticks may be varied. Transmission by salivary secretions during the feeding process is the most common form of transmission,[27-29] and Babesia and Theileria are transmitted in this manner.[28] Fecal contamination of the bite wound is also an effective mechanism, especially for the transmission of rickettsiae.[27,29,43,44] Feces containing Coxiella burneti can remain infective for over a year, thus increasing the possibility of transmission.[43] Regurgitation of gut contents is thought to occur with the transmission of Cowdria ruminantium because this parasite has not been found to pass into the hemocoel of the tick.[27,35,44] Some pathogens, such as spirochetes and viruses may be transmitted by contamination of the bite by coxal fluid excreted during the process of feeding by argasid ticks.[29,45] Contact of body fluids from crushed ticks with abraded skin or the accidental ingestion of an infected tick may likewise contribute to transmission;[28,44,46] this is known to occur with Rickettsia rickettsii.[44,46]

Transmission of disease organisms may be affected by changes in temperature. Low ambient temperatures can inhibit the transmission of Babesia to cattle.[47,48] Placement of ticks at elevated temperatures experimentally has been shown to influence the rate of salivary gland infection with Theileria parva as well as to stimulate the development of infective stages of this parasite.[49,50] Infective stages of Theileria annulata and Babesia bovis in the salivary glands of ticks can also be produced by incubation of unfed ticks at 37 C.[51,52] Avirulent Rickettsia rickettsii

can be reactivated, i.e., stimulated to become virulent, by incubation at 37 C.[53,54]

The processes of tick feeding and molting may also influence the transmission of disease agents. Development of mature infective forms of *Theileria parva* in salivary glands has been shown to be synchronized with the feeding process of the tick, with mature infective stages being most numerous from 4 to 5 days after attachment.[55] Infective forms of *Babesia bigemina* have been demonstrated to develop only in prefed ticks.[56] Increased virulence of *R. rickettsii* is also stimulated by tick feeding.[54] The development in the gut of stages of *Theileria* capable of invading the salivary glands has been demonstrated to be coordinated with the process of tick molting.[57] The virulence of *R. rickettsii* has been shown to decrease immediately after molting, suggesting that molting hormones play a role in modifying virulence.[29,54,58]

Anaplasma marginale in Arthropods

Various arthropods have been shown in field and laboratory studies to be possible vectors of *A. marginale*. Transmission by these vectors can occur by either mechanical or biological means.

Mechanical transfer is an important mechanism of transmission of *A. marginale*. Mechanical transmission of this disease agent can occur via contaminated surgical instruments, such as dehorners and syringe needles.[59] This disease organism is also frequently spread by the contaminated mouthparts of hematophagous arthropods. Horseflies (*Tabanus* sp.), considered to be the major insects to vector anaplasmosis,[60-62] have been shown under experimental conditions to be capable of transmitting infection for up to two hours after feeding on an infected animal, and

at times they appear to be the primary vectors of this disease.[61] Stableflies (Stomoxys sp.),[63] mosquitoes (primarily Psorophora sp.),[60,64] and deerflies (Chrysops sp.) have also been implicated as mechanical vectors.[65] Only female horseflies, deerflies, and mosquitoes feed on blood, and therefore only females are capable of mechanical transmission. However, male and female stableflies are bloodfeeders, and thus both are capable of mechanical transmission.[46]

Numerous species of ixodid ticks have been demonstrated experimentally to be capable to biological transmission of A. marginale, and ticks appear to be the only proven biological vectors.[1,59,66,67] Because both male and female ticks are bloodfeeders, either sex can transmit infection.[68] Successful transfer of A. marginale by male ticks suggests that males, with their capability of feeding on multiple hosts, may play an important role in transmission of this pathogen under natural conditions.[69,70] Dermacentor andersoni and D. occidentalis have been shown to transmit anaplasmosis in both laboratory and field studies, and are considered to be the most important vectors of this disease in the western United States.[67,69-73] Dermacentor occidentalis is recognized to be important in transferring infections from black-tailed deer to cattle.[74] Other vectors in the United States include D. variabilis and D. albipictus.[1,69,75] In tropical and subtropical regions, Boophilus annulatus and B. microplus are also important vectors.[2,76]

Transstadial transmission of A. marginale by ticks, including Dermacentor sp., has been demonstrated in several investigations.[69,70,75,77-79] Inoculation of susceptible animals with a homogenate of guts from adult ticks infected as nymphs has also produced infections.[78,79] Successful

transovarial transmission was described in one instance,[80] but could not be duplicated by other researchers.[69,70,79]

Inoculation studies have shown that transmission of A. marginale by ticks can be influenced by temperature or by the process of feeding. Gut homogenates collected from infected ticks that had been incubated at 37 C or from ticks fed for a period of 6 days prior to gut removal produced shorter prepatent periods when inoculated into susceptible calves than those from unfed adult ticks.[79] Incubation of ticks for 2.5 days at 37 C and subsequent inoculation of their gut homogenates into susceptible animals has also been shown to produce shorter prepatent periods.[81] Both feeding and incubation stimulate an increase in tick body temperatures which most likely cause changes in metabolic processes. Thus, the development of A. marginale within the tick may be influenced by an increase in its own metabolism or that of the tick due to elevated temperatures.[79]

The level of parasitemia of donor cattle has been shown to influence A. marginale transmission. Gut homogenates from ticks fed on calves with high parasitemias resulted in shorter prepatent periods when inoculated into susceptible animals than comparable homogenates from ticks fed on calves with low parasitemias.[82] Also, transmission by ticks fed on a carrier animal with an undetectable parasitemia resulted in long prepatent periods.[79]

To detect the presence of A. marginale in D. andersoni, the fluorescent-antibody (FA) technique has been used. Anaplasma marginale, including forms with tail-like appendages, has been found in smears of the gut contents and excreta of adult ticks.[83] Organisms have also been observed in the gut contents and Malpighian tubules of nymphs for short periods after detachment.[84,85] Recently, antibody fluorescent objects were

observed in tissue sections of gut and salivary glands from feeding adult ticks[78] and in the hemocytes of incubated and feeding adults[86] suggesting the presence of A. marginale. Fluorescence was also demonstrated in gut tissue from incubated adult ticks that were known to harbor colonies of A. marginale.[87]

Other labeled antibody studies have been used to identify the presence of A. marginale in Dermacentor species. The peroxidase-antiperoxidase (PAP) technique has been employed to label organisms within colonies from the gut tissues of incubated adult ticks.[88] Ferritin-labeled antibody has also been used to demonstrate the presence of organisms in gut homogenates from feeding adult ticks.[89]

Morphology of Anaplasma marginale in Ticks

The ultrastructure of A. marginale has been described in midgut epithelial cells of adult D. andersoni infected as nymphs, and in replete nymphal ticks. Individual organisms similar to those in infected bovine erythrocytes were observed in gut tissues of replete nymphs, feeding adult ticks, and unfed, unincubated adult ticks.[90] Colonies of A. marginale, in which organisms were enclosed by a limiting membrane, have been demonstrated in unfed, incubated adult D. andersoni and the morphologic characteristics of these colonies described.[91] Small electron-dense particles were observed to be associated with individual organisms within colonies.[90] Incubation of unfed adult ticks at 37 C was found to maximize colony development.[90,92] Permitting ticks to feed on infected calves with high parasitemias also produced larger numbers of colonies than when ticks fed on calves with few parasites in the peripheral blood.[82] The identity of organisms within colonies has been verified by fluores-

cent-antibody (FA)[87] and peroxidase-antiperoxidase (PAP) techniques.[88] Anaplasma marginale has not been observed in salivary glands by electron microscopy,[90] but salivary gland homogenates have been shown to produce infections when inoculated into susceptible cattle.[78]

Colonies of A. marginale have been described in D. andersoni[90,91] and in Rhipicephalus simus.[93] The colonies were most commonly observed adjacent to the basement membrane in midgut epithelial cells. Five morphologic types categorized at the light microscopy level appear to represent stages in a developmental sequence. Type 1 colonies were round with densely packed organisms and contained little empty space. Type 2 colonies were similar except that an open area or halo separated the compact group of organisms from the colony's limiting membrane. In type 3 colonies the organisms were arranged in clumps with open spaces between. Individual organisms were difficult to distinguish in these colonies using light microscopy. The type 4 colonies contained many individual organisms. The colony shape was often irregular and the size frequently much larger than types 1, 2, or 3. Finally, type 5 colonies contained fewer but larger individual organisms than type 4 and more empty space was present; the colony shape was also frequently irregular.[91]

Different forms of A. marginale have been observed within colonies by electron microscopy: electron-dense forms, reticulated forms, pleomorphic reticulated forms, and small particles. These forms were similar in some respects to stages described in the developmental cycle of Chlamydia. Electron-dense forms were present in type 1 and type 2 colonies. The type 3 colony contained both electron-dense and reticulated forms. Type 4 and type 5 colonies contained only reticulated forms. A developmental sequence from electron-dense to reticulated forms has been

suggested. Pleomorphic reticulated forms were only present in type 5 colonies, and small particles were observed in colony types 3, 4, and 5.[91]

The different morphologic forms of *A. marginale* observed in colonies from adult ticks appear to resemble developmental stages of *Chlamydia*. In the chlamydial life cycle a small electron-dense elementary body which is the infective stage enters the cell and reorganizes through an intermediate dispersing form into a larger reticulated body. Reticulated bodies are noninfective and divide by binary fission. Reorganization of reticulated bodies into elementary bodies takes place through intermediate condensing forms. Chlamydiae also form membrane-bound colonies[94] similar to those of *A. marginale*. However, it is not known at present whether the morphologic forms found in chlamydiae and those seen in *A. marginale* are related.

Determining the number of colonies of *A. marginale* per unit area of gut tissue is useful for comparing the gut tissues from different groups of ticks. The mean colony density, which is the number of colonies per 0.1 mm^2 gut tissue examined, was described for unfed, incubated *D. andersoni* adults. Incubation at 37 C for 2.5 days produced the highest mean colony density. Although all colony types were present, the type 3 colony was the most abundant.[92] In *Rhipicephalus simus* adults, the colony density was higher for unfed, unincubated ticks than in unfed, incubated or prefed ticks; this was different from the results found for *D. andersoni*.[93]

The Research Problem

Although *A. marginale* has been under investigation for many years, the life cycle of this organism in the bovine host and in tick vectors

has yet to be described fully. Further knowledge of the developmental cycle of *A. marginale* would aid in attempts to control this important disease organism. The purpose of the present study was to determine the time when feeding nymphal *D. andersoni* become infected with *A. marginale* and also to study the development of colonies of *A. marginale* in feeding and replete nymphal ticks that had fed on an infected calf.

CHAPTER II

MATERIALS AND METHODS

Experimental Animals

Ten splenectomized dairy calves (4, 9 to 10 months old, and 6, 2 to 3 months old), that tested negative with the anaplasmosis complement-fixation (CF) test[a], were used for the present study. One donor calf was infected with the Virginia isolate of *Anaplasma marginale* by adult feeding *Dermacentor andersoni* that acquired infection as nymphs. When the donor calf developed a parasitemia of 3 to 5 percent, it was used as a source of infection for the feeding of nymphal *D. andersoni*. The remaining 9 calves were used for transfer, animal inoculation, and control studies. All calves were tested twice each week for infections with *A. marginale* by examination of Wright's-stained blood smears and by measurement of packed cell volume (PCV). Once marginal bodies were observed in the red blood cells calves were monitored daily. Infected animals were treated with 11 mg of oxytetracycline/kg of body weight once the parasitemia levels began to decrease, but none were treated while ticks were feeding. Calves that did not become infected were challenged exposed with infected blood from a known carrier cow between 120 and 140 days after tick feeding. During tick feeding, animals were confined to individual stalls equipped with head stanchions. The stalls were located in a building maintained at a temperature of 25 C.

Laboratory Propagation and Infection of Ticks

Dermacentor andersoni nymphs used in these experiments were raised by the Entomology Tick Laboratory of Oklahoma State University.[95] Larval ticks were fed on rabbits to facilitate development to the nymphal stage. Approximately 1300 nymphs were placed in orthopedic stockinettes glued to the sides of a calf infected with *A. marginale* when the parasitemia level was 3 to 5%. Replete nymphs were collected and stored in a humidity chamber until used for experiments. The humidity chamber was maintained at 90 to 98% relative humidity at 25 C with a 14 hour photophase period.

Tissue Collection and Electron Microscopy

Gut homogenates consisting of 50 unfed, incubated adult ticks from each transfer group were prepared. The gut tissues were dissected from each tick, pooled in RPMI 1640[b] medium, and placed in a ground glass tissue homogenizer to be homogenized for inoculation.

Gut tissues from the feeding and replete nymphs and adults were collected and processed for electron microscopy. Gut tissue was dissected free of the body cavity and fixed immediately in chilled 2.0% glutaraldehyde buffered with 0.27 M sodium cacodylate and post-fixed in 2.0% osmium tetroxide, also buffered with 0.27 M cacodylate. The fixed tissue was washed several times in the buffer, dehydrated with a graded series of ethanols (50, 70, 90, 95, and 100%), and infiltrated with Dow Epoxy Resin 736 (DER) utilizing propylene oxide as the intermediate solvent. Thick (1.5 μm) and thin (silver-reflective) sections of embedded tissue were cut with an ultramicrotome.[c] Thick sections were stained with

Mallory's stain[96] and thin sections were stained with uranyl acetate and lead citrate.[97] Thin sections were prepared with a diamond knife,[d] collected on 300 mesh copper grids, and examined and photographed in an electron microscope[e] operated at 60 kilovolts.

Typing and Measurement of Colonies and Statistical Analysis

Thick sections of gut tissue were examined by light microscopy for the presence of colonies of _A. marginale_. The number of colonies per unit area of gut tissue examined was determined. Colony areas were computed from measurements of the width and length of each colony that were measured with a calibrated ocular micrometer. Colonies in adult ticks were assigned a type number (1 to 5) on the basis of morphologic characteristics as described by Kocan et al.[91] The data were analyzed statistically.

Experimental Design

Study of Transferred Nymphal Ticks

Approximately 600 unfed nymphal _D. andersoni_ were placed in an orthopedic stockinette attached to the donor calf infected with _A. marginale_. Nymphs that had not attached by 48 hours were removed and discarded. At each day of feeding, approximately 75 attached nymphs were removed from the infected calf and transferred to an uninfected (CF negative) calf and permitted to finish feeding. A separate stockinette for each transfer day (6 days altogether) was provided on uninfected calves. When nymphs had fed to repletion, they were collected and stored in a

humidity chamber and allowed to molt to the adult stage. At one month post-molting, the adults were used for histologic examination and animal inoculation.

The adult ticks were incubated for 2.5 days at 37 C to maximize colony development.[90] Gut tissues were dissected from 10 male and 10 female ticks for each day that nymphal transfers were made; the tissues were processed for electron microscopy as described previously. Gut homogenates were prepared from 50 adult ticks representing each of the six transfer days and material representing each day was inoculated into one of six susceptible calves. The calves were monitored for patent infections as described previously (Figures 1 and 2).

Study of Feeding Nymphal Ticks

Approximately 300 unfed nymphal D. andersoni were placed in an orthopedic stockinette attached to the donor calf infected with A. marginale. Unattached ticks were removed after 48 hours. Ten nymphs were removed from the calf on each day of feeding, and unfed nymphs and replete nymphs from the sixth day of feeding were also collected. Control tissues from nymphs that fed on an uninfected calf were likewise collected on each day of feeding. Gut tissues were dissected from each tick and processed individually for electron microscopy. The gut tissue from each tick was sectioned and examined by light microscopy (Figure 3).

Study of Replete Nymphal Ticks

Approximately 400 unfed nymphal D. andersoni were placed in an orthopedic stockinette attached to the donor calf infected with A. marginale. Unattached ticks were removed after 48 hours. The nymphs were

Figure 1. Experimental design for study of transferred nymphal ticks. Experimental Study 1.

600 nymphs placed on calf infected with *Anaplasma marginale* at 3 to 5% parasitemia

↓

At each day of feeding 75 nymphs removed from infected calf and transferred to uninfected calf to allow completion of feeding

Transfer of Nymphal Ticks

Day of Feeding on Infected Calf	Transfer	Uninfected Calf & Cell No.
(1) 1 Day	→	Calf 1 - Cell 1
(2) 2 Day	→	Calf 1 - Cell 2
(3) 3 Day	→	Calf 1 - Cell 3
(4) 4 Day	→	Calf 2 - Cell 4
(5) 5 Day	→	Calf 2 - Cell 5
(6) 6 Day	→	Calf 2 - Cell 6

↓

Replete nymphs collected from uninfected calves and allowed to molt to adults; at 1 month post-molting: (a) collected 10 males and 10 females from each transfer day, gut tissue dissected from each tick and processed individually for electron microscopy; (b) a gut homogenate of 50 ticks prepared for each transfer day to inoculate 6 susceptible calves

Tissue Collected, Examined, or Used as Inoculum

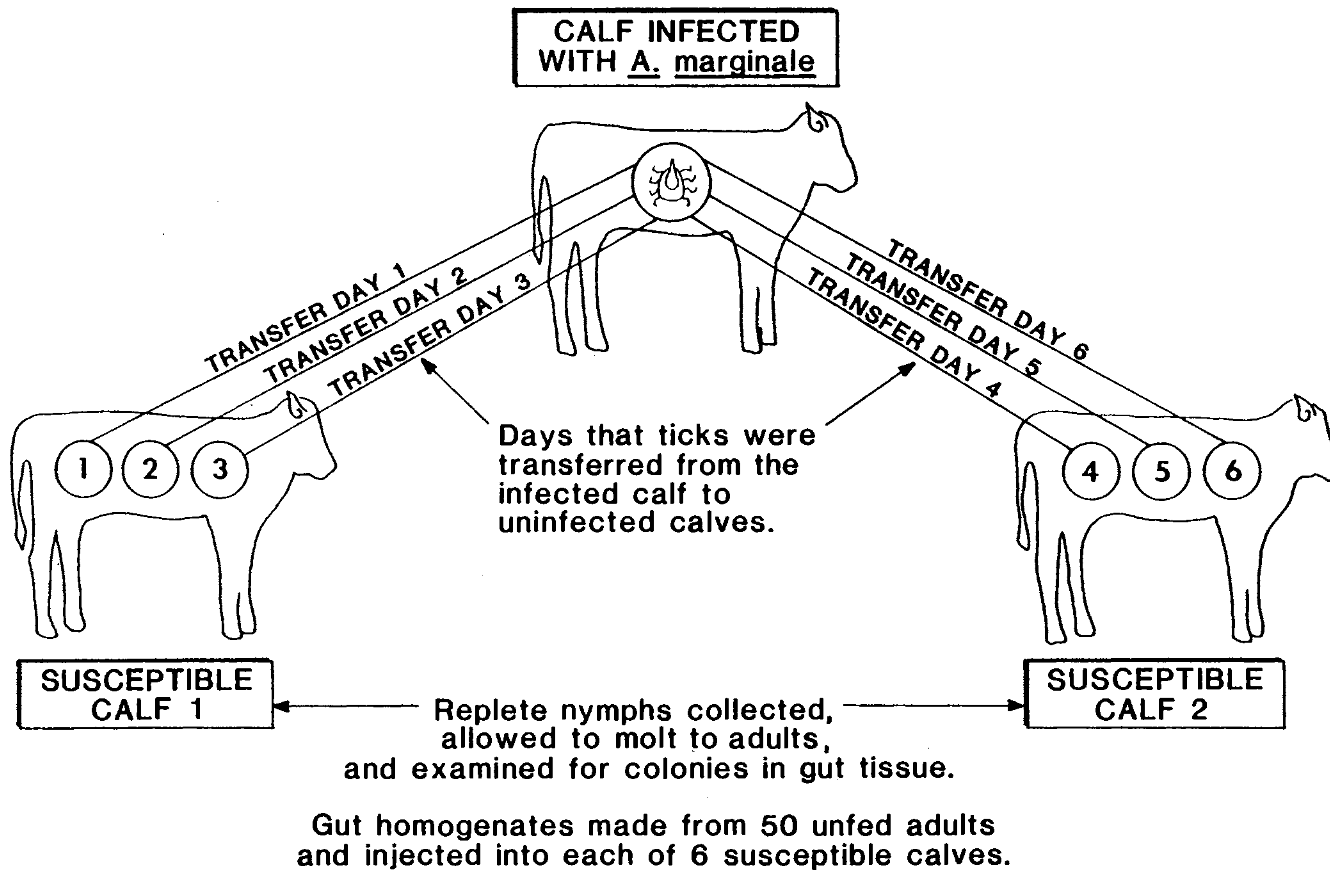
Nymphal Transfer Day	No. of Ticks Collected	Calf Inoculated With Gut Homogenate
(1) Day 1	10 males, 10 females	Calf 3
(2) Day 2	10 males, 10 females	Calf 4
(3) Day 3	10 males, 10 females	Calf 5
(4) Day 4	10 males, 10 females	Calf 6
(5) Day 5	10 males, 10 females	Calf 7
(6) Day 6	10 males, 10 females	Calf 8

↓

Gut tissue from each of the 120 ticks sectioned and examined individually by light microscopy

Calves monitored for infection

Figure 2. Experimental design for nymphal transfer studies. Experimental Study 1. Transfer of nymphs at each day of feeding from calf infected with *Anaplasma marginale* to susceptible calves.



CALF INFECTED WITH A. marginale
TRANSFER DAY 1
TRANSFER DAY 2
TRANSFER DAY 3
TRANSFER DAY 4
TRANSFER DAY 5
TRANSFER DAY 6
1
2
3
4
5
6
Days that ticks were transferred from the infected calf to uninfected calves.
SUSCEPTIBLE CALF 1
SUSCEPTIBLE CALF 2
Replete nymphs collected, allowed to molt to adults, and examined for colonies in gut tissue.
Gut homogenates made from 50 unfed adults and injected into each of 6 susceptible calves.

Figure 3. Experimental design for study of feeding nymphal ticks. Experimental Study 2.

300 nymphs placed on calf infected with *Anaplasma marginale* at 3 to 5% parasitemia

↓

At each day of feeding 10 nymphs removed from calf, gut tissue dissected from each tick and processed individually for electron microscopy (6 days required to complete feeding)

Tissue Collected & Examined

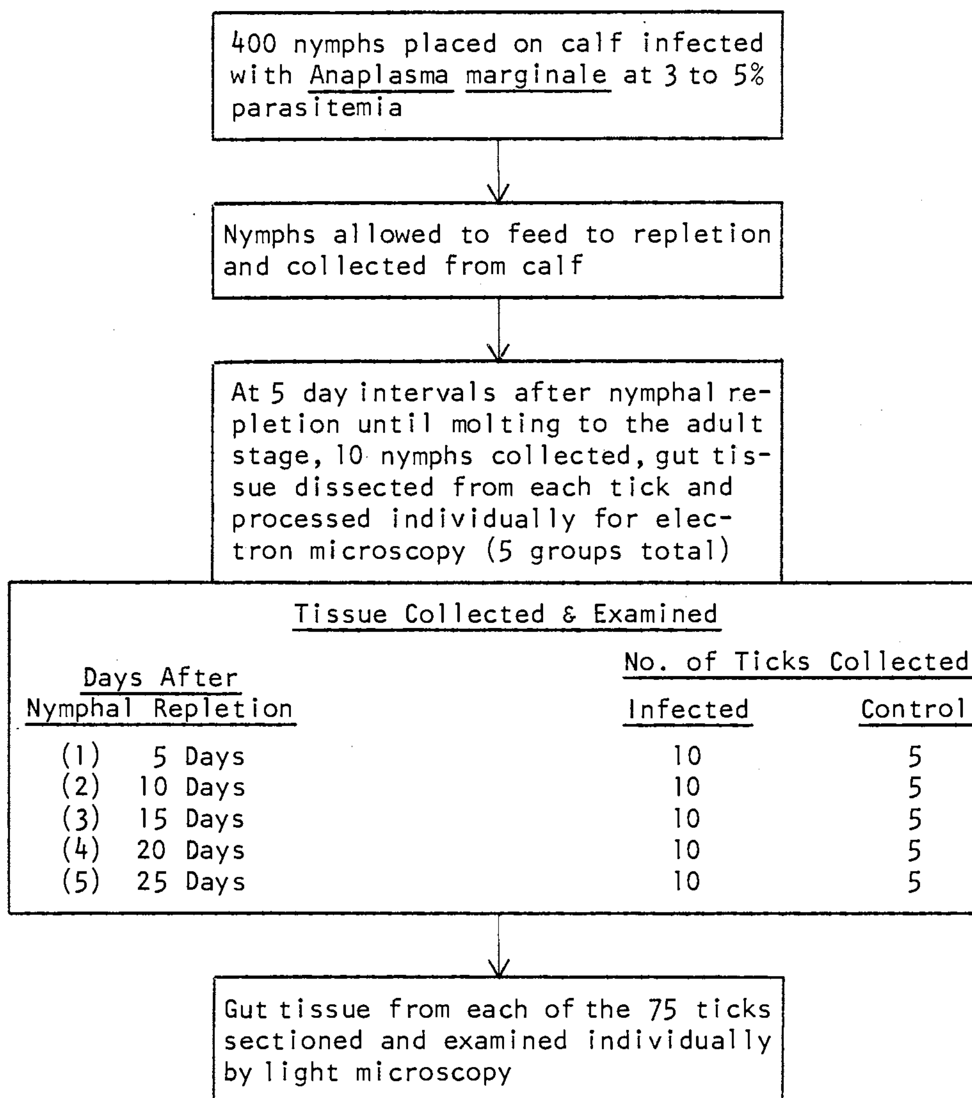
	Nymphal Days of Feeding	No. of Ticks Collected	
		Infected	Control
(1)	0 Days (Unfed Nymphs)	10	5
(2)	1 Day	10	5
(3)	2 Days	10	5
(4)	3 Days	10	5
(5)	4 Days	10	5
(6)	5 Days	10	5
(7)	6 Days	10	5
(8)	Replete Nymphs (From Day 6)	10	5

↓

Gut tissue from each of the 120 ticks sectioned and examined individually by light microscopy

allowed to feed to repletion and were collected from the calf and maintained in a humidity chamber. At five day intervals (5 to 25 days post-repletion, a total of 5 groups) ten ticks were selected at random from among the replete nymphs for histologic examination. Control tissues from nymphs that fed on an uninfected calf were also collected for each of the 5 post-repletion days. The gut tissues were dissected from each tick and processed individually for electron microscopy. The gut tissues were sectioned and examined by light and electron microscopy as described previously (Figure 4).

Figure 4. Experimental design for study of replete nymphal ticks. Experimental Study 3.


400 nymphs placed on calf infected with Anaplasma marginale at 3 to 5% parasitemia
Nymphs allowed to feed to repletion and collected from calf
At 5 day intervals after nymphal repletion until molting to the adult stage, 10 nymphs collected, gut tissue dissected from each tick and processed individually for electron microscopy (5 groups total)
Tissue Collected & Examined
Days After Nymphal Repletion
No. of Ticks Collected
Infected
Control
(1) 5 Days 10 5
(2) 10 Days 10 5
(3) 15 Days 10 5
(4) 20 Days 10 5
(5) 25 Days 10 5
Gut tissue from each of the 75 ticks sectioned and examined individually by light microscopy

NOTES

[a]CF test done by the Oklahoma Animal Disease Diagnostic Laboratory, Stillwater, Oklahoma.

[b]Gibco Laboratories, Grand Island, New York.

[c]Sorvall MT-5000, Sorvall Inc., Newton, Connecticut.

[d]DuPont Diamond Knife, DuPont Instruments, Wilmington, Delaware.

[e]Philips EM-200, Philips Electronic Instruments, Mount Vernon, New York.

CHAPTER III

RESULTS

Transfer of Feeding Nymphal Ticks From Infected to Susceptible Calves

Light and Electron Microscopy

Colonies of *Anaplasma marginale* were observed in cross-sections of gut tissue from one month old adult *Dermacentor andersoni* that fed for 3 to 6 days as nymphs on an infected calf. Colonies of *Anaplasma* organisms were similar to those previously described in adult ticks[91] and consisted of organisms surrounded by a limiting membrane (Figures 5a and b). All five morphologic types (1 to 5) were observed with light microscopy in Mallory's stained gut tissue. However, most colonies observed (89%) were type 3 colonies (Figures 5 and 6).

An electron micrograph of a type 3 colony (Figure 6a) that was observed in gut tissue from transfer day 6 contained aggregates of organisms. Forms of *A. marginale* found in type 3 colonies included electron-dense forms, reticulated forms, and small particles (Figure 6b).

Tick Transmission and Inoculation With Tick Gut Homogenates

Parasitemia of Donor Calf During Tick Feeding. The *A. marginale* parasitemias of the donor calf during tick feeding are listed in Table I.

Figure 5. Light micrographs of type 3 colonies of *A. marginale* in the midgut epithelial cells of an adult female *D. andersoni* from transfer day 6. The colonies (C) contain individual organisms surrounded by a limiting membrane. (a) x 1900; (b) x 1900.

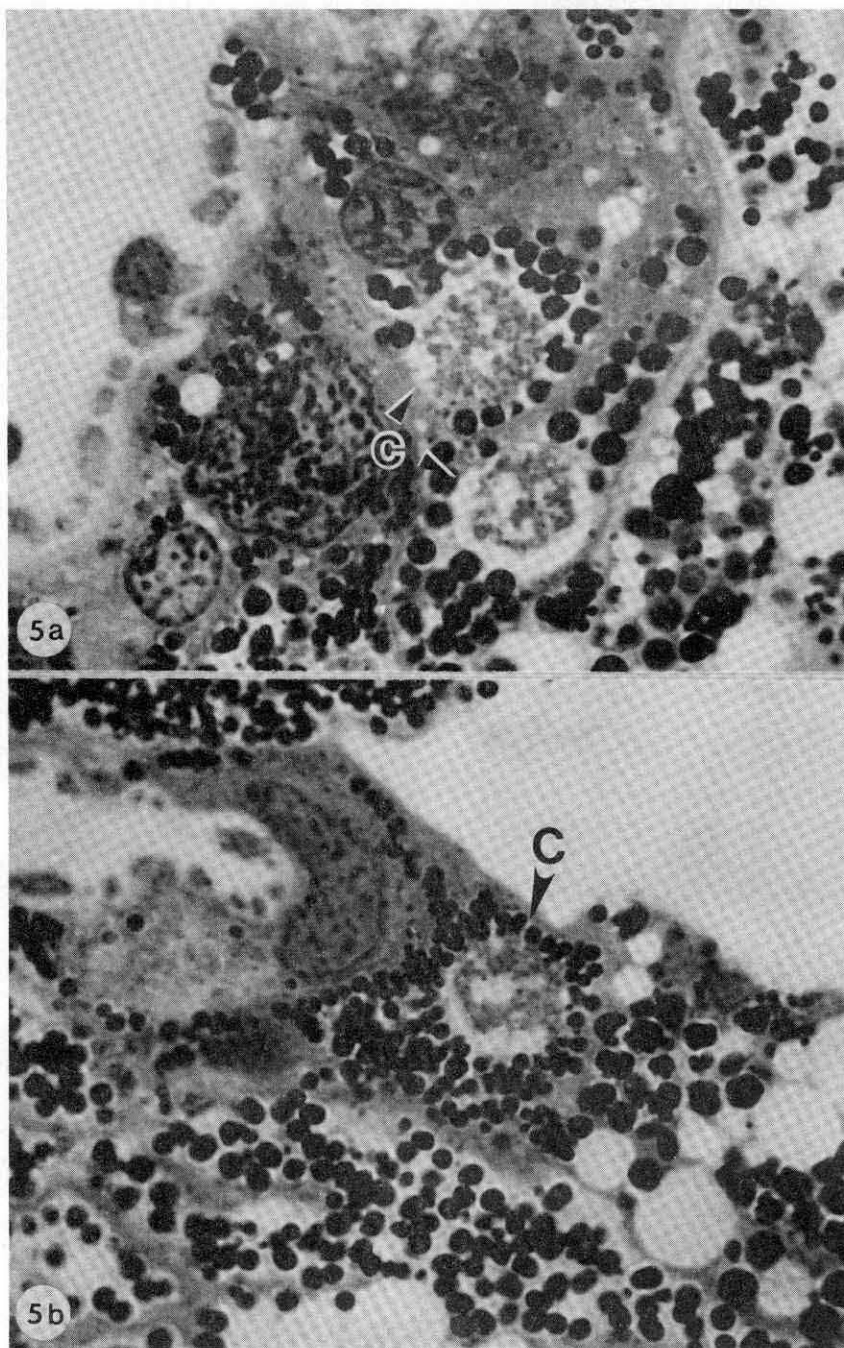
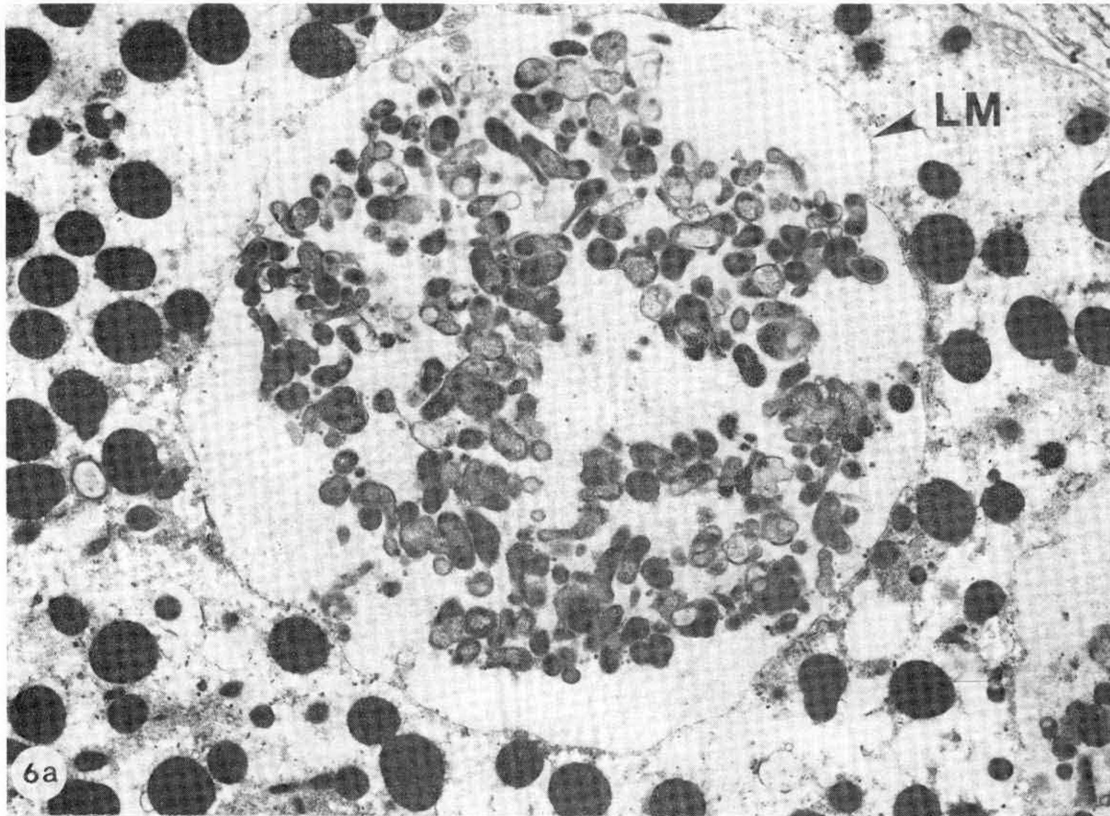

5a
C
5b
C

Figure 6. Electron micrographs of a type 3 colony of *A. marginale* in the midgut epithelial cells of an adult female *D. andersoni* from transfer day 6.

(a) Aggregates of organisms are seen surrounded by a limiting membrane (LM); x 9600.

(b) Forms of *A. marginale* include electron-dense forms (EF), reticulated forms (RF), and small particles (P); x 20,600.


LM
6a

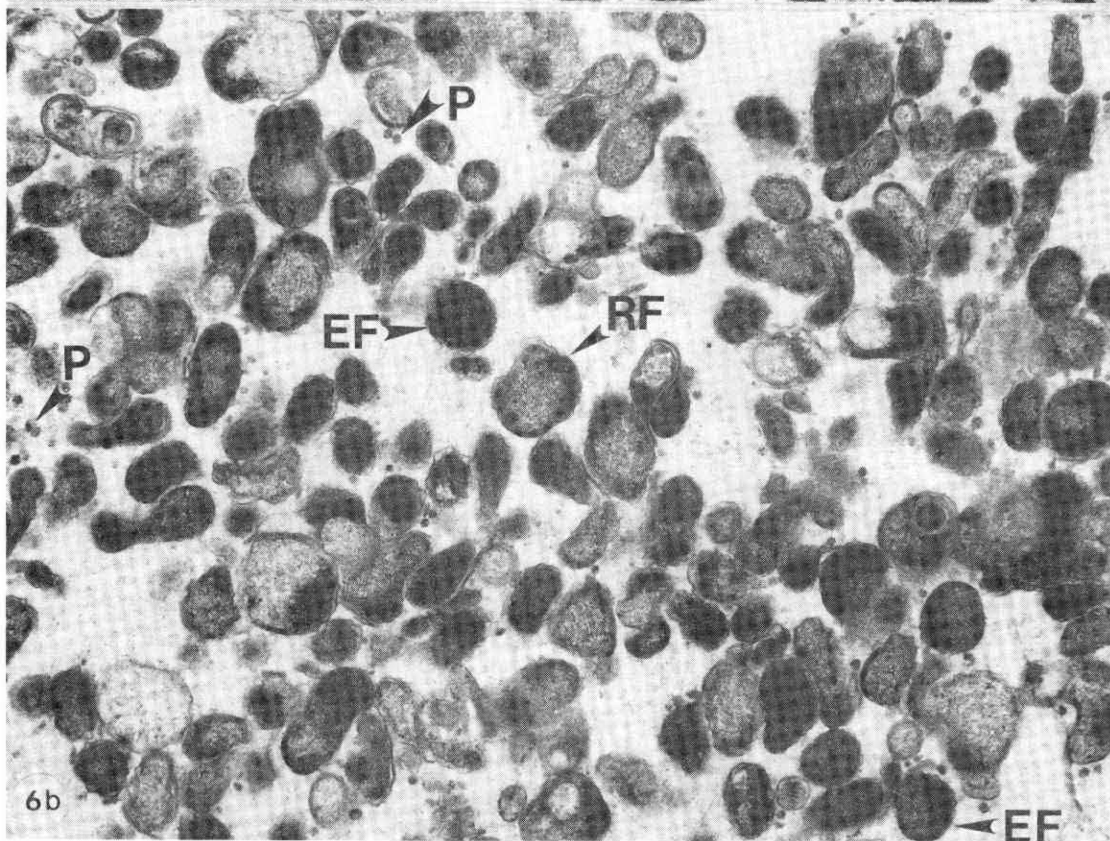

P
EF
RF
P
6b
EF

TABLE I

ANAPLASMA MARGINALE PARASITEMIAS IN DONOR CALF 386 DURING TICK FEEDING

	Day of Tick Feeding								
	0	1	2	3	4	5	6	7	8
Percent Parasitemia	4.4	9.8	15.8	27.2	37.8	48.2	45.6	26.4	26.4

The highest parasitemias of the donor calf coincided with the last two days of nymphal feeding (days 5 and 6).

Transmission of *Anaplasma marginale* to Susceptible Transfer Calves by Feeding Ticks. The susceptible calf on which the transfer nymphs 5 and 6 completed feeding developed anaplasmosis after 153 days (Table II). The calf to which ticks were transferred after feeding 1 through 4 days on an infected calf did not develop anaplasmosis. This calf was challenged exposed with infected blood 120 days after tick feeding and proved to be susceptible.

Transmission of *Anaplasma marginale* to Susceptible Calves by Injection of Tick Gut Homogenates. Gut homogenates made from adult *D. andersoni* that were allowed to feed on an infected calf for 2 to 6 days as nymphs produced anaplasmosis when inoculated into susceptible calves (Table III). Shortest prepatent periods resulted from inoculation of homogenates from adult ticks transferred after 5 and 6 days of feeding as nymphs on an infected calf. Transfer day 6 inoculum produced an infection with the shortest prepatent period of 23 days, which was 10 to 15 days shorter than the other prepatent periods observed. One exception was transfer day 4 inoculum that produced an infection with a prepatent period of 63 days. With the exception of transfer day 4, the prepatent periods decreased from transfer day 2 to day 6. Peak parasitemias observed in inoculated calves varied, and did not appear to be related to the number of days of nymphal feeding.

Statistical Analysis of Colony Density

Mean colony densities (number of colonies per 0.1 mm^2 gut tissue

TABLE II

TRANSMISSION OF <u>ANAPLASMA MARGINALE</u> TO SPLENECTOMIZED CALVES BY NYMPHAL <u>DERMACENTOR ANDERSONI</u> THAT WERE TRANSFERRED TO SUSCEPTIBLE CALVES AFTER FEEDING FOR 1 TO 6 DAYS ON A CALF INFECTED WITH <u>A. MARGINALE</u>

		Appearance of Anaplasmosis in Calves	
Calf No.	Transfer Days	Prepatent Period (Days)	Peak Parasitemia (Percent)
394*	1-4	---	---
392	5-6	153	15.2

*Calf subsequently challenged exposed and found to be susceptible.

TABLE III

TRANSMISSION OF *ANAPLASMA MARGINALE* TO SPLENECTOMIZED CALVES BY INJECTION OF GUT HOMOGENATES FROM 50 UNFED, ADULT *DERMACENTOR ANDERSONI* THAT WERE ALLOWED TO FEED AS NYMPHS FOR 1 TO 6 DAYS ON A CALF INFECTED WITH *A. MARGINALE*

		Appearance of Anaplasmosis in Calves	
Transfer Day	Calf No.	Prepatent Period (Days)	Peak Parasitemia (Percent)
T1	RH-9*	---	---
T2	RH-10	38	18.0
T3	RH-11	37	41.4
T4	RH-12	63	44.6
T5	RH-13	34	50.0
T6	409	23	43.8

*Calf subsequently challenged exposed and found to be susceptible.

examined) in midgut epithelial cells of adult ticks infected as nymphs are listed in Table IV. Colonies were first seen in tissue sections of gut from transfer day 3 ticks. Mean colony densities for the combined groups of males and females increased from 0.01 for transfer day 3 to 0.73 for day 6. In transfer day 3 and 4 gut tissue, males had a higher mean colony density; but in transfer day 5 and 6 gut tissue, females had a higher mean colony density. The high mean colony density in females from transfer day 6 was due to a large number of colonies found in one individual. Colonies were seen in 13 of 20 gut cross-sections examined for transfer day 5 and were present in 17 of 20 for day 6. However, only one colony was seen in gut tissue from transfer day 3 ticks and only 6 of 20 individual ticks contained colonies from transfer day 4. The value for the standard error of the mean (SEM) was higher for female ticks in transfer day 6 than for the other transfer day groups.

A graph of the mean colony densities for the combined tick groups (males and females) along with the average prepatent periods produced by injection of gut homogenates from ticks from each transfer day into susceptible calves is displayed in Figure 7. The mean colony density increased as the nymphs fed for longer periods on an infected calf. The transfer day 6 group had the highest mean colony density. The prepatent periods decreased as the colony densities increased except for transfer day 4 which exhibited an unusually long prepatent period. The shortest prepatent period was exhibited by ticks removed on day 6 as did the highest mean colony density. Although a calf inoculated with a gut homogenate from transfer day 2 ticks developed anaplasmosis, no colonies of *A. marginale* were observed in transfer day 2 tissue sections.

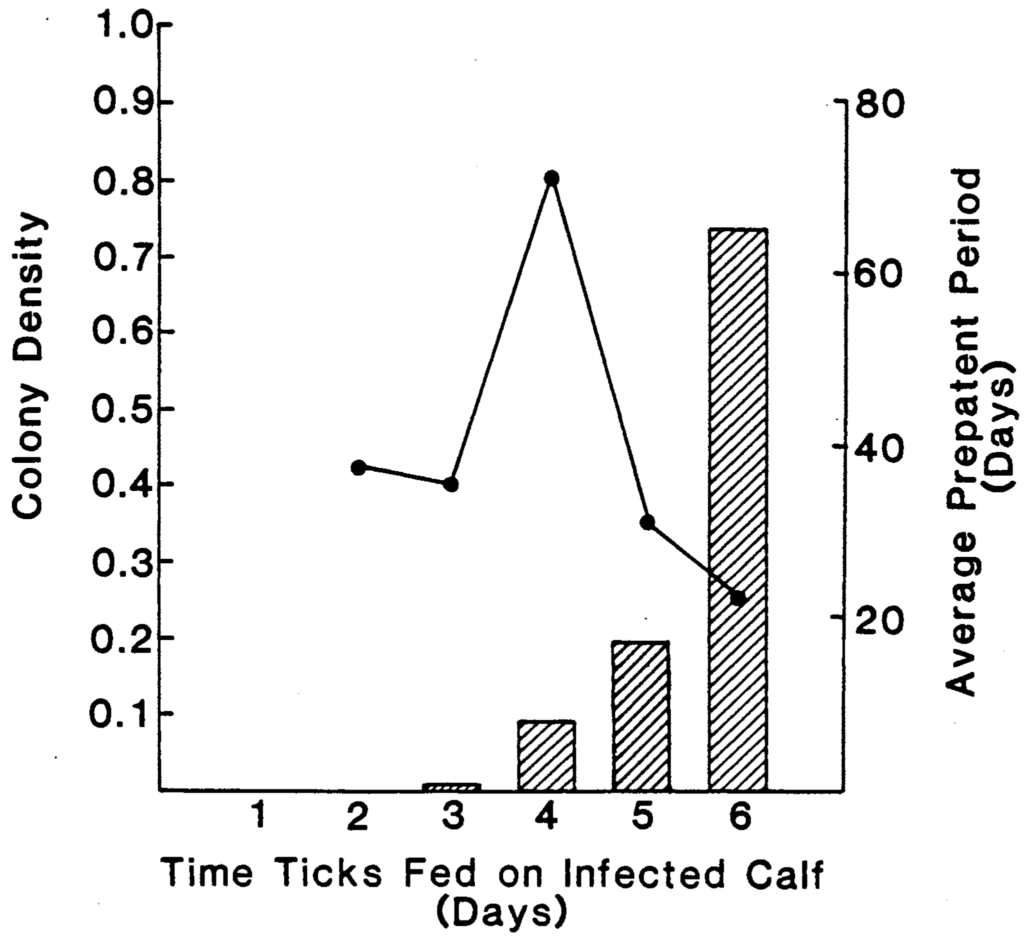
TABLE IV

MEAN COLONY DENSITY* IN ADULT *DERMACENTOR ANDERSONI* THAT WERE ALLOWED TO FEED AS NYMPHS FOR 1 TO 6 DAYS ON A CALF INFECTED WITH *ANAPLASMA MARGINALE*

Transfer Day	Sex	No. of Ticks Per Group	Mean Colony Density	Range	SEM
T1	Male	10	0	---	
	Female	10	0	---	
	Combined	20	0	---	
T2	Male	10	0	---	
	Female	10	0	---	
	Combined	20	0	---	
T3	Male	10	0.01	0.00-0.13	0.01
	Female	10	0	---	
	Combined	20	0.01	0.00-0.13	
T4	Male	10	0.12	0.00-0.60	0.07
	Female	10	0.06	0.00-0.47	0.05
	Combined	20	0.09	0.00-0.60	
T5	Male	10	0.13	0.00-0.63	0.06
	Female	10	0.26	0.00-0.95	0.09
	Combined	20	0.20	0.00-0.95	
T6	Male	10	0.29	0.00-0.86	0.09
	Female	10	1.17	0.00-5.83	0.54
	Combined	20	0.73	0.00-5.83	

*Mean colony density (number of colonies per 0.1 mm^2 gut tissue examined).

Figure 7. Comparison of the mean colony density (number of colonies per 0.1 mm^2 gut tissue examined) from the combined groups of male and female *D. andersoni* that were allowed to feed as nymphs for 1 to 6 days on a calf infected with *A. marginale* with the average prepatent period produced by gut homogenates injected into uninfected calves.


Colony Density
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
80
60
40
20
Average Prepatent Period
(Days)
1
2
3
4
5
6
Time Ticks Fed on Infected Calf
(Days)

The analysis of variance table of the mean colony densities for transferred nymphal ticks is depicted in Table V. The interaction between sex and transfer day was not significant. Hence, the difference in mean colony densities between sexes was not significant. However, the difference in mean colony densities among transfer days was found to be statistically significant ($P < 0.05$). Mean colony densities increased with longer feeding time to 0.73 by day 6. In a pairwise comparison of the mean differences, the mean colony density for transfer day 6 was significantly higher ($P < 0.01$) than the colony densities for days 3, 4, and 5; but the means for days 3, 4, and 5 did not differ significantly from each other.

Feeding Nymphal Ticks

The increasing size of nymphal ticks on each day of feeding is illustrated in Figure 8. Colonies of *A. marginale* were not observed in the cross-sections of gut tissue from infected or control nymphal ticks on days 1 to 6 of feeding. However, groups of rickettsia-like organisms, believed to be symbiotes, were seen in approximately half of the cross-sections from day 3 through day 6 of feeding, as well as in nymphs that were replete on day 6 (Figure 9a). Similar aggregates of organisms were seen in control tissues from feeding nymphs (Figure 9b). Electron microscopy confirmed the rickettsial nature of the organisms (Figure 10). The large organisms had two cell membranes surrounding a filamentous cytoplasm that contained dense granular material.

TABLE V

ANALYSIS OF VARIANCE OF THE NUMBER OF COLONIES PER 0.1 MM^2 GUT TISSUE EXAMINED FROM ADULT *DERMACENTOR ANDERSONI* THAT WERE ALLOWED TO FEED AS NYMPHS FOR 1 TO 6 DAYS ON A CALF INFECTED WITH *ANAPLASMA MARGINALE*

Source of Variation	DF	Sum of Squares	Mean Square	F	P Value
Total	79	0.39	---	---	---
Transfer Day	3	0.06	0.020	5.3	0.003
Sex	1	0.01	0.010	2.8	0.099
Transfer Day X Sex	3	0.03	0.010	2.4	0.073
Error	72	0.29	0.004	---	---

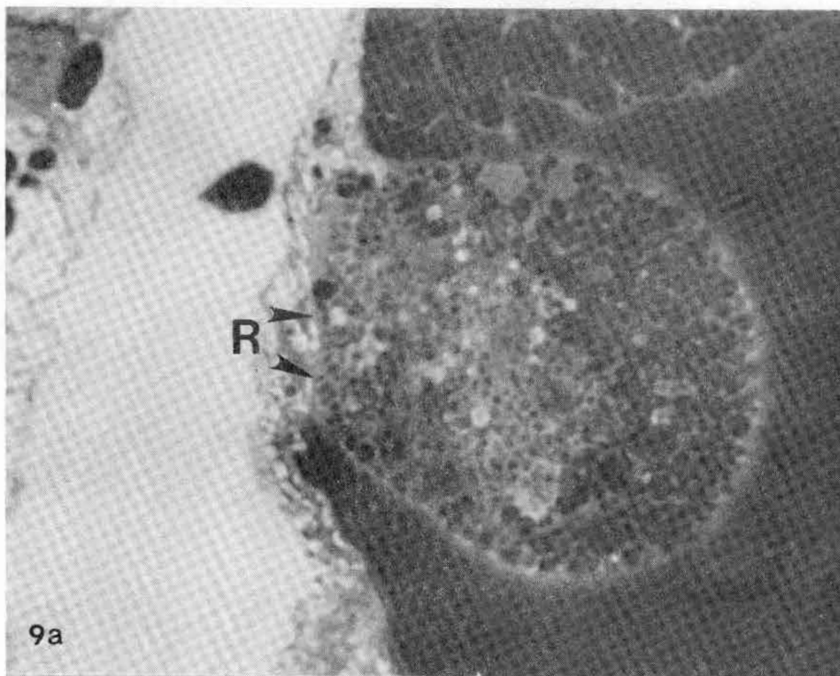
Figure 8. Nymphal *D. andersoni* ticks collected on each day of feeding until replete. Ticks illustrated are the unfed nymph (U), nymphs from day 1 through day 6 of feeding (1-6), and an adult male tick (A) recently molted from the nymphal stage; x 2

Nymphal Ticks
U 1 2 3 4 5 6 A
Day of Feeding
8

Figure 9. Light micrographs of groups of rickettsia-like organisms, believed to be symbiotes, in midgut epithelial cells of feeding nymphal D. andersoni.

(a) Aggregates of organisms (R) are seen in a nymph that became replete on the 6th day after feeding on an infected calf; x 1900.

(b) Aggregates of organisms (R) are seen in a nymph that fed for 3 days on an uninfected calf; x 1800.


R
9a

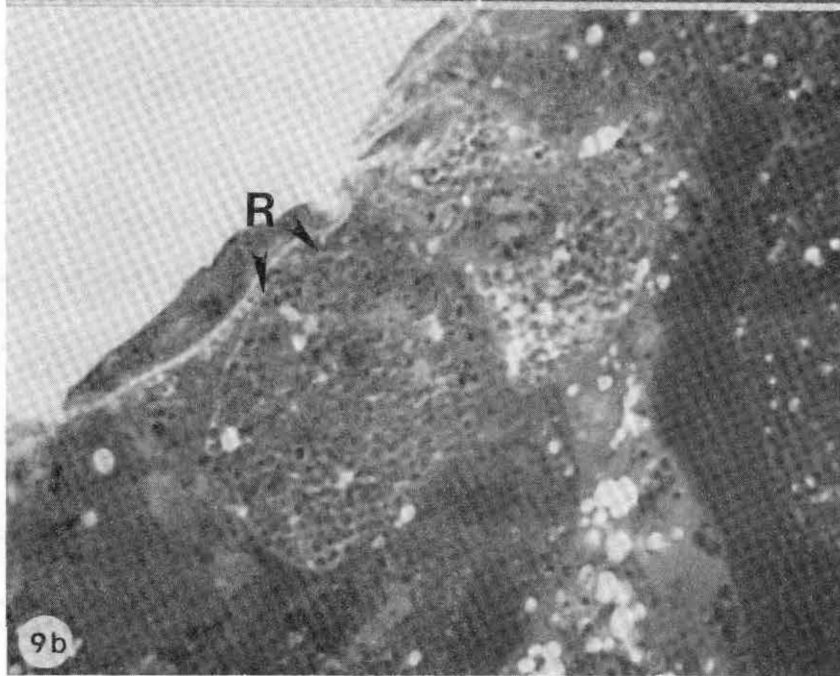
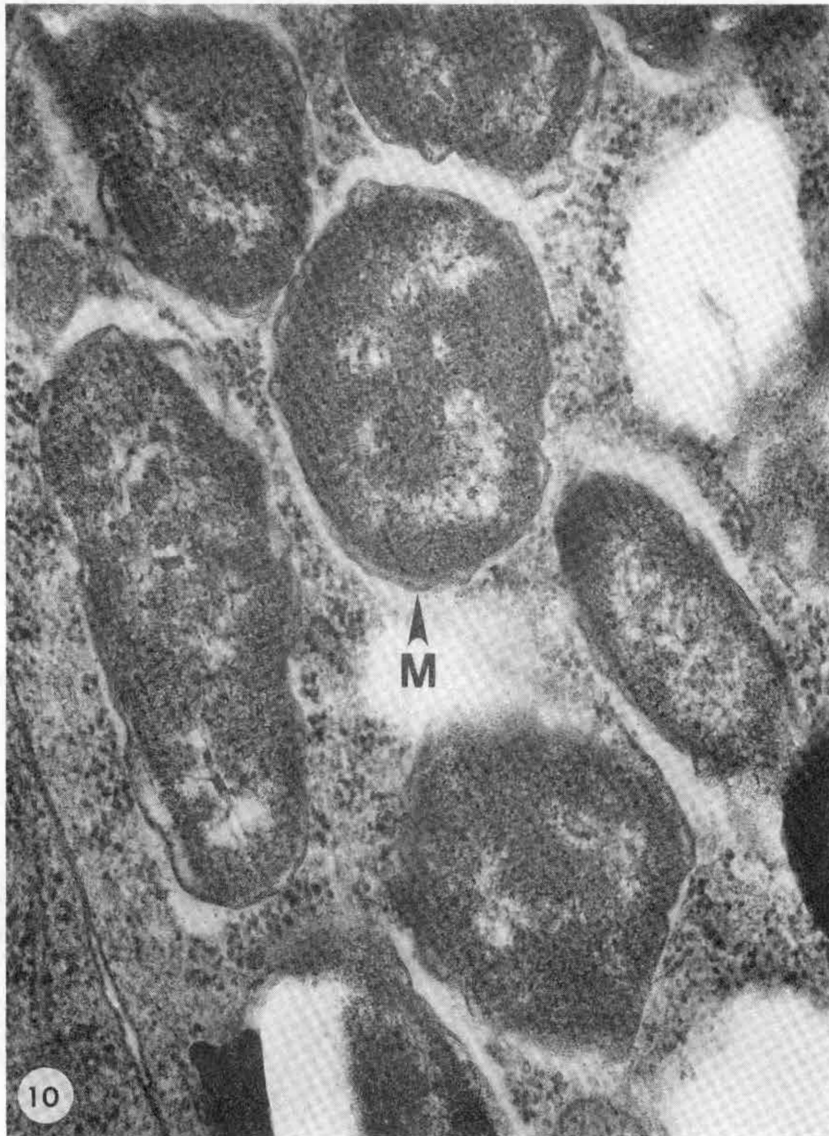

R
9b

Figure 10. Electron micrograph of rickettsia-like organisms found in the midgut epithelial cells of a nymph that fed for 4 days on an infected calf. The organisms have filamentous cytoplasm surrounded by two cell membranes (M). x 66,600.


M
10

Replete Nymphal Ticks

Light Microscopy

Colonies of A. marginale were seen in cross-sections of gut tissue from nymphal D. andersoni at 5 to 25 days post-repletion using light microscopy. No colonies were observed in sections of associated control tissues collected at the same times. Most colonies were located adjacent to the basement membrane of the midgut epithelial cells and occasionally in the Malpighian tubules; some were also observed in the interior of the epithelial cells.

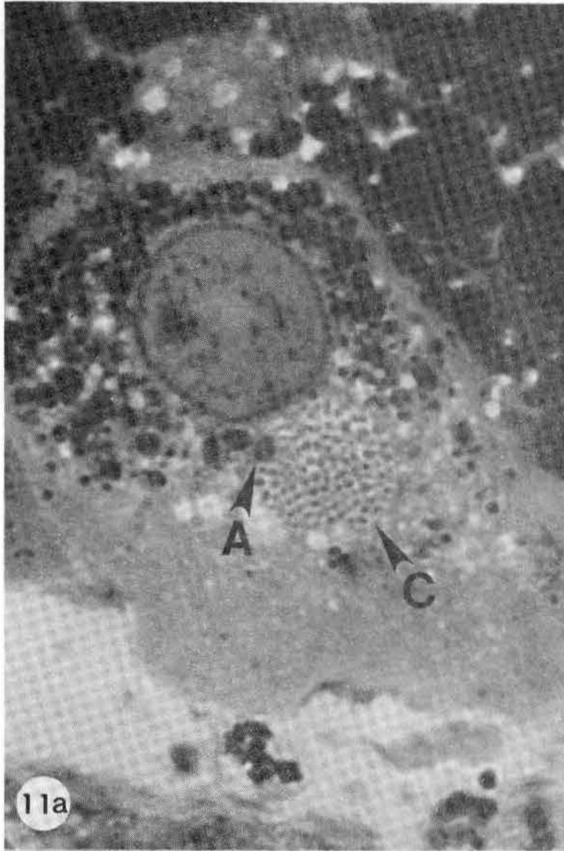
The morphology of A. marginale colonies in nymphal ticks was different from that for colonies described in the gut of adults. The colonies ranged from circular to oblong and varied in size within each repletion group. Aggregates of small colonies that included as many as 20 colonies were occasionally observed in all groups of replete nymphs. There appeared to be a decrease in the size of the Anaplasma organisms from 5 to 20 days post-repletion. The shape of organisms within colonies also changed from round to rod-shaped during this period. The individual organisms occurred separately, and the open spaces between organisms appeared to increase with increasing repletion time.

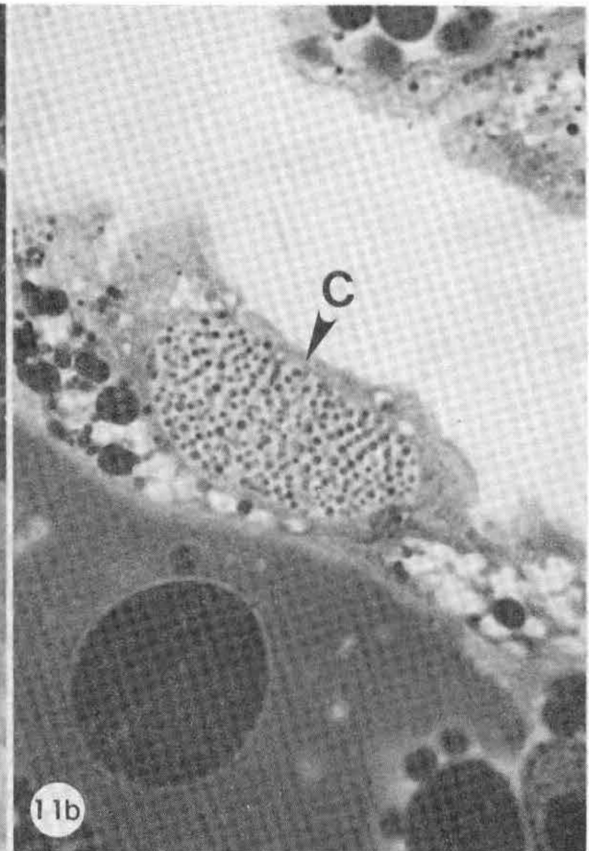
At 5 days post-repletion (Figures 11a-d), colonies of A. marginale were first observed and contained large, separated organisms. The individual organisms within colonies were either approximately uniform in size (Figures 11a and b) or varied somewhat in size (Figures 11c and d). Organisms were generally close to one another and evenly dispersed within the colony. Unusually large organisms were occasionally seen within colonies near the margin of the limiting membrane (Figure 11a).

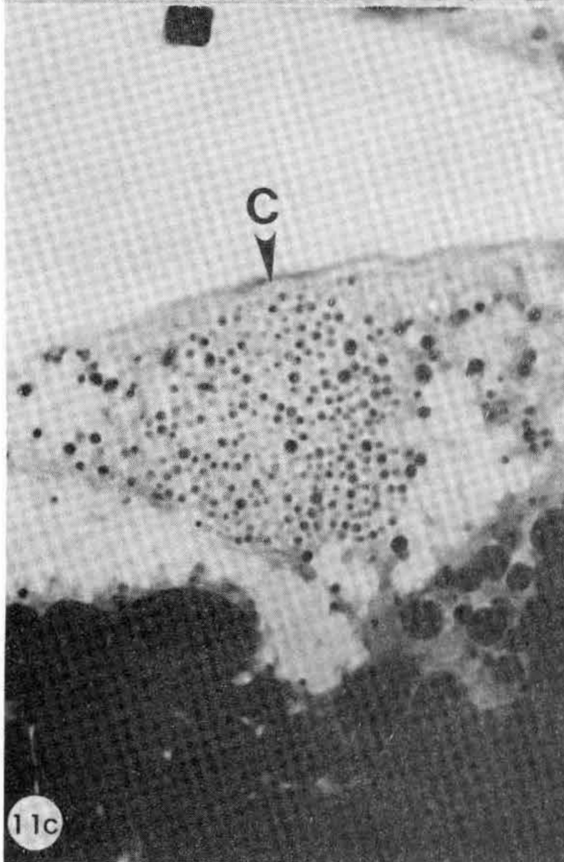
Figure 11. Light micrographs of colonies of _A. marginale_ (C) in the midgut epithelial cells of nymphal _D. andersoni_ at 5 days post-repletion. Colonies contain large, separated organisms.

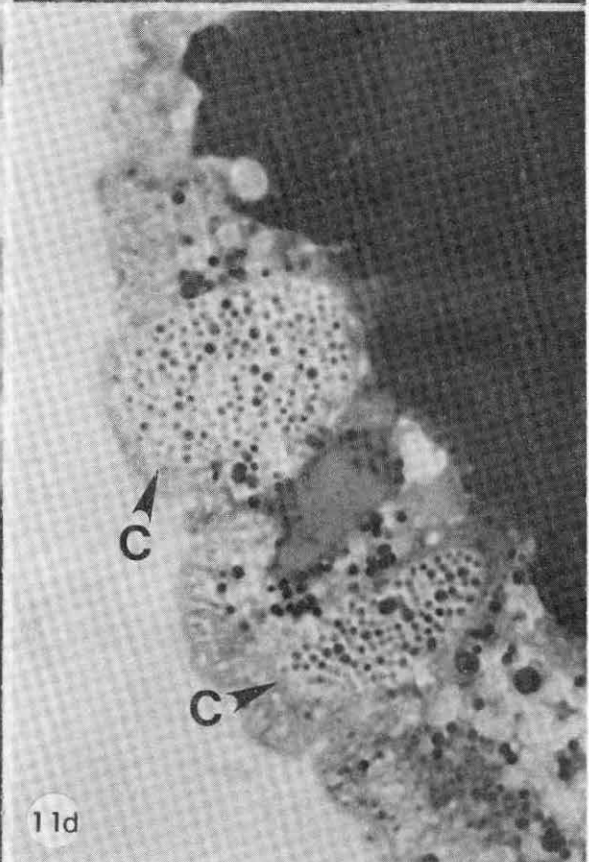
(a) and (b) Organisms are approximately uniform in size. An unusually large organism (A) is seen near the margin of a colony. (a) x 2000; (b) x 1800.

(c) and (d) Organisms vary somewhat in size. (c) x 1900; (d) x 1800.


A
C
11a


C
11b


C
11c


C
C
11d

Colonies seen at 10 days post-repletion (Figures 12a-d) were similar to those seen in the 5 day repletion group. The colonies were either located within the interior of the epithelial cells (Figures 12a and b) or near the basement membrane (Figure 12c), and aggregates of small colonies were sometimes seen (Figure 12d). Also, some unusually large organisms were seen occasionally within colonies (Figure 12d). The individual organisms were either closely packed within the colony (Figures 12a and c) or were separated by open spaces (Figure 12b).

At 15 days post-repletion (Figures 13a-d), colonies contained *Anaplasma* organisms that were generally smaller (Figures 13a and b) and appeared to vary more in size than those seen in the 10 day group. Some colonies had open spaces separating the organisms (Figure 13a), whereas others contained closely packed organisms (Figures 13b-d). Unusually large organisms located within some colonies and aggregates of small colonies were also observed (Figure 13b); these findings were similar to those described previously in ticks collected from the 5 and 10 day repletion groups.

In colonies observed at 20 days post-repletion (Figures 14a-d), the organisms within the *Anaplasma* colonies appeared to be smaller when compared with those seen at 5 days post-repletion, and most organisms were rod-like in shape (Figures 14a and b). Some colonies contained small round organisms (Figures 14c and d) that sometimes occurred in clusters (Figure 14c). Wide empty spaces were also more apparent between organisms (Figures 14c and d) than in previous repletion groups.

Colonies of *A. marginale* observed at 25 days post-repletion (Figures 15a-d) were similar to those seen at 20 days. The individual organisms were small and most were rod-shaped (Figures 15a-d).

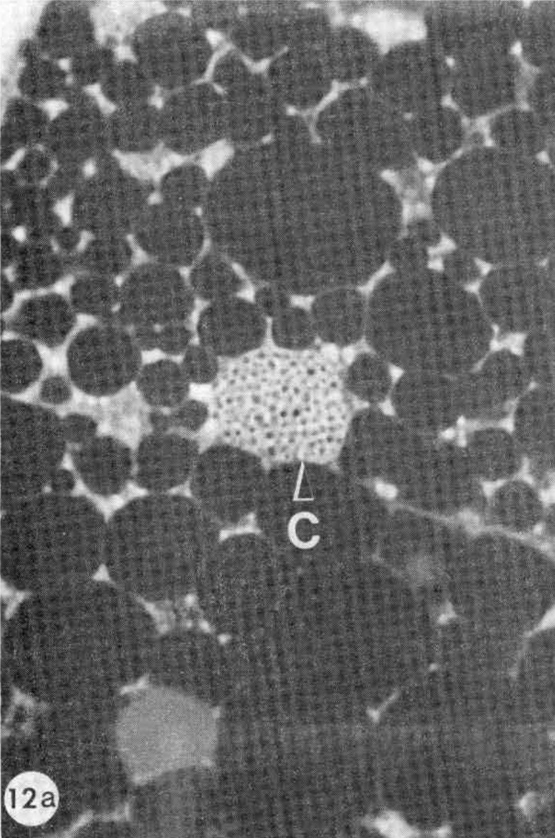
Figure 12. Light micrographs of colonies of _A. marginale_ (C) in the midgut epithelial cells of nymphal _D. andersoni_ at 10 days post-repletion.

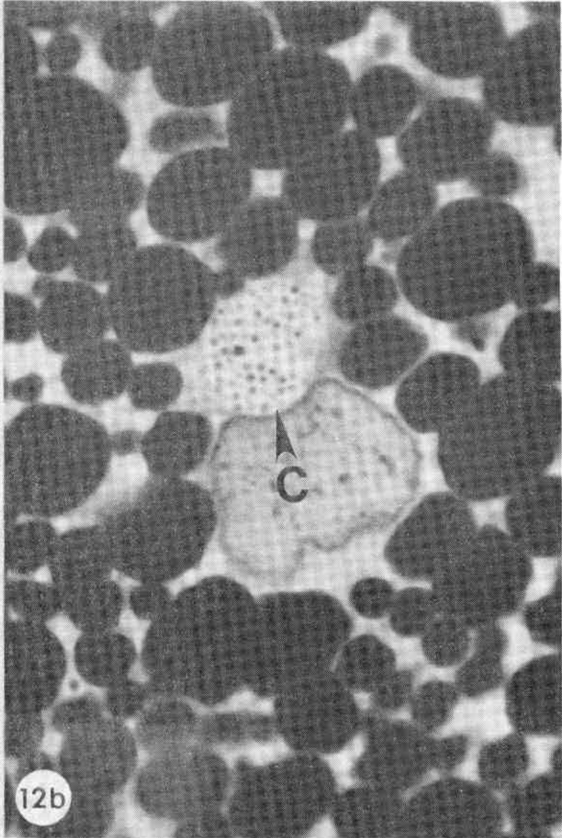
(a) Some colonies are located within the interior of the epithelial cells. Organisms are packed closely together within the colony. x 1800.

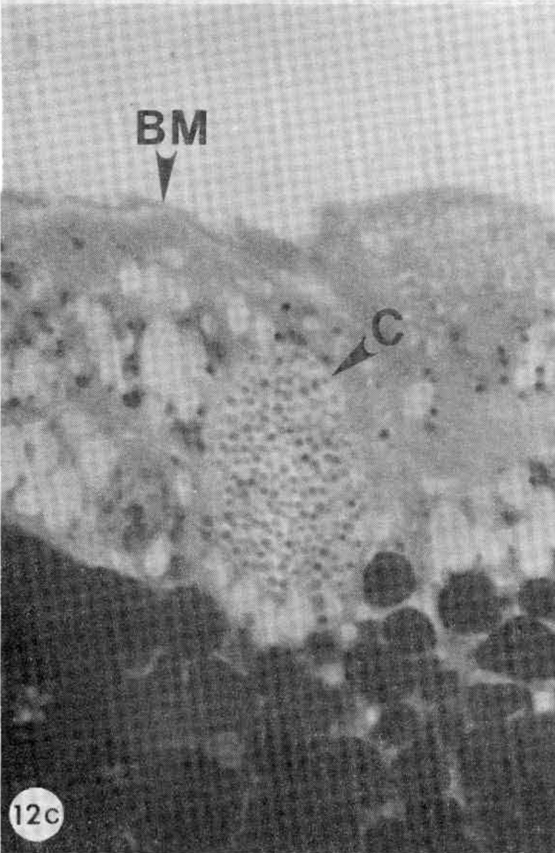
(b) Some colonies are located within the interior of the epithelial cells. Organisms are separated by open spaces. x 1900.

(c) Other colonies are located near the basement membrane (BM). Organisms are packed closely together within the colony. x 2000.

(d) Aggregates of small colonies are sometimes seen. Unusually large organisms (A) are seen occasionally. x 1800.


C
12a


C
12b


BM
C
12c

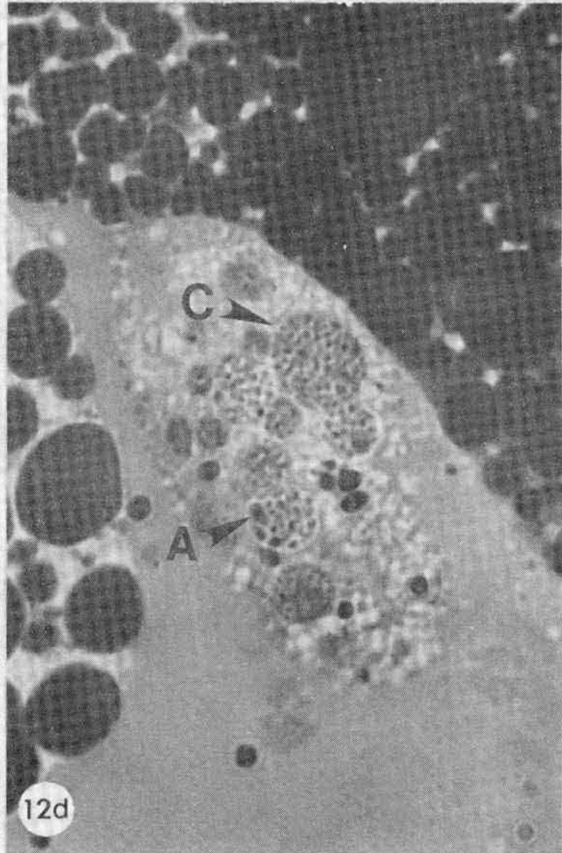

C
A
12d

Figure 13. Light micrographs of colonies of *A. marginale* (C) found in the midgut epithelial cells of nymphal *D. andersoni* at 15 days post-repletion. Organisms appear to vary more in size than those seen previously in the 10 day group.

(a) Organisms are generally smaller than those seen in ticks from previous repletion groups. Some colonies have open spaces separating the organisms. x 1800.

(b) An aggregate of small colonies is seen containing some unusually large organisms (A). Organisms are generally smaller than those seen previously in the 5 and 10 day repletion groups and are closely packed within the colony. x 1900.

(c) and (d) Organisms are closely packed within the colony.

(c) x 1900.

(d) x 2000.

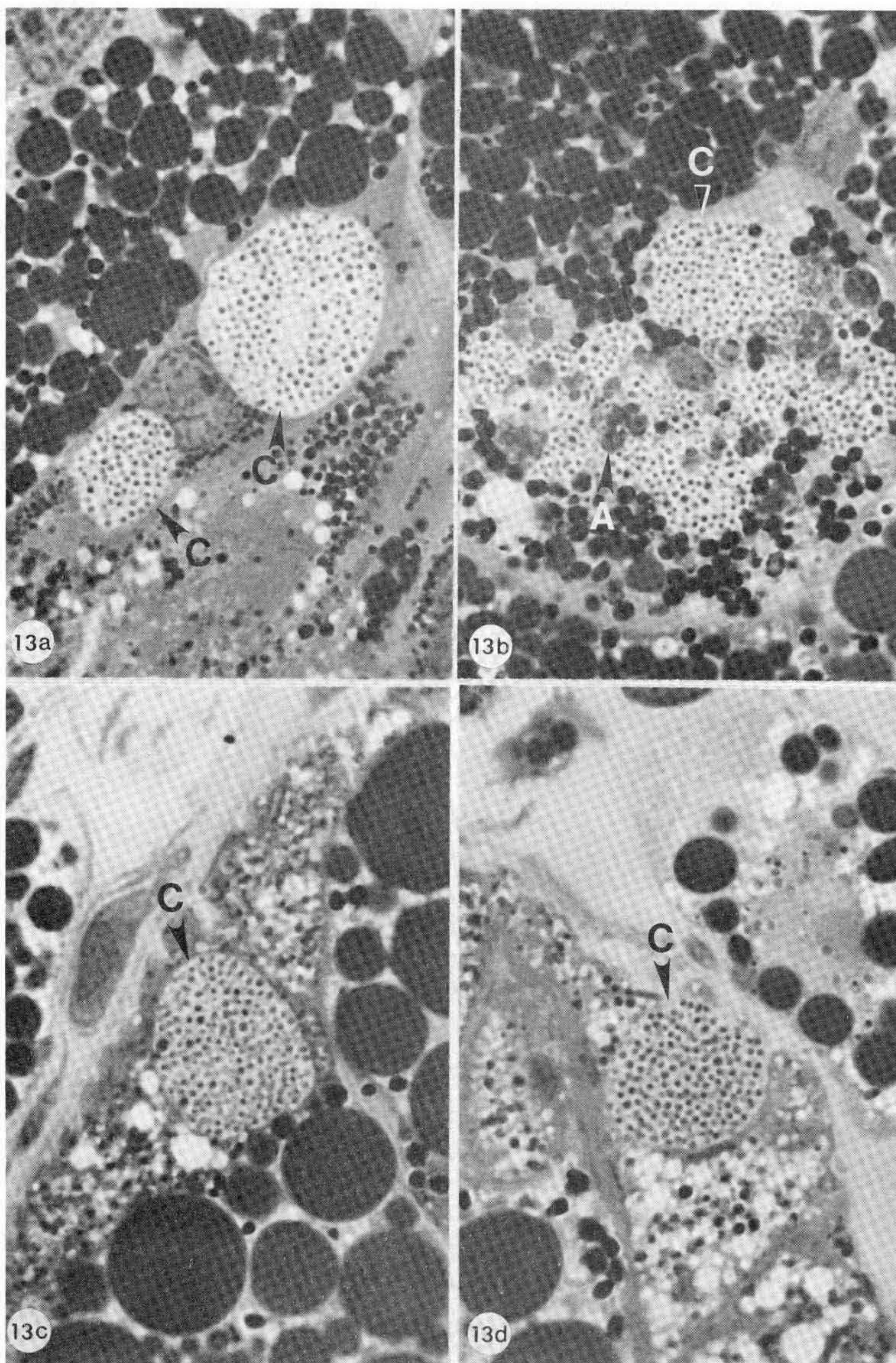

C
C
13a
C
A
13b
C
13c
C
13d

Figure 14. Light micrographs of colonies of *A. marginale* (C) found in the midgut epithelial cells of nymphal *D. andersoni* at 20 days post-repletion. The organisms within colonies appear to be smaller when compared with those seen at 5 days post-repletion.

(a) and (b) Most organisms are rod-like in shape.

(a) x 1900.

(b) x 2000.

(c) Some colonies contain small round organisms separated by wide empty spaces. Organisms sometimes occur in clusters. x 1900.

(d) Some colonies contain small round organisms. Wide empty spaces between organisms are more apparent than in ticks from previous repletion groups. x 1900.

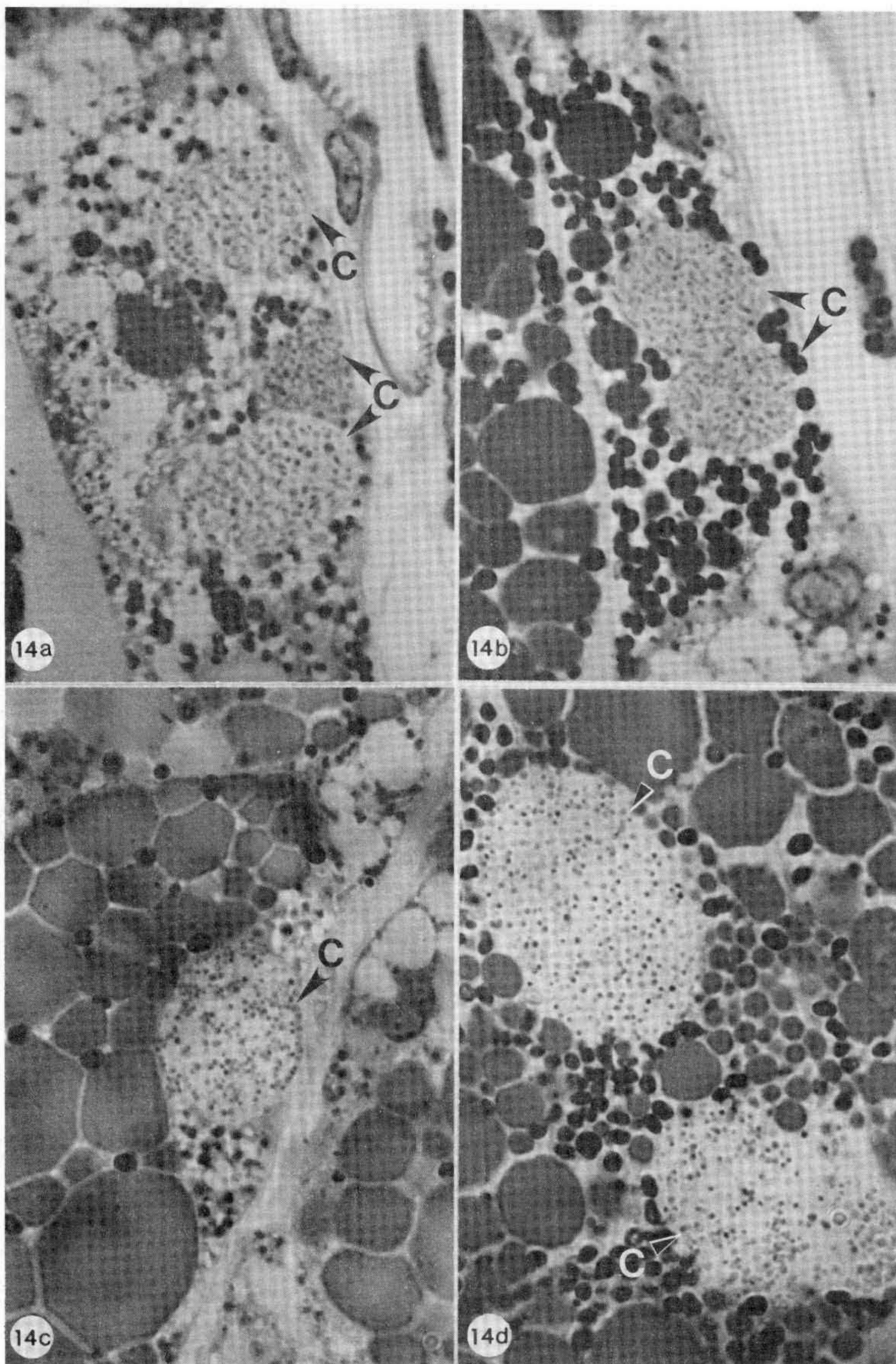
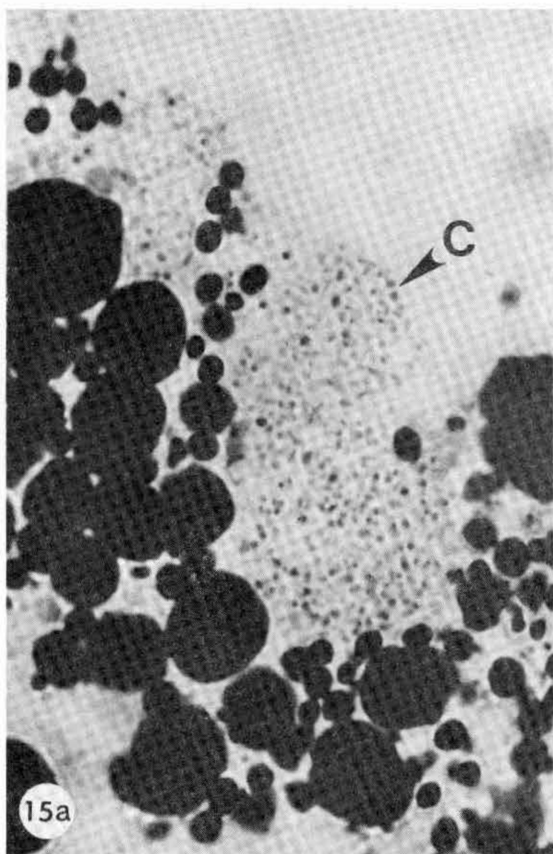
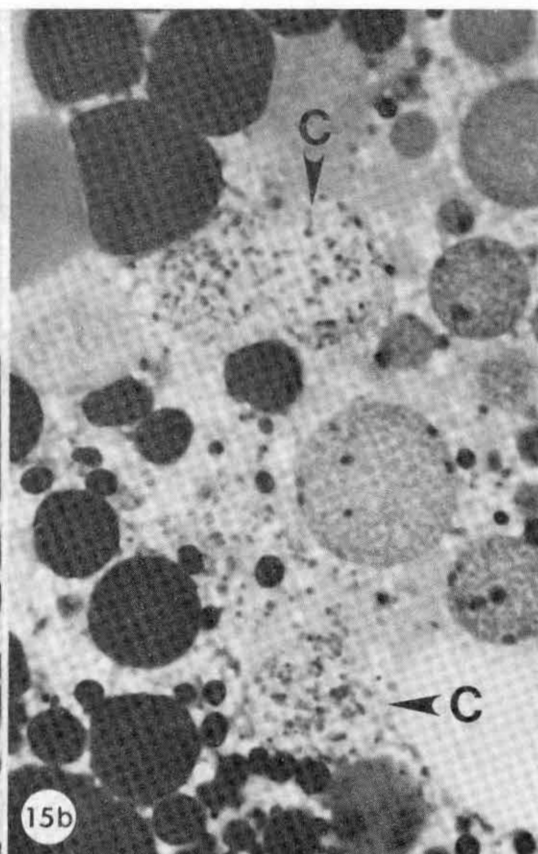
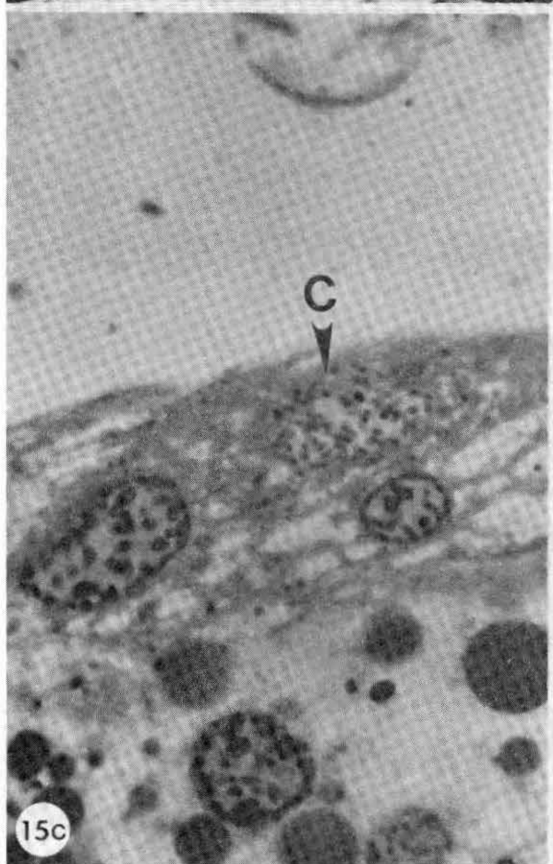
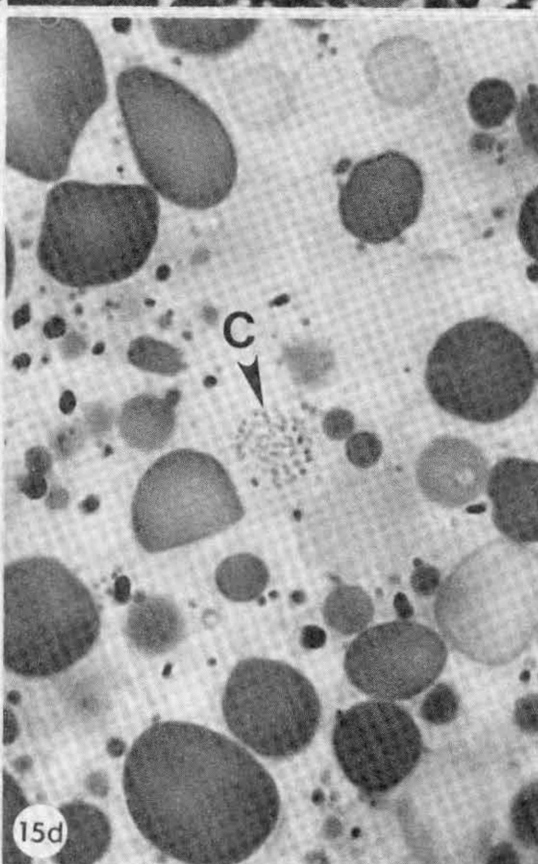

C
C
C
14a
14b
14c
14d

Figure 15. Light micrographs of colonies of *A. marginale* (C) found in the midgut epithelial cells of nymphal *D. andersoni* at 25 days post-repletion. The colonies contain small, rod-shaped organisms. (a) x 1900. (b) x 2100. (c) x 1900. (d) x 1900.


C
15a


C
C
15b


C
15c


C
15d

Electron Microscopy

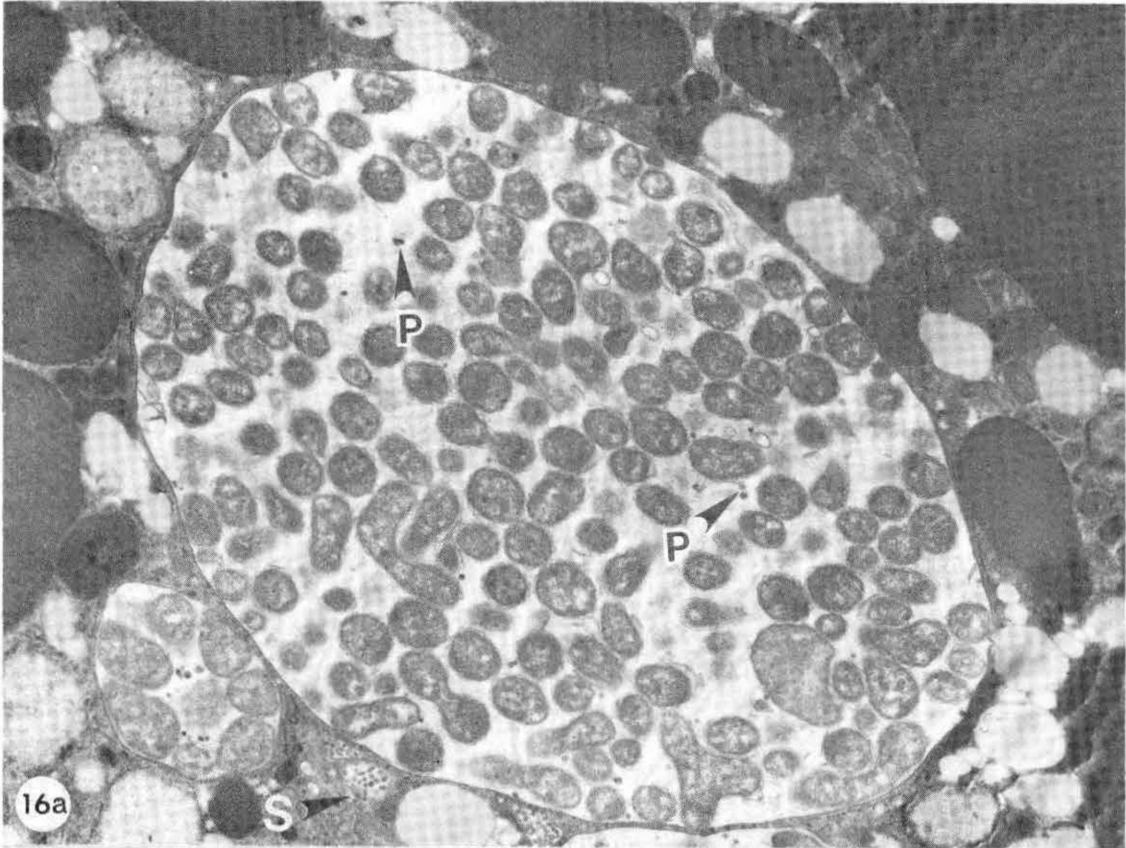
Colonies of *A. marginale* visualized by electron microscopy confirmed the light microscopy observations. *Anaplasma* organisms within colonies appeared to gradually decrease in size in the period from 5 to 20 days post-repletion. The shape of the organisms changed from round at 5, 10, and 15 days to rod-shaped at 20 and 25 days post-repletion. Individual organisms were separate from each other and the open spaces between organisms gradually increased with increasing repletion time. All of the *Anaplasma* organisms observed were reticulated forms. Small electron-dense particles were also seen in colonies from ticks dissected at each day of repletion. Electron-dense forms were not observed in any of the replete nymphs studied.

At 5 days post-repletion, colonies contained round to oblong-shaped organisms (Figures 16a and b). The margins of the limiting membrane of these organisms had a typically wavy appearance (Figure 16b). Some of the organisms within the colonies appeared to be undergoing binary fission (Figure 16b). Small electron-dense particles were present in some of the colonies (Figures 16a and 17a) in the spaces between *Anaplasma* organisms. A grouping of small particles within a limiting membrane outside a colony was also observed (Figure 16a). Likewise, small particles were occasionally present within the limiting membrane of the reticulated forms (Figure 17b). A few *Anaplasma* organisms within colonies were enclosed by membrane whorls (Figure 17b). Some colonies contained a large clump of organisms (Figure 18a), a finding that appeared to correspond to the unusually large organisms occasionally seen at the light microscopy level (Figure 11a).

Figure 16. Electron micrographs of a colony of *A. marginale* in the midgut epithelial cells of nymphal *D. andersoni* at 5 days post-repletion. Colonies contain round to oblong-shaped organisms.

(a) Small electron-dense particles (P) are present in the spaces between organisms. A grouping of small particles within a limiting membrane (S) outside the colony is also present. x 12,300.

(b) The margins of the limiting membrane of these organisms has a typically wavy appearance. Some organisms appear to be undergoing binary fission (F). x 35,000.


P
P
S
16a

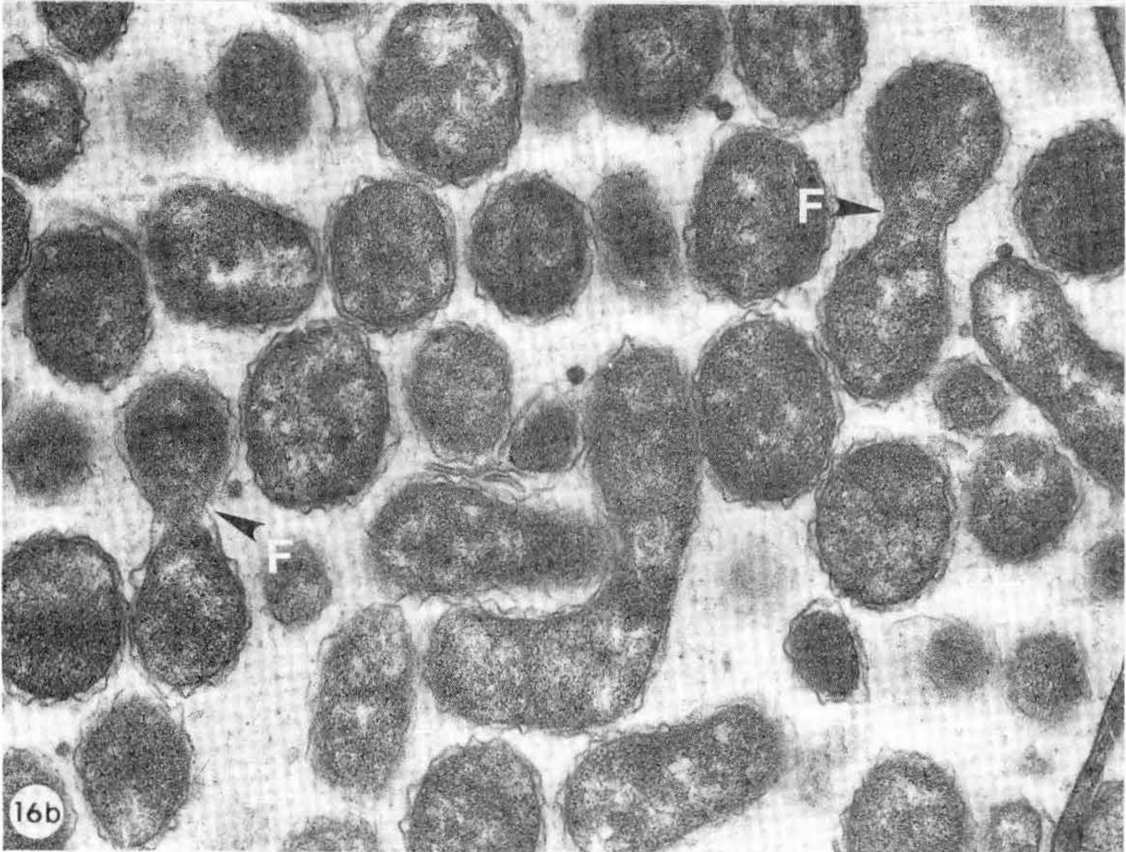
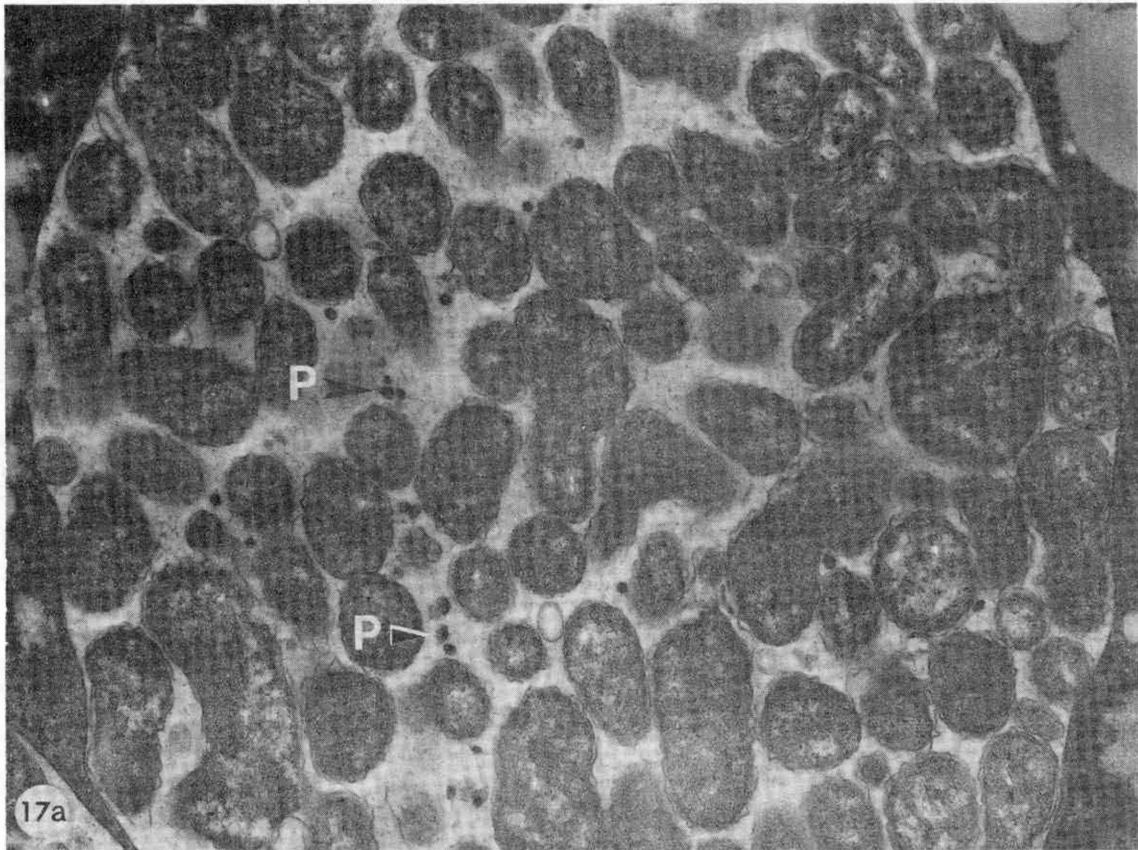

F
F
16b

Figure 17. Electron micrographs of _A. marginale_ organisms within colonies from nymphal _D. andersoni_ at 5 days post-repletion.

(a) Small electron-dense particles (P) are present in the spaces between organisms. x 22,200.

(b) Small electron-dense particles (P) are occasionally present within the limiting membrane of the organisms. A few organisms are enclosed by membrane whorls (W). x 18,200.


P
P
17a

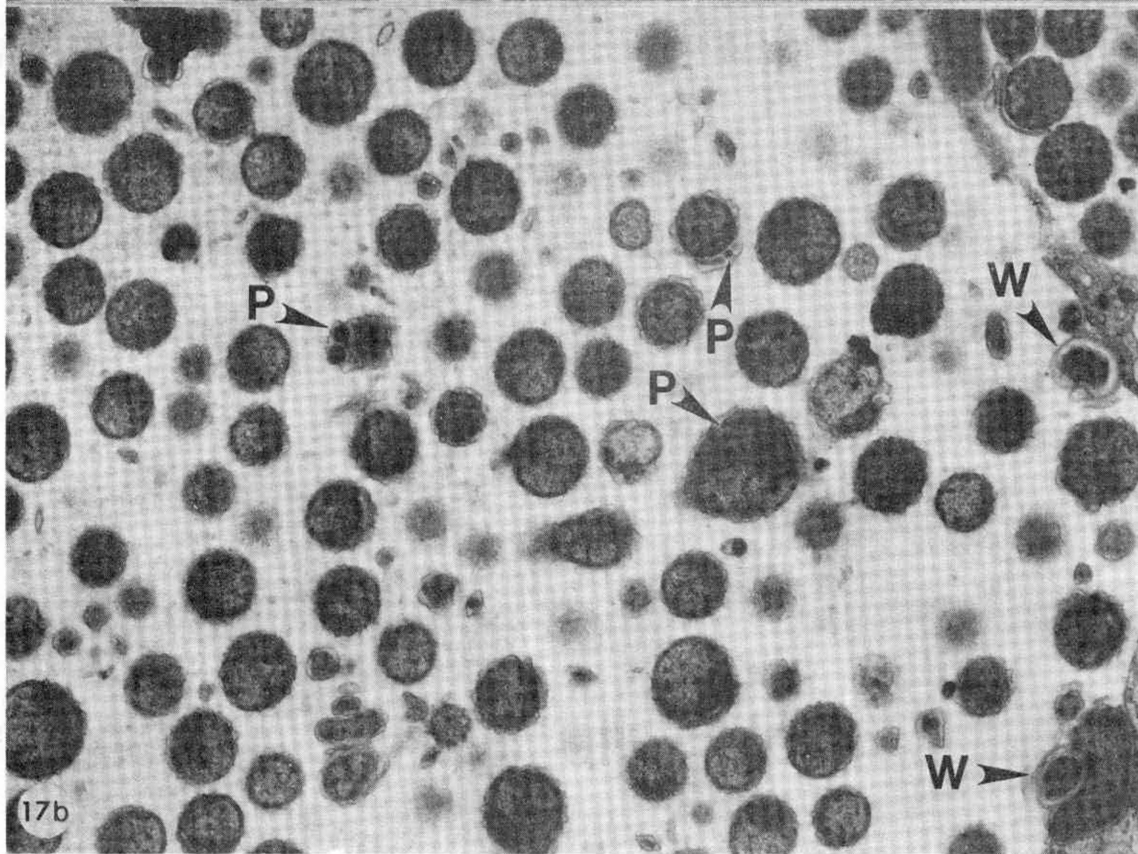
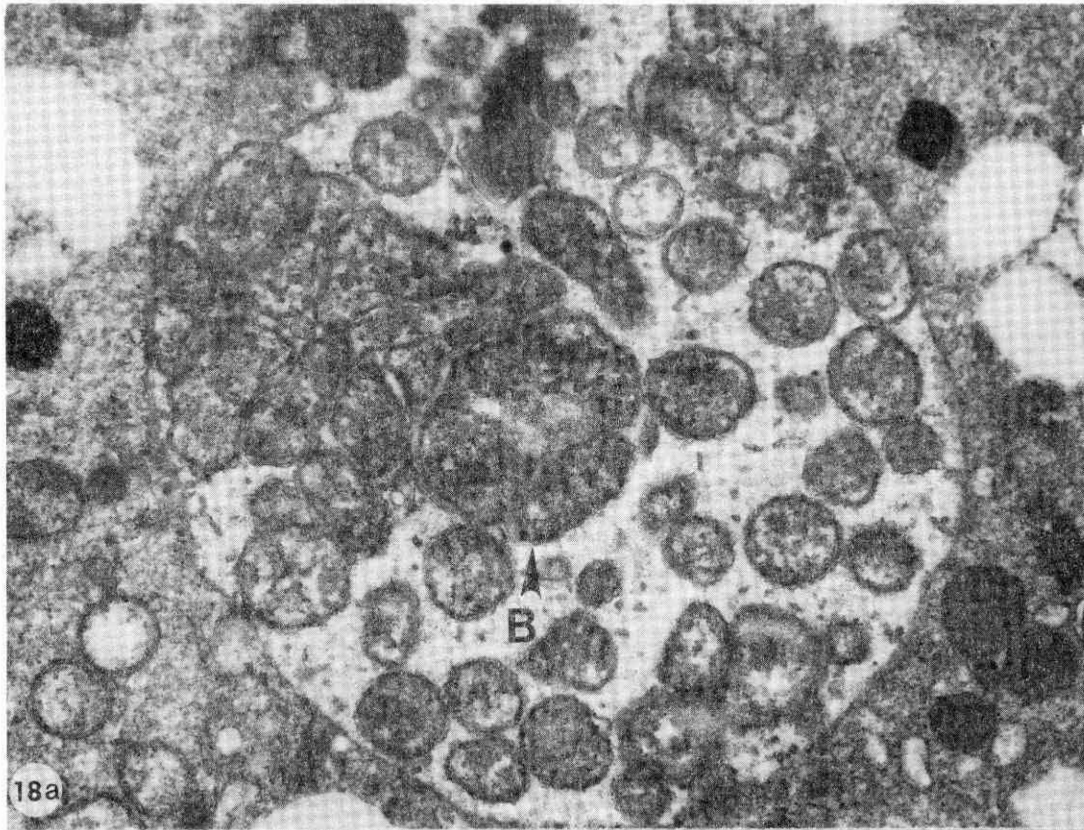
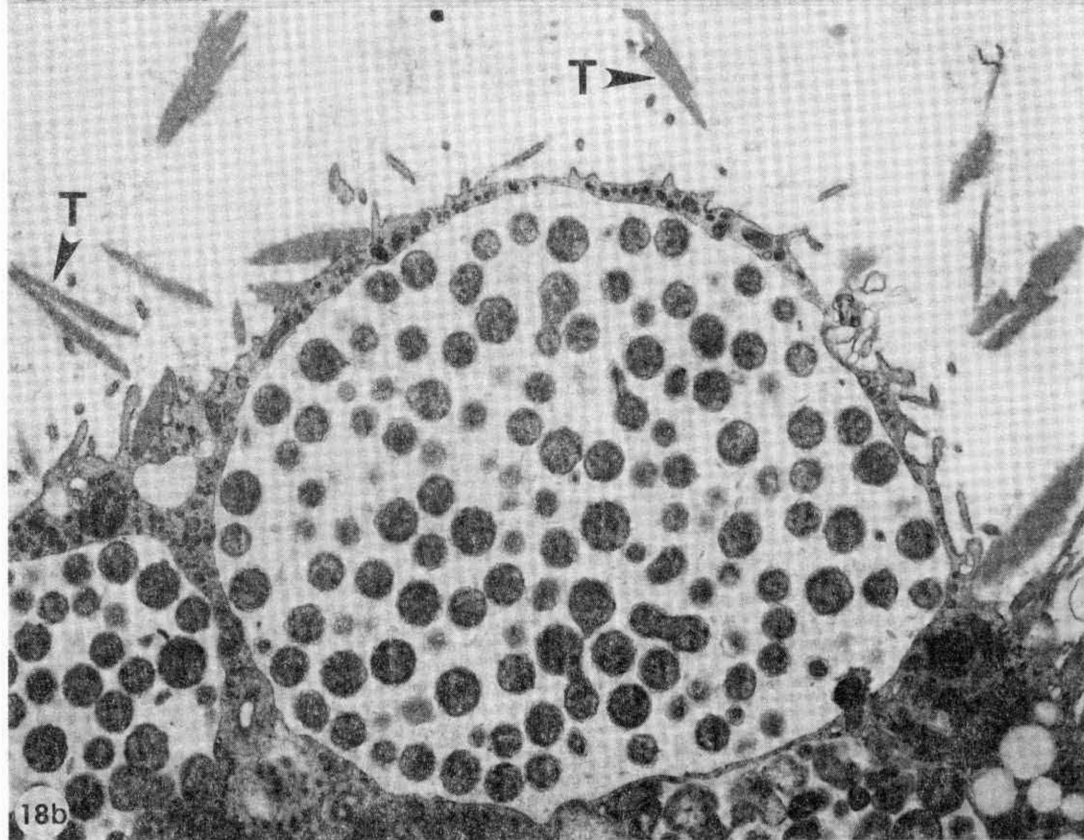

P
P
P
W
W
17b

Figure 18. Electron micrographs of colonies of *A. marginale* in the midgut epithelial cells of nymphal *D. andersoni* at 5 days post-repletion.

(a) A large clump of organisms (B) is present within the colony. x 19,800.

(b) Tail-like appendages (T) are present in the gut lumen near a few colonies. x 10,400.


B
18a


T
T
18b

Tail-like appendages were observed in the lumen of the gut near a few colonies at 5 days post-repletion (Figure 18b). Appendages were either attached to or interdigitated with the luminal surface of the host cell membrane (Figures 19a and b) or were free in the gut lumen (Figure 20a). These appendages displayed longitudinal and transverse periodicity (Figure 20a). One appendage was also observed within the epithelial tissue and was enclosed by a limiting membrane (Figure 20b). However, initial bodies were not seen attached to or associated with these appendages.

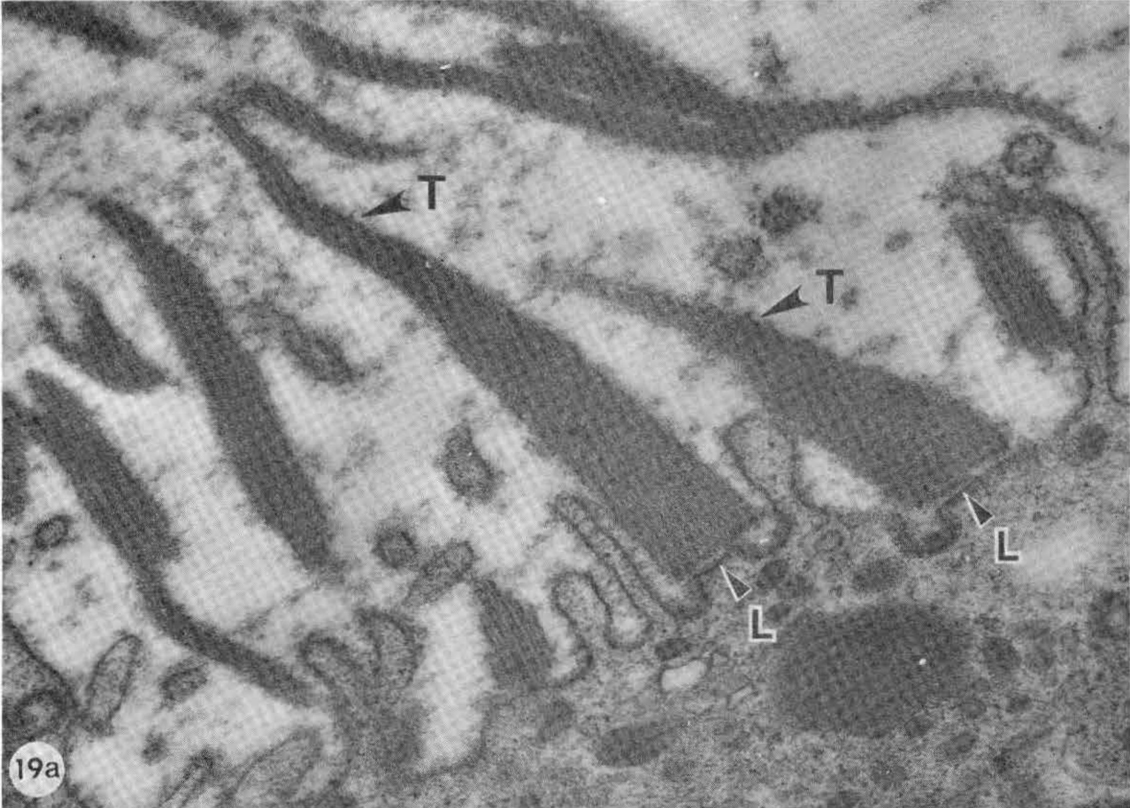
In the 10 day post-repletion group, colonies of _A. marginale_, when compared with the 5 day repletion group, contained fewer organisms with more open space between individual organisms (Figures 21a and b). Fewer oblong-shaped organisms were seen and few were observed that appeared to be dividing by binary fission. Small electron-dense particles were frequently seen within the limiting membrane of the _Anaplasma_ organisms (Figures 22a and b). Some of the organisms were subdivided on their margin by alternating light and dark bands (Figures 22a and b). Small particles were also seen in the spaces between organisms (Figure 23a). Some organisms were occasionally seen with clumps of small particles expanding within their limiting membranes (Figure 23a). Clumps of organisms that contained membrane whorls around the individual organisms were also observed in some colonies (Figure 23b).

Colonies seen at 15 days post-repletion (Figures 24a and b) were similar to those seen at 10 days. Most _Anaplasma_ organisms were round with open spaces separating the individual organisms. In some colonies, organisms exhibited irregular shapes (Figure 25a). Small electron-dense particles and the previously described subdivisions of light and dark

Figure 19. Electron micrographs of tail-like appendages found associated with the midgut epithelial cells in nymphal *D. andersoni* at 5 days post-repletion.

(a) Some appendages (T) are attached to the luminal surface (L) of the host cell membrane. x 45,100.

(b) Some appendages (T) are interdigitated with the luminal surface (L) of the host cell membrane. x 37,300.


T
T
L
L
19a

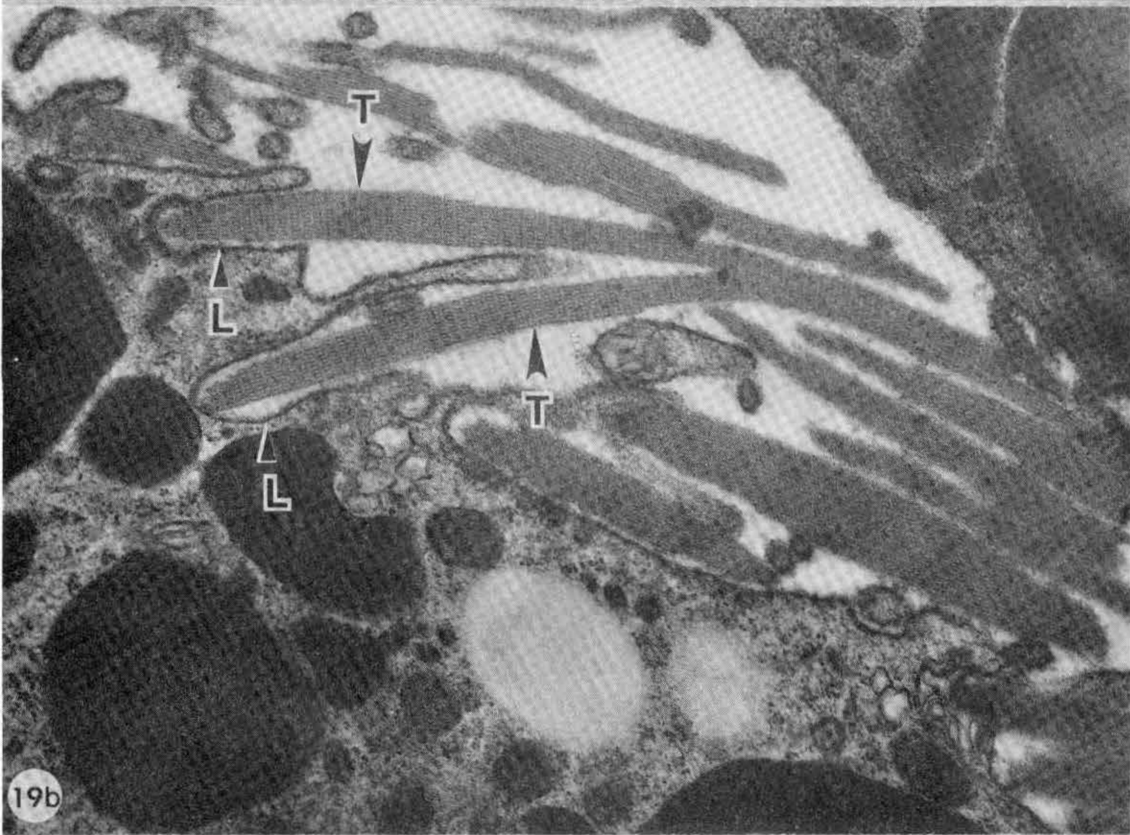
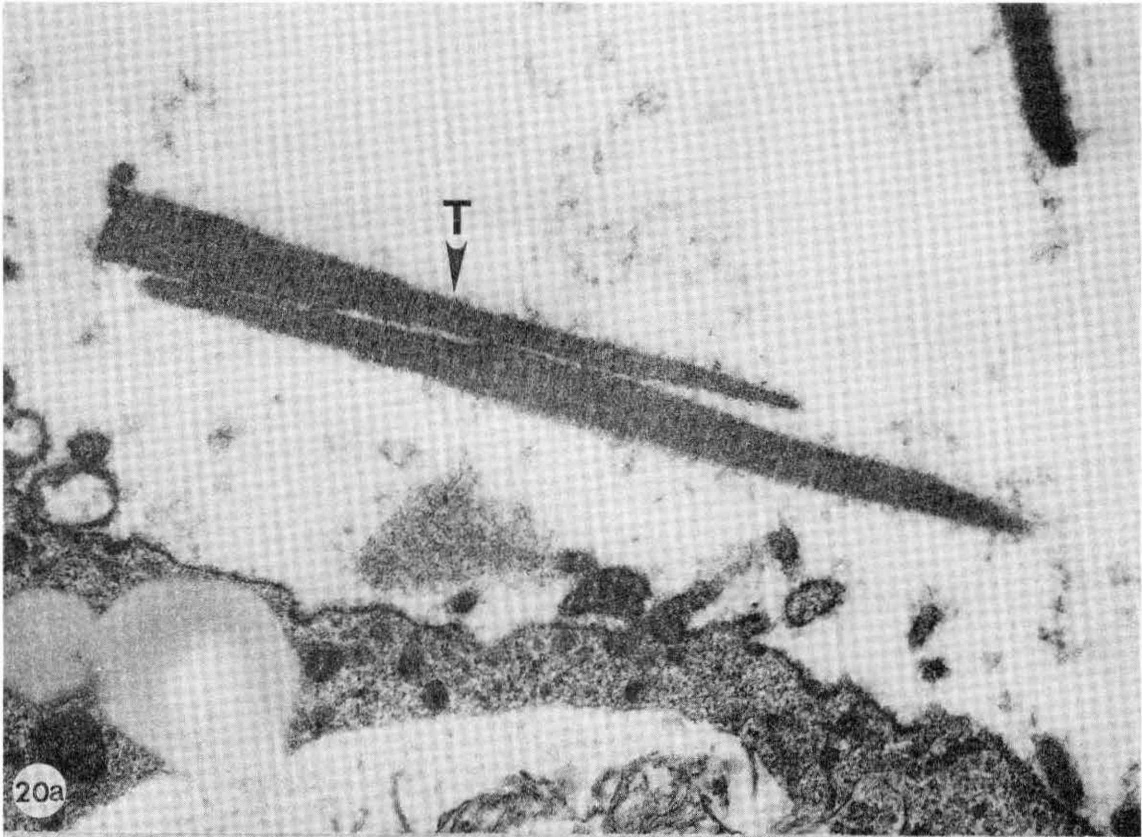

T
L
T
L
19b

Figure 20. Electron micrographs of tail-like appendages found associated with the midgut epithelial cells in nymphal *D. andersoni* at 5 days post-repletion.

(a) Some appendages (T) appear to be free in the gut lumen. Appendages display longitudinal and transverse periodicity. x 34,300.

(b) One appendage (T) appears to be in the epithelial cell and enclosed by a limiting membrane (LM). x 31,800.


T
20a

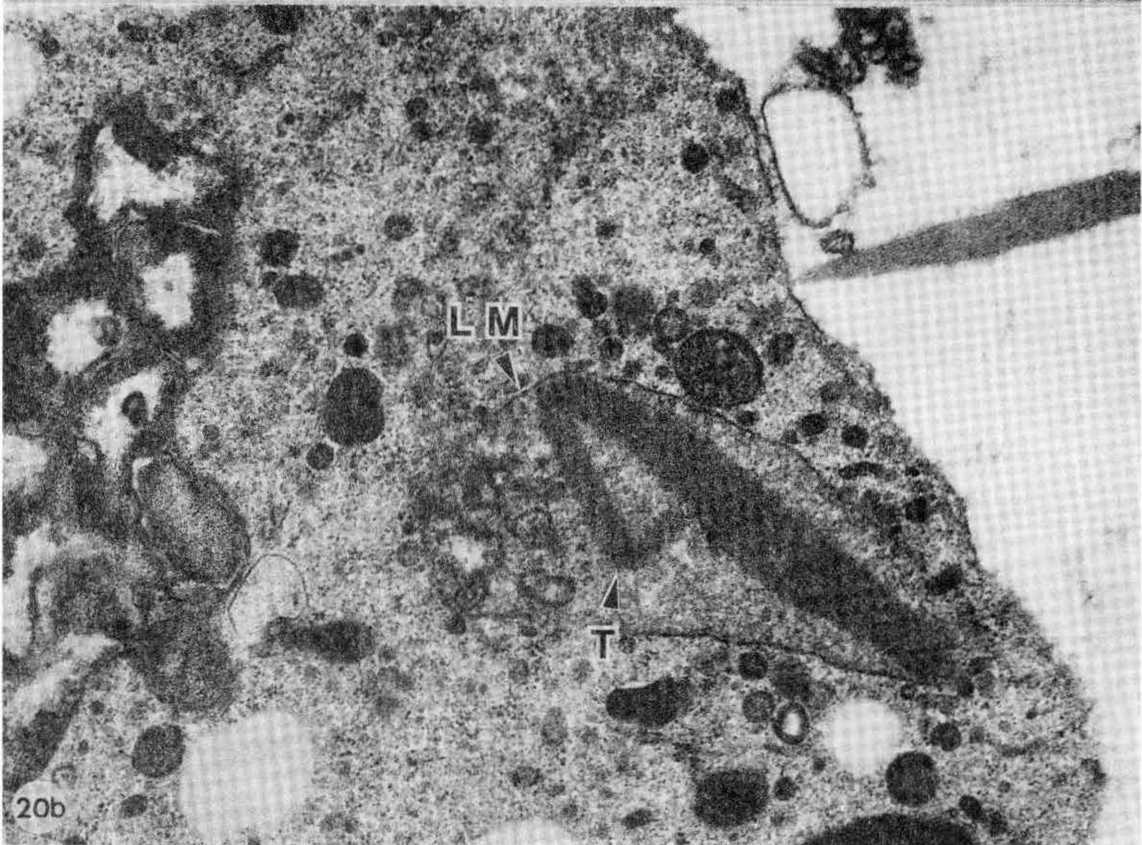
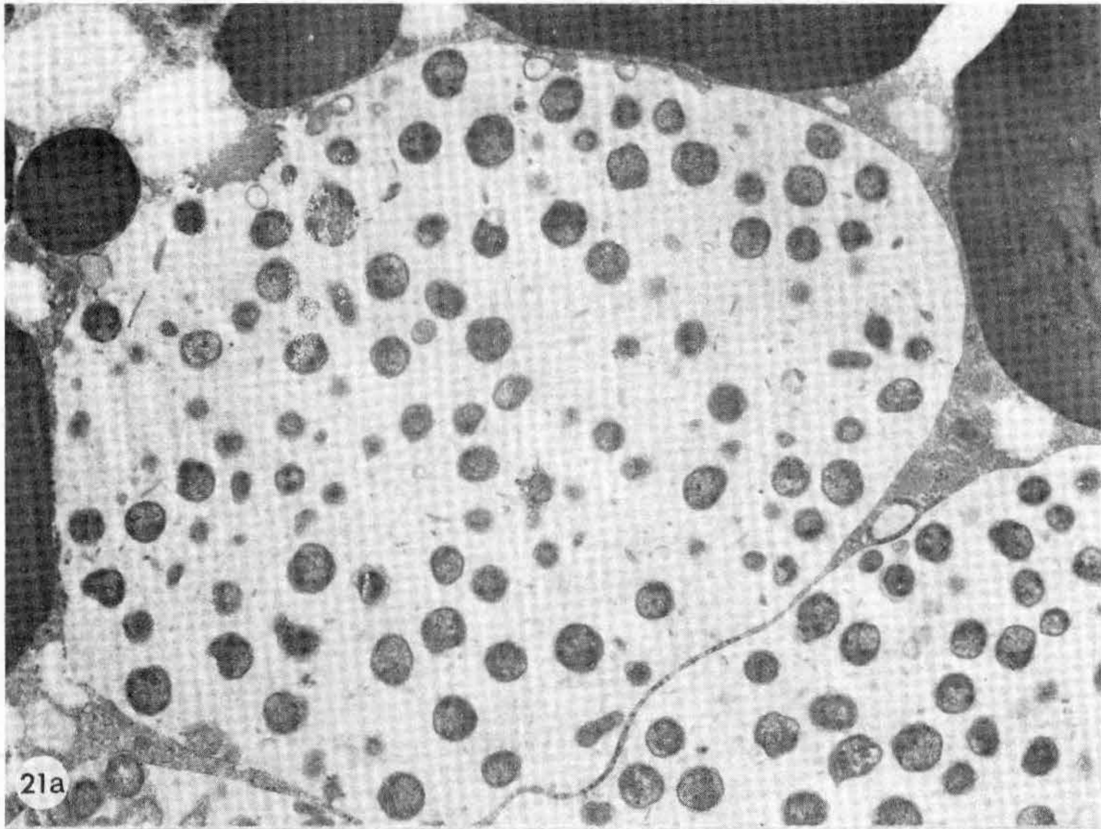

LM
T
20b

Figure 21. Electron micrographs of colonies of *A. marginale* in the midgut epithelial cells of nymphal *D. andersoni* at 10 days post-repletion. Colonies contain fewer organisms with more open space between individual organisms when compared with the 5 day repletion group. (a) x 9600. (b) x 9700.


21a

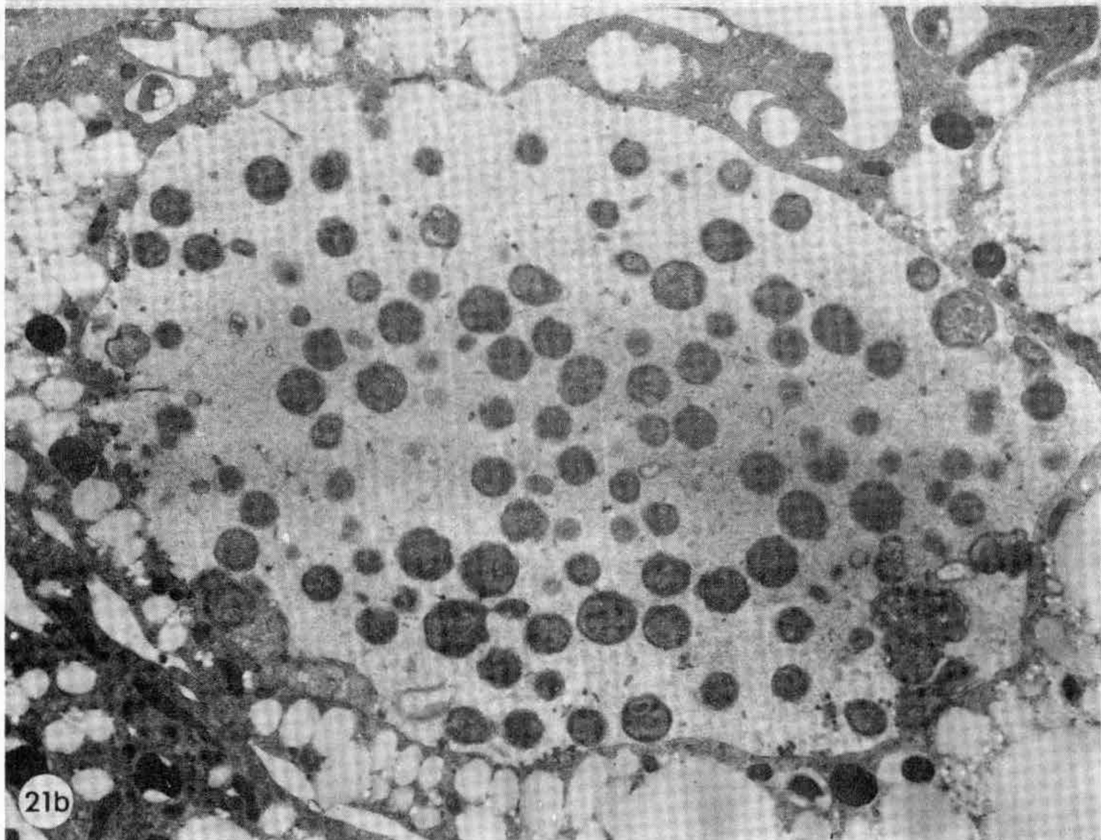
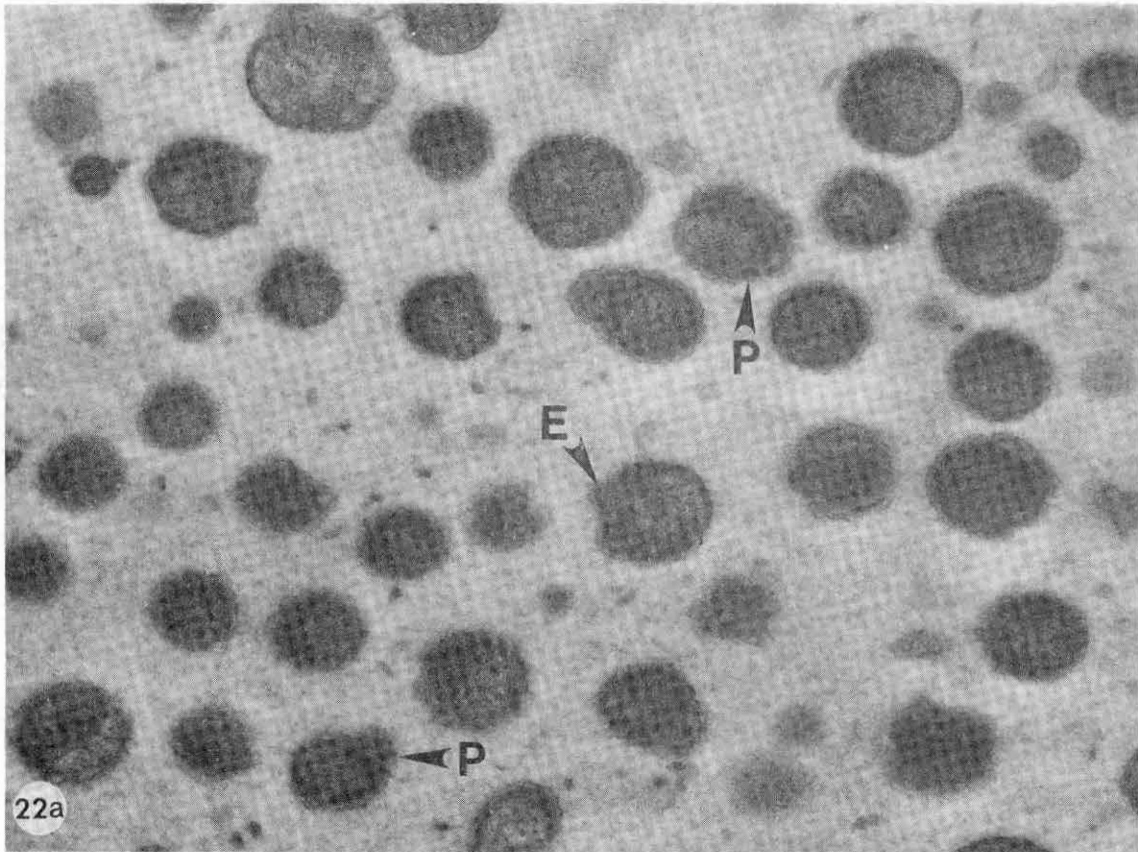

21b

Figure 22. Electron micrographs of *A. marginale* organisms within colonies from nymphal *D. andersoni* at 10 days post-repletion. Small electron-dense particles (P) are seen within the limiting membrane of organisms. Some organisms are subdivided on their margin by alternating light and dark bands (E).
(a) x 25,200. (b) x 22,100.


P
E
P
22a

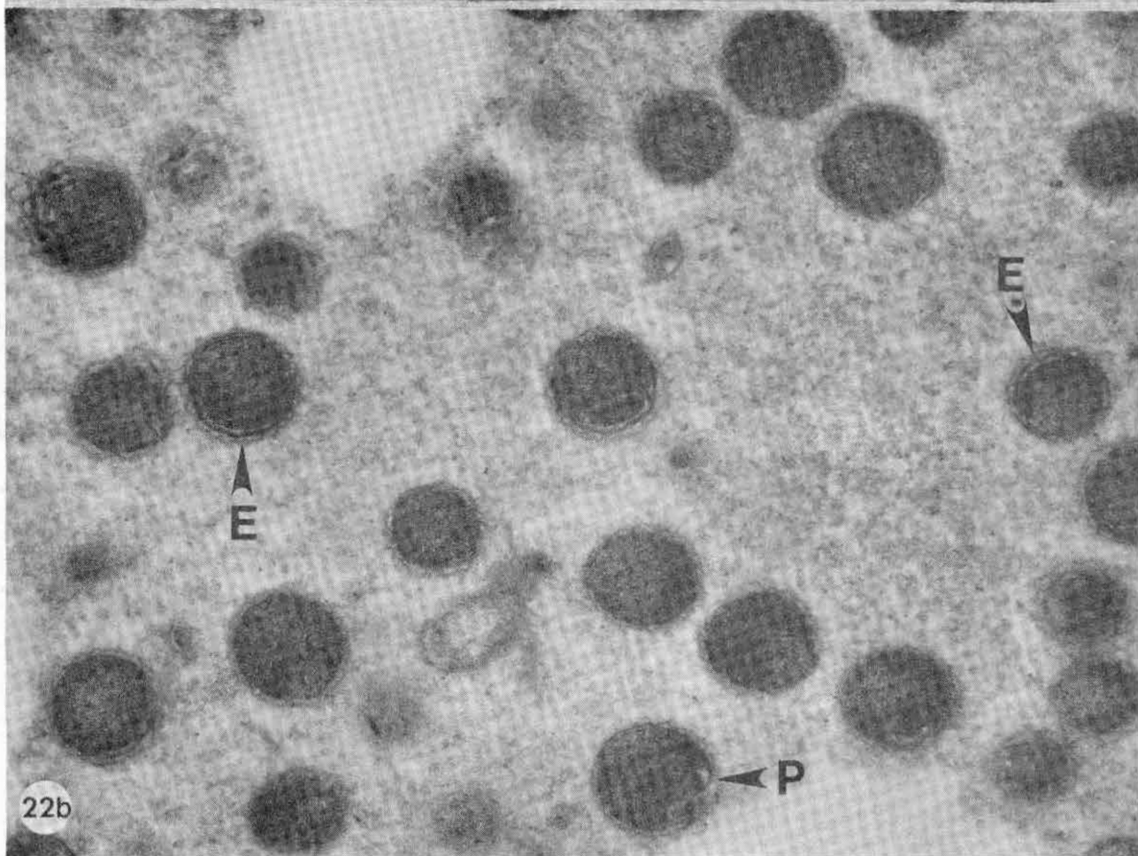
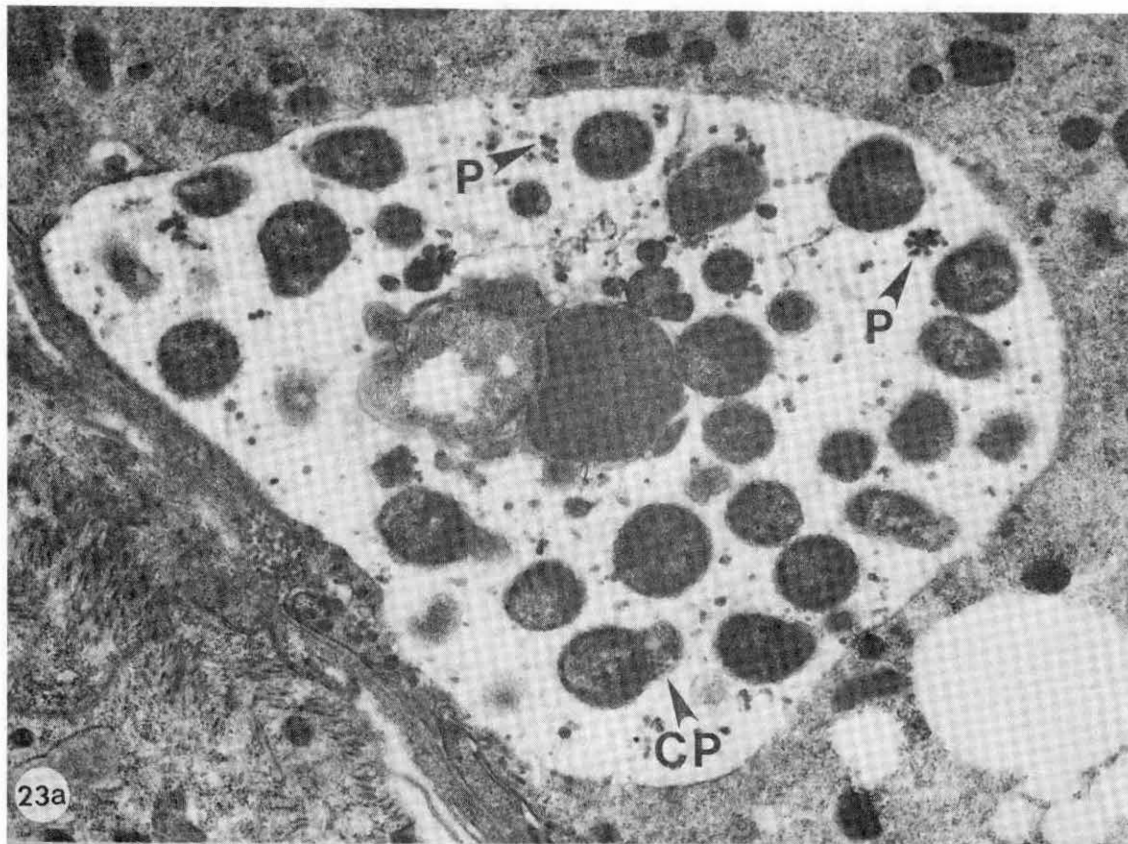

E
E
P
22b

Figure 23. Electron micrographs of colonies of *A. marginale* in the midgut epithelial cells of nymphal *D. andersoni* at 10 days post-repletion.

(a) Small electron-dense particles (P) are seen in the spaces between organisms. Some organisms contain clumps of small particles (CP) expanding within the limiting membrane. x 21,400.

(b) A clump of organisms (B) contains membrane whorls around the individual organisms (W). x 20,600.


P
P
CP
23a

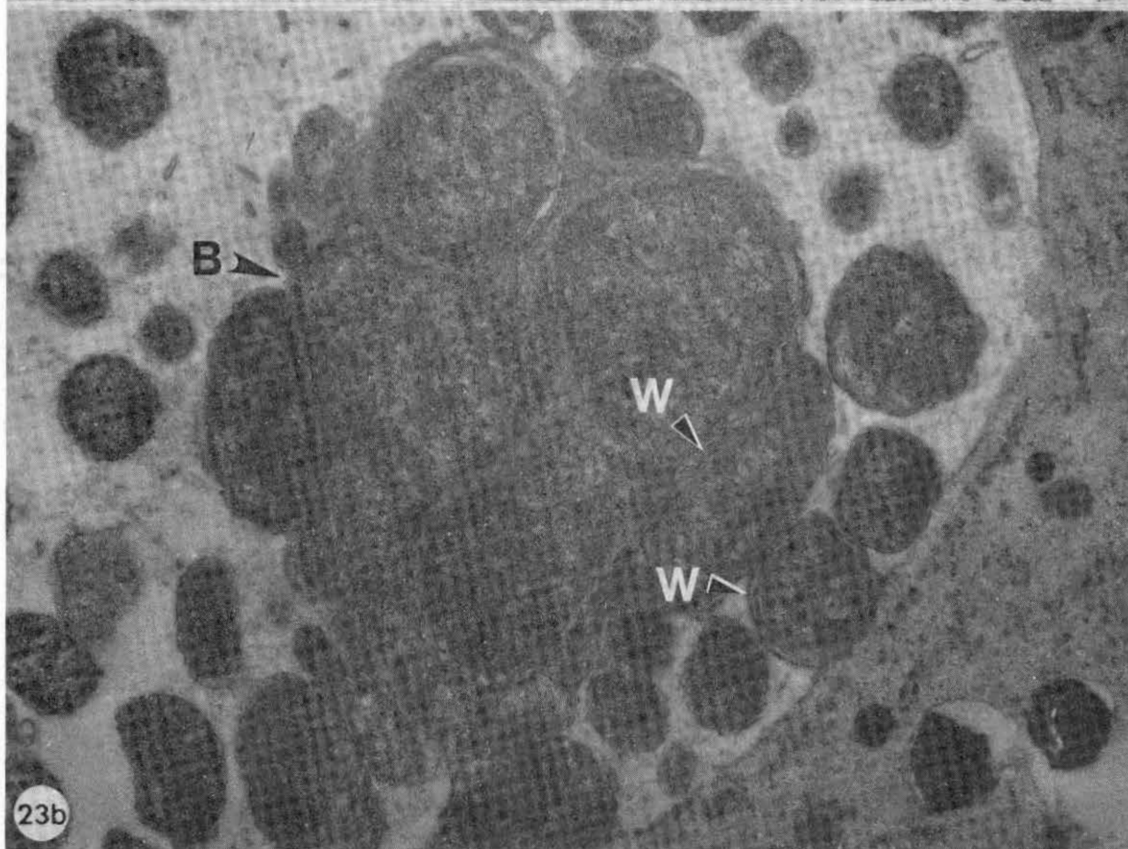
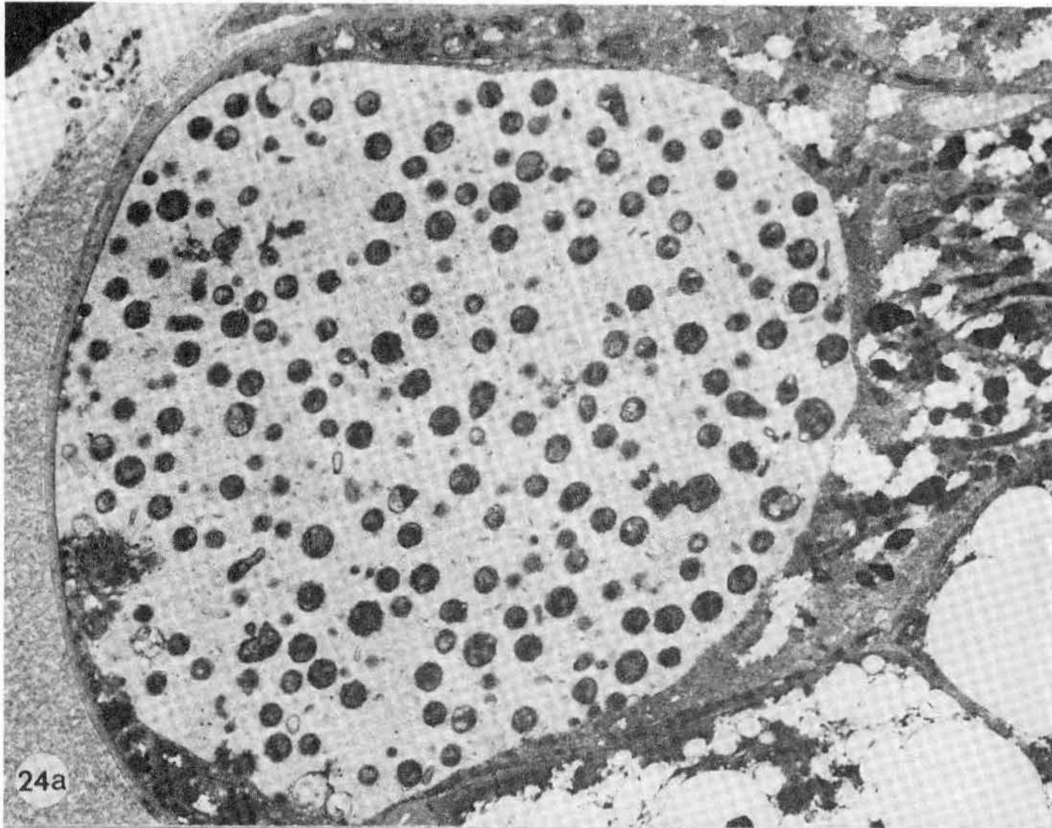

B
W
W
23b

Figure 24. Electron micrographs of colonies of _A. marginale_ in the midgut epithelial cells of nymphal _D. andersoni_ at 15 days post-repletion. The organisms are round with open spaces separating individual organisms. (a) x 6100. (b) x 8900.


24a

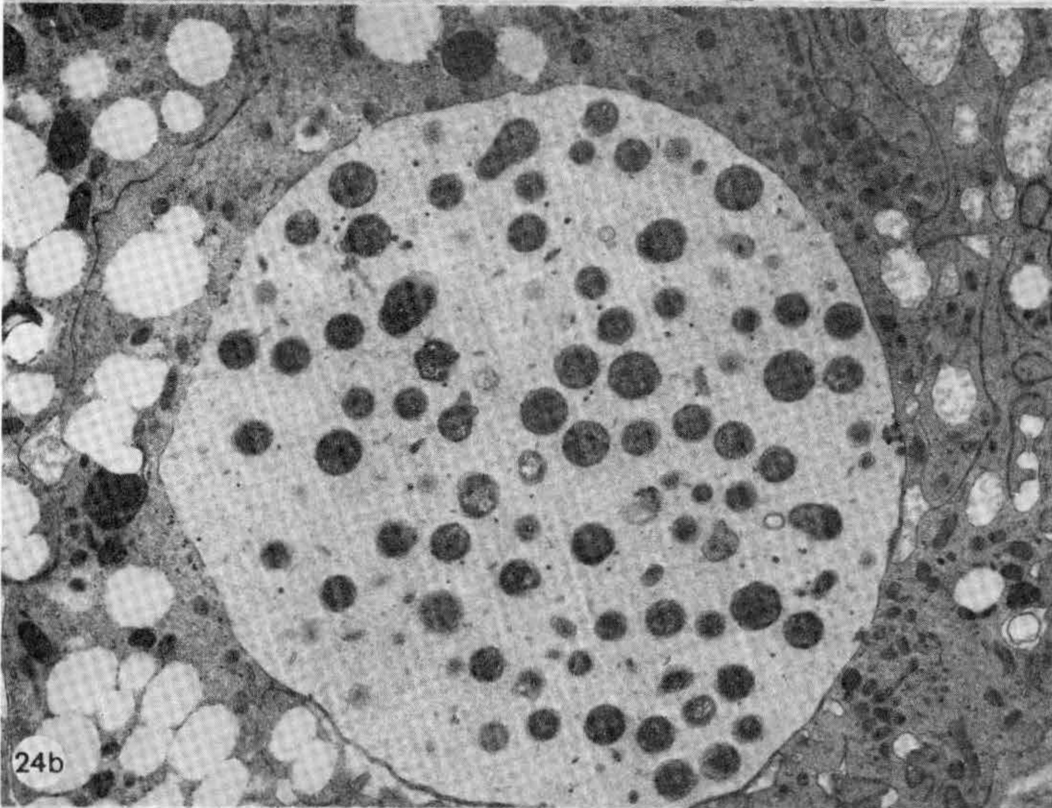
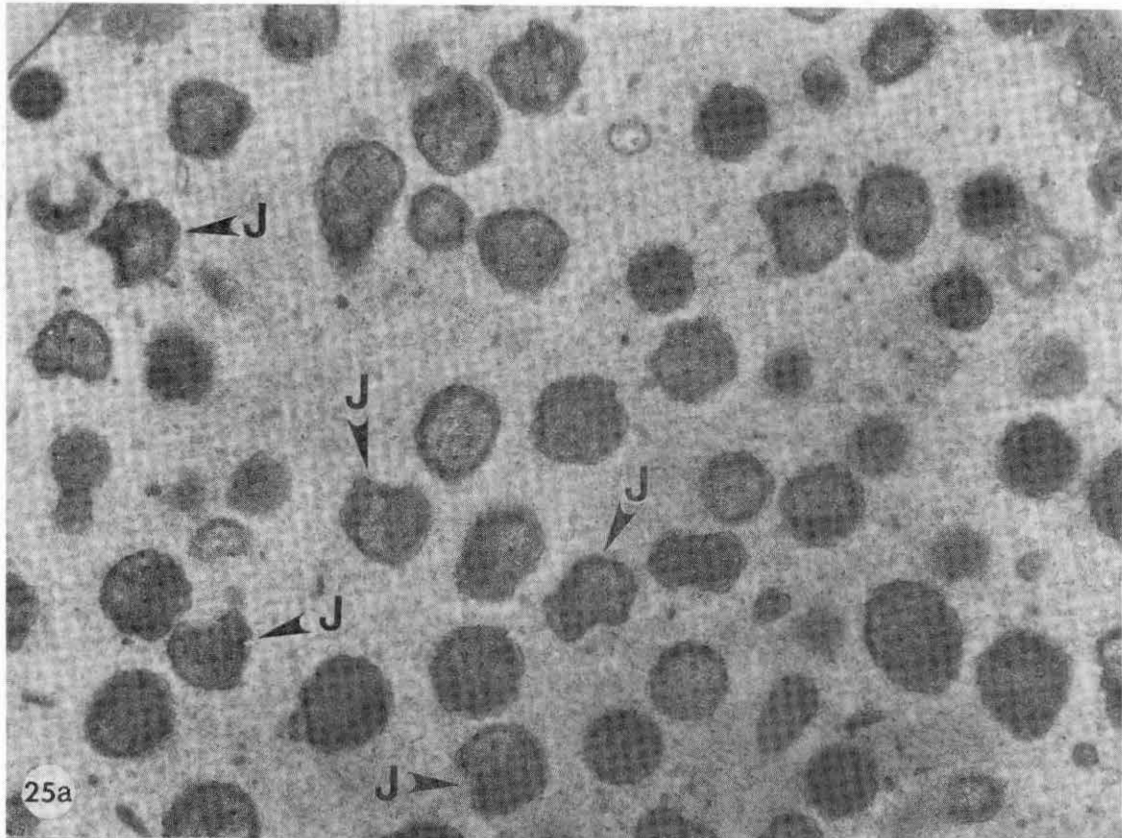
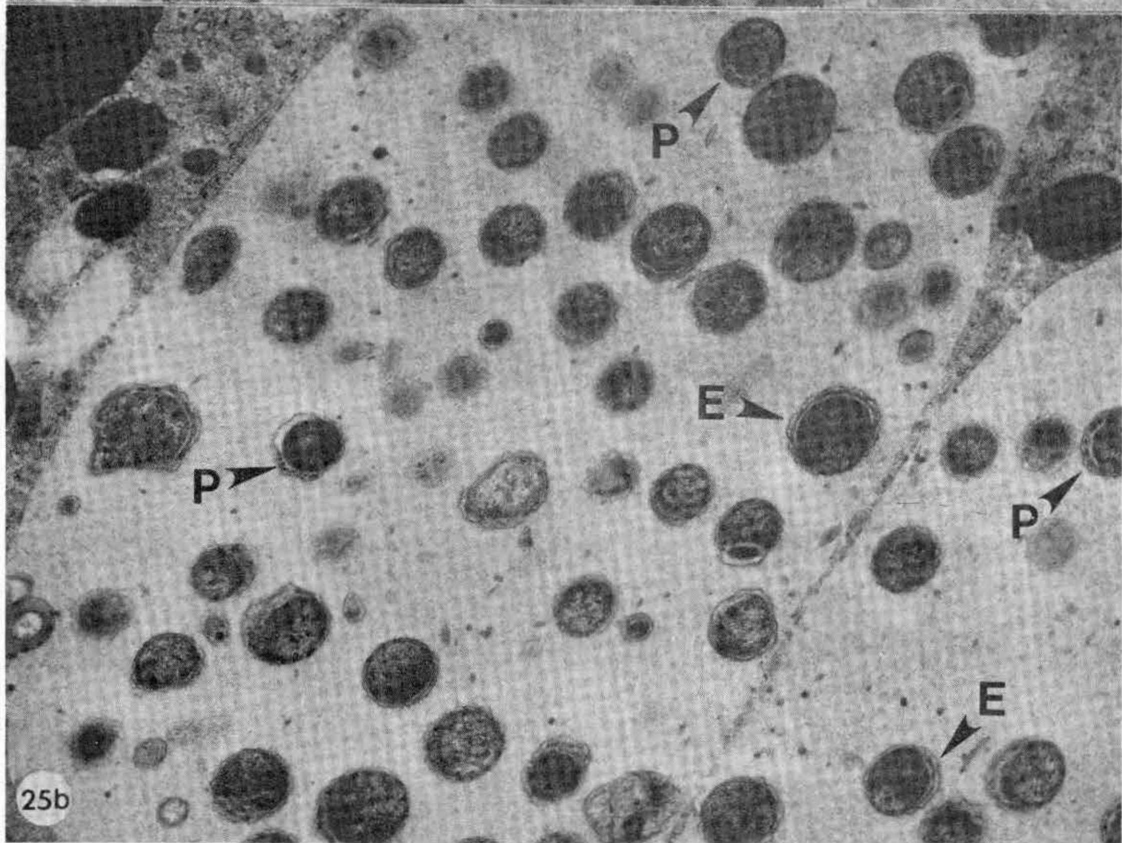

24b

Figure 25. Electron micrographs of _A. marginale_ organisms within colonies from nymphal _D. andersoni_ at 15 days post-repletion.

(a) The organisms (J) in some colonies exhibit irregular shapes. x 20,000.

(b) Small electron-dense particles (P) are seen within the limiting membrane of organisms. Organisms are subdivided on their margin by alternating light and dark bands (E). x 16,900.


J
J
J
J
J
25a


P
E
P
P
E
25b

bands were frequently seen within the limiting membrane of the organisms (Figure 25b). Small particles were also occasionally seen in the spaces between organisms. Clumps of organisms were likewise seen within certain colonies and these contained membrane whorls (Figure 26a). Organisms with clumps of small particles expanding within their limiting membranes were also observed (Figure 26b).

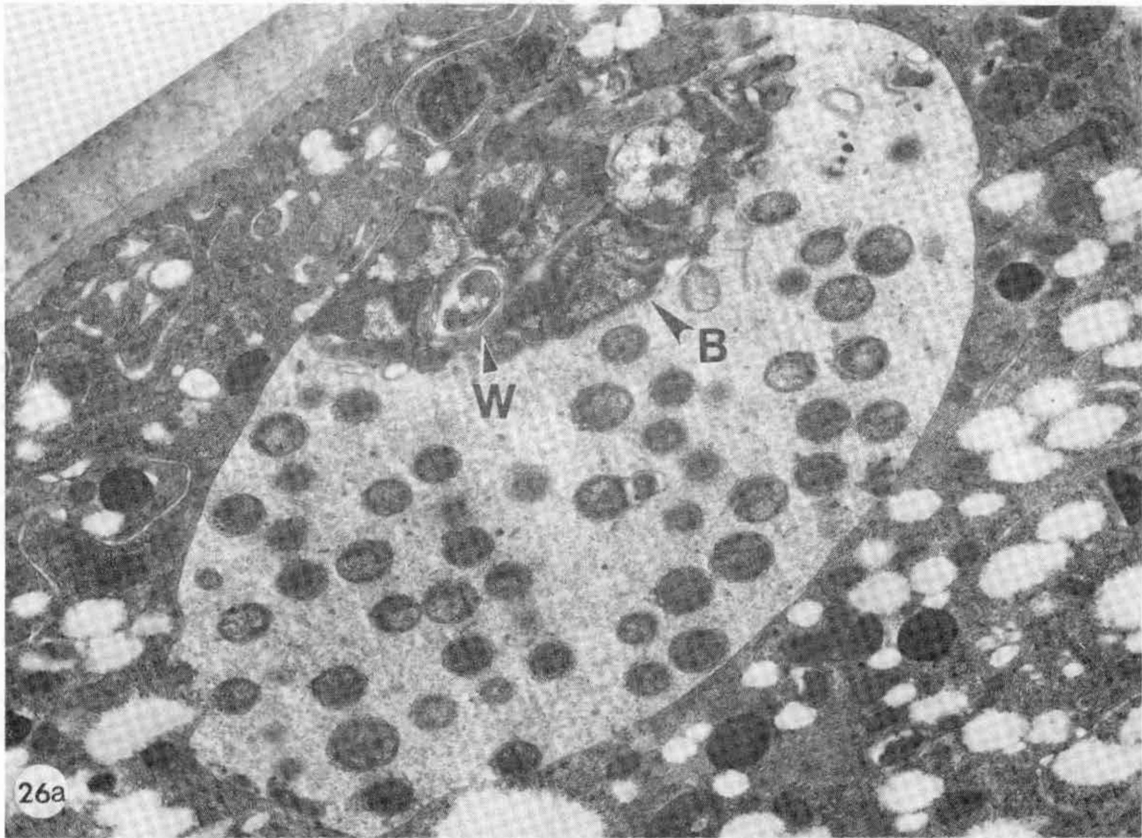
At 20 days post-repletion, colonies of A. marginale contained organisms that were different from those seen in ticks from groups that were replete for up to 15 days. Many of these organisms had an elongated, rod-shaped appearance (Figures 27a and b). Some colonies contained few organisms that were usually clustered in one area (Figure 28a). Others contained large clumps of organisms in which individual organisms were surrounded by membrane whorls (Figures 28b and 29a). In general, the organisms appeared to be smaller than those seen in ticks from earlier repletion groups. Most of the rod-shaped organisms had two cell membranes (Figures 29b and 30a), whereas others were surrounded by more than two cell membranes (Figure 30b). Some colonies contained more compact and irregularly-shaped organisms than usually seen (Figures 31a and b). Small electron-dense particles were seen within the limiting membrane of some organisms (Figures 31a and b, 32a and b) or between organisms (Figure 28b). Internal subdivisions of light and dark bands were also seen in certain organisms (Figures 32a and b). The margins of the limiting membranes of most organisms were smoother than those seen in ticks from groups that were replete for a shorter time.

Anaplasma organisms seen within colonies in the 25 day post-repletion group were similar to those seen at 20 days. These organisms were either elongated (Figure 33a) or were more compact and irregularly shaped

Figure 26. Electron micrographs of colonies of _A. marginale_ in the midgut epithelial cells of nymphal _D. andersoni_ at 15 days post-repletion.

(a) A clump of organisms (B) is seen that contains membrane whorls (W). x 13,000.

(b) Some organisms contain clumps of small electron-dense particles (CP) expanding within the limiting membrane. x 31,100.


B
W
26a

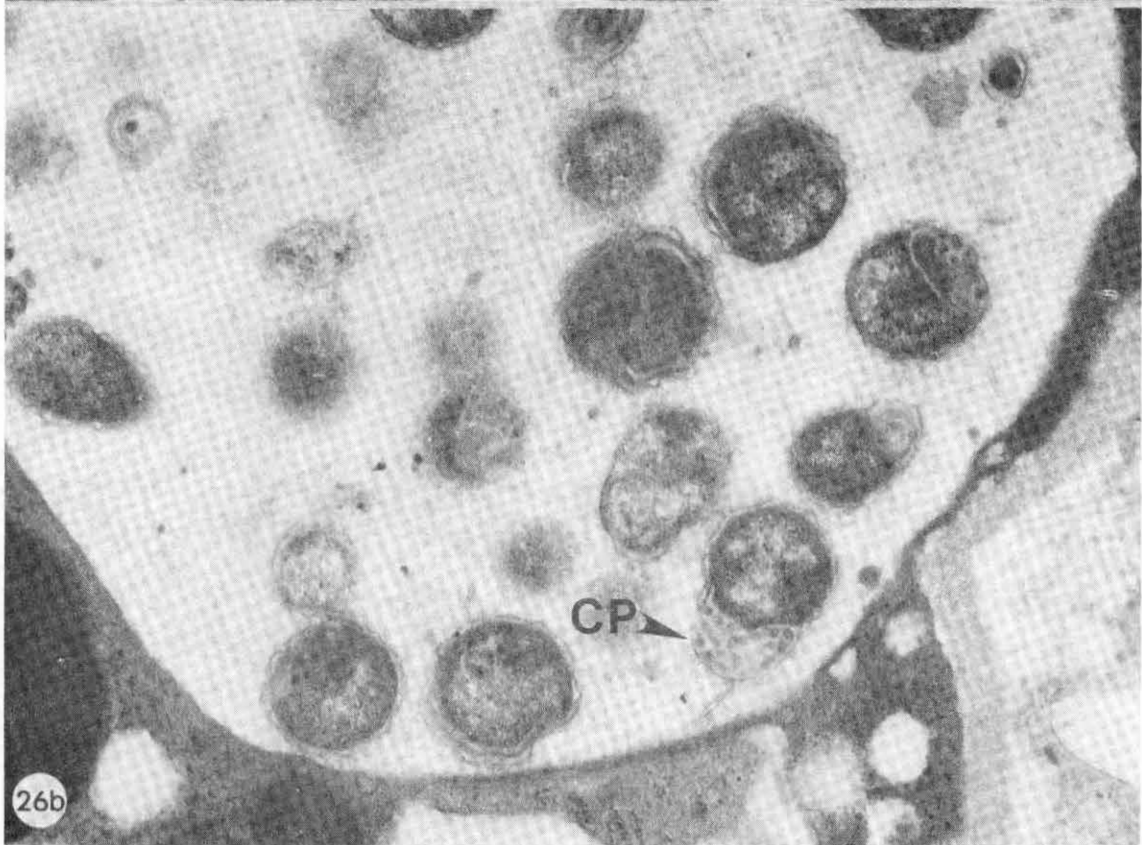
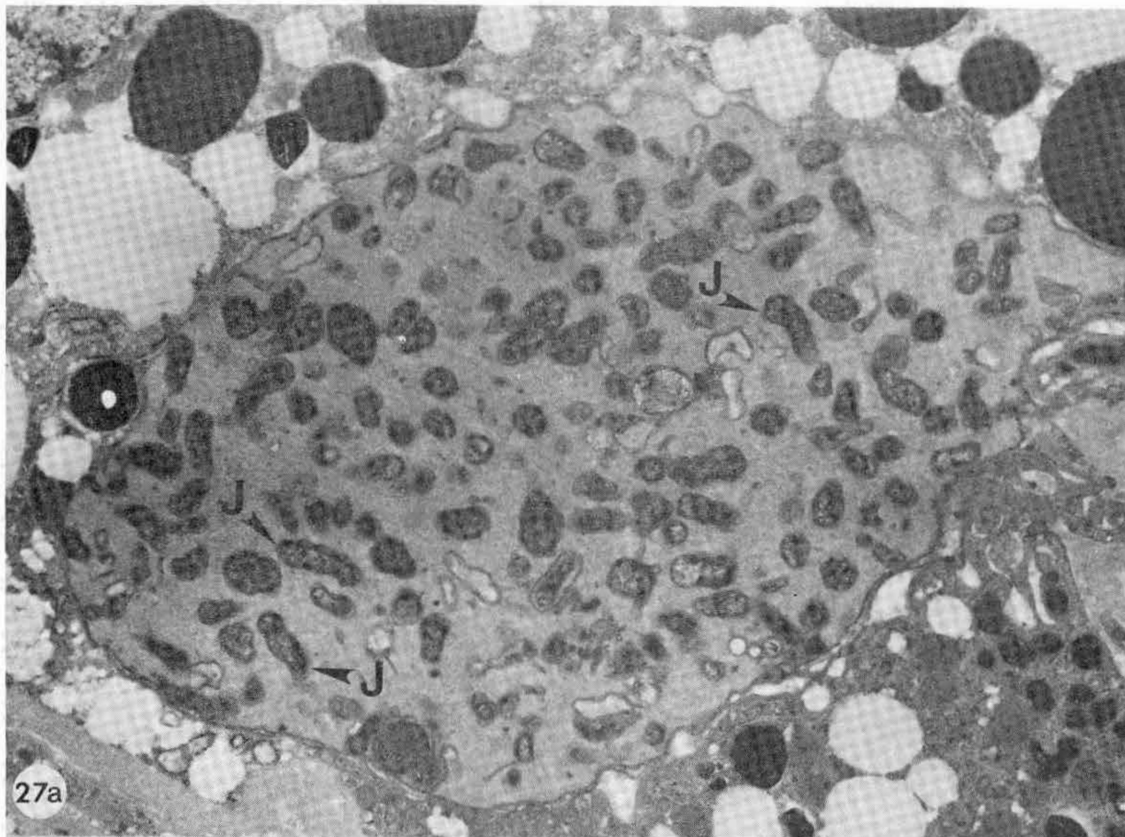

CP
26b

Figure 27. Electron micrographs of a colony of _A. marginale_ in the midgut epithelial cells of nymphal _D. andersoni_ at 20 days post-repletion. Many of the organisms (J) have an elongated, rod-shaped appearance. (a) x 10,000. (b) x 17,300.


J
J
J
27a

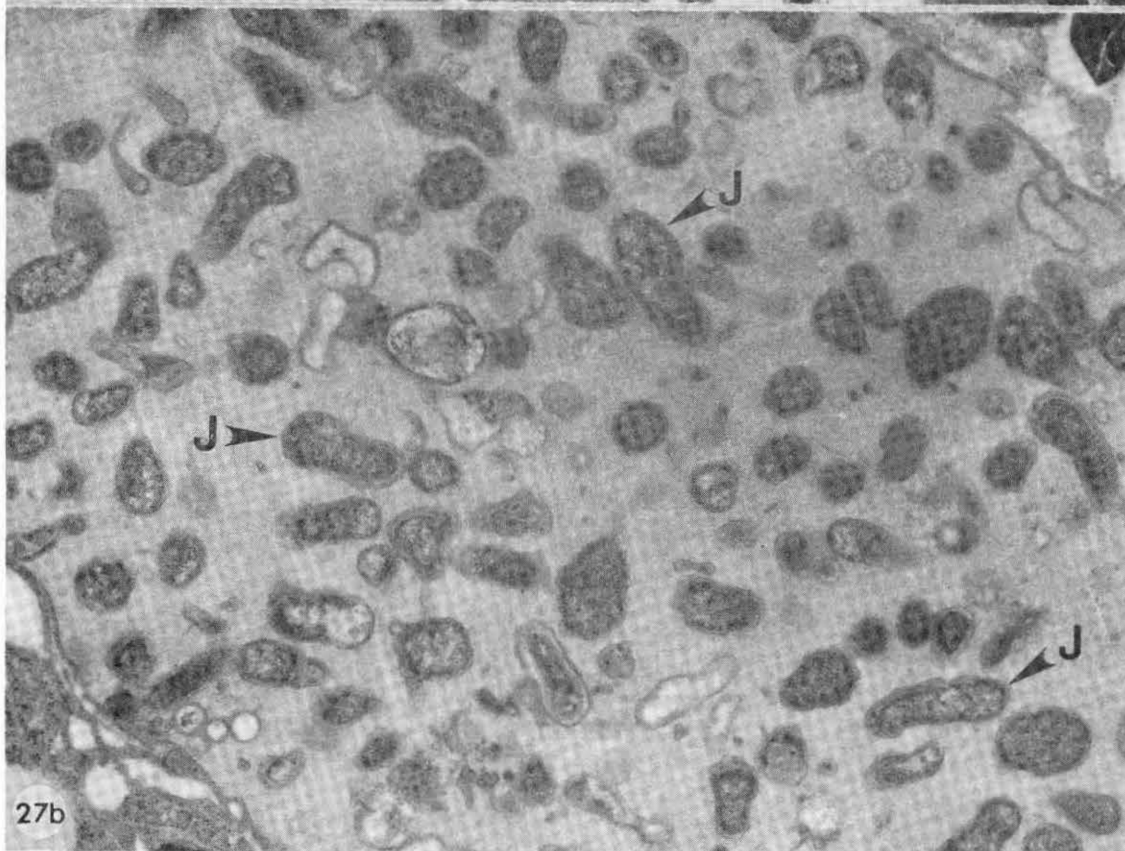
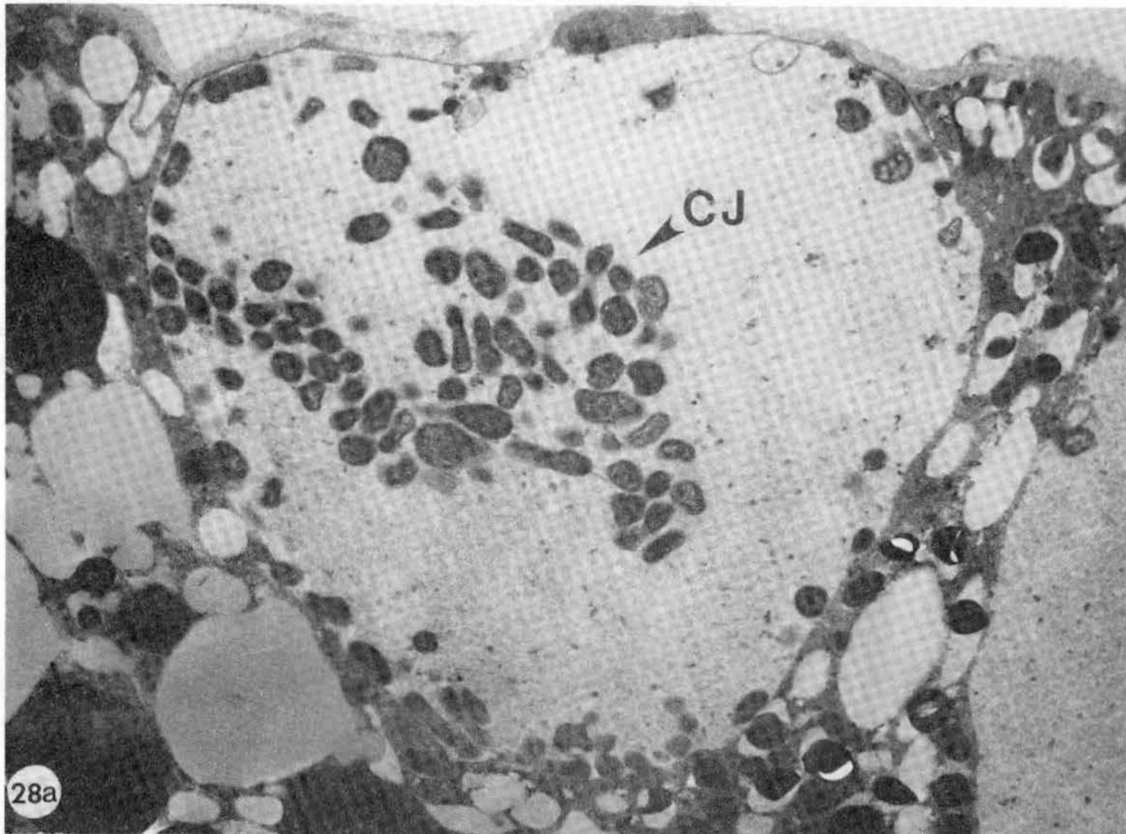

J
J
J
27b

Figure 28. Electron micrographs of colonies of *A. marginale* in the midgut epithelial cells of nymphal *D. andersoni* at 20 days post-repletion.

(a) Some colonies contain a few organisms clustered in one area (CJ). x 17,100.

(b) A large clump of organisms within a colony contains individual organisms surrounded by membrane whorls (W). Small electron-dense particles (P) are seen between organisms. x 17,000.


CJ
28a

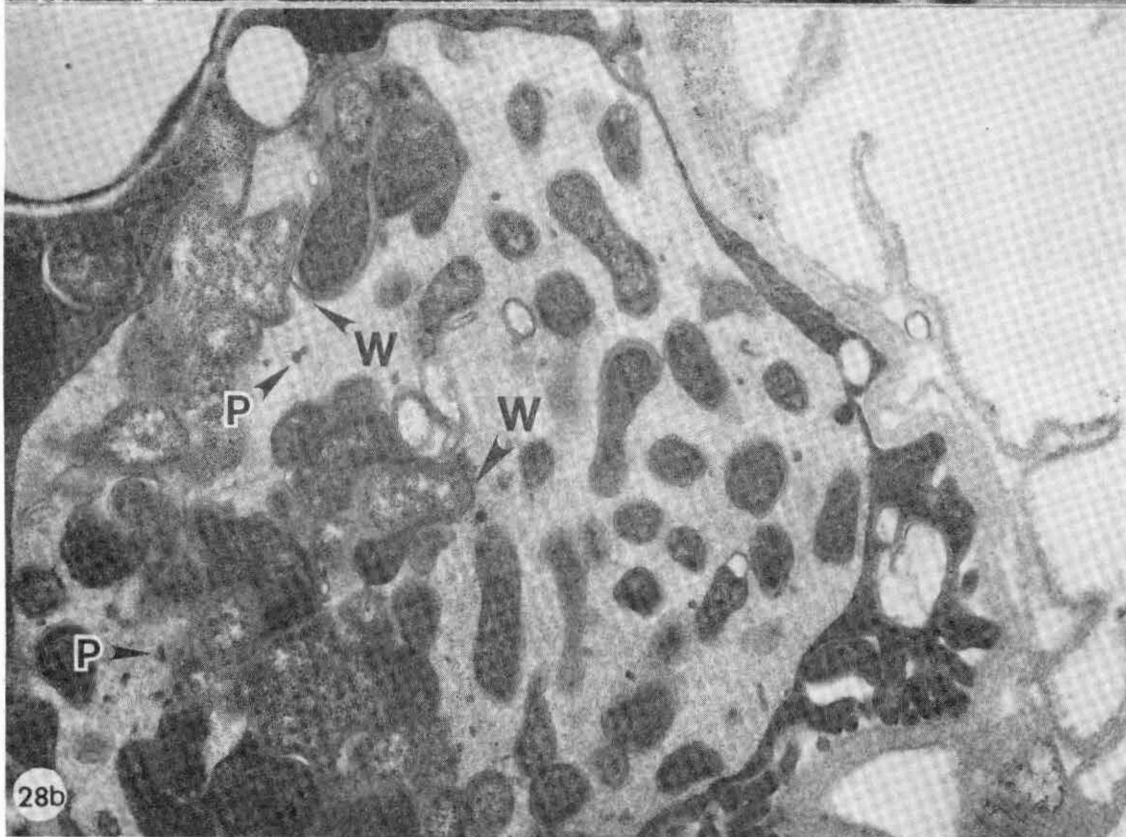
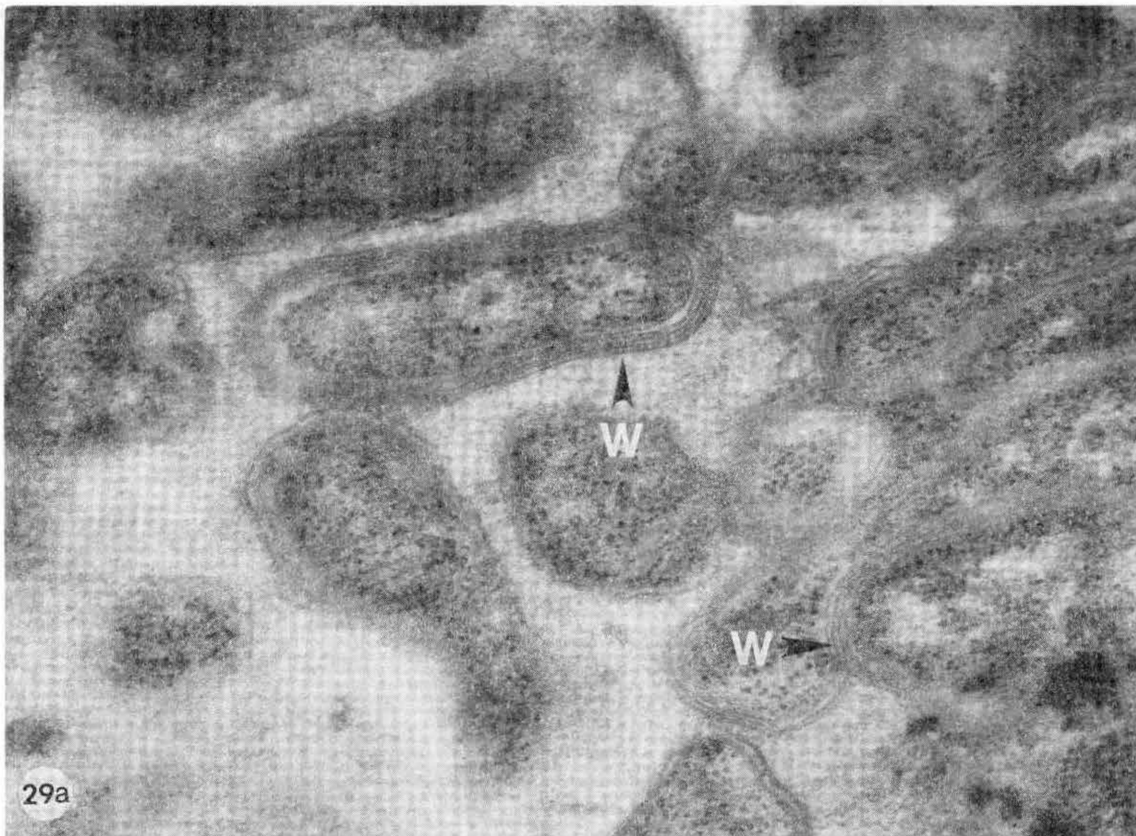

W
P
W
P
28b

Figure 29. Electron micrographs of *A. marginale* organisms within colonies from nymphal *D. andersoni* at 20 days post-repletion.

(a) Individual organisms from a clump are surrounded by membrane whorls (W). x 53,600.

(b) Most rod-shaped organisms are surrounded by two cell membranes (M). x 21,800.


W
W
29a

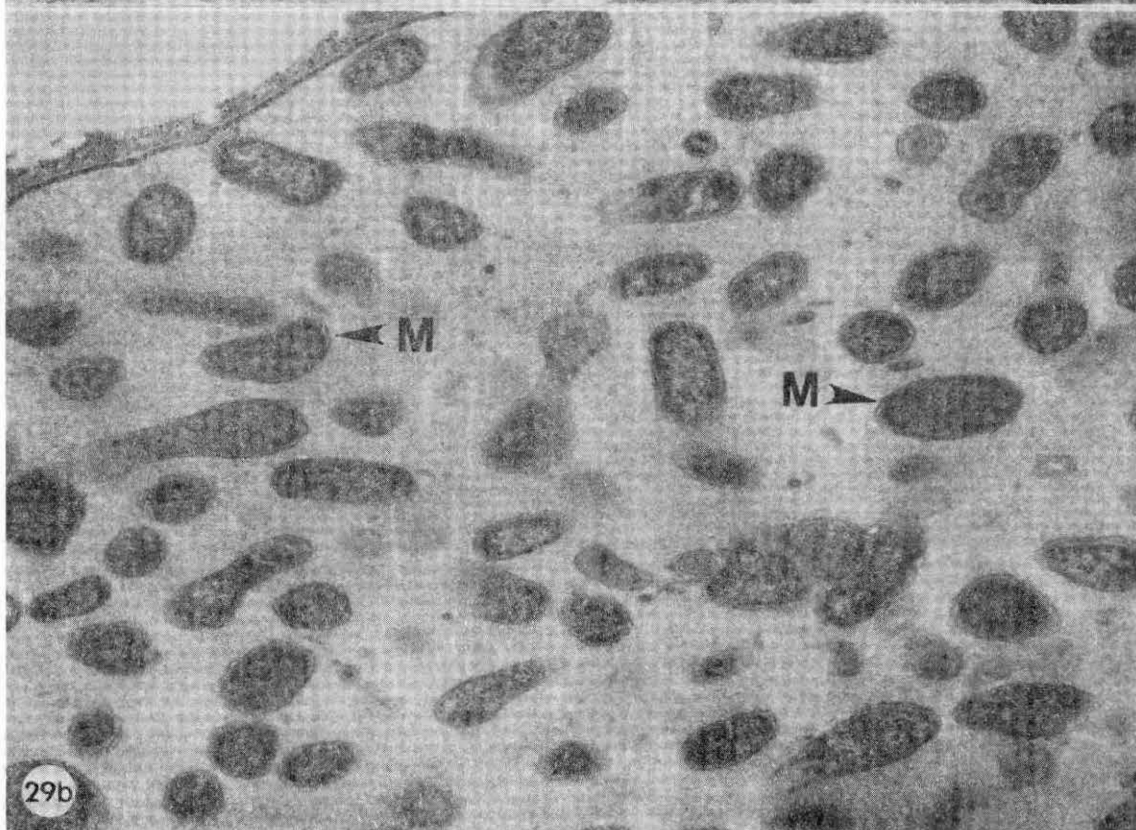
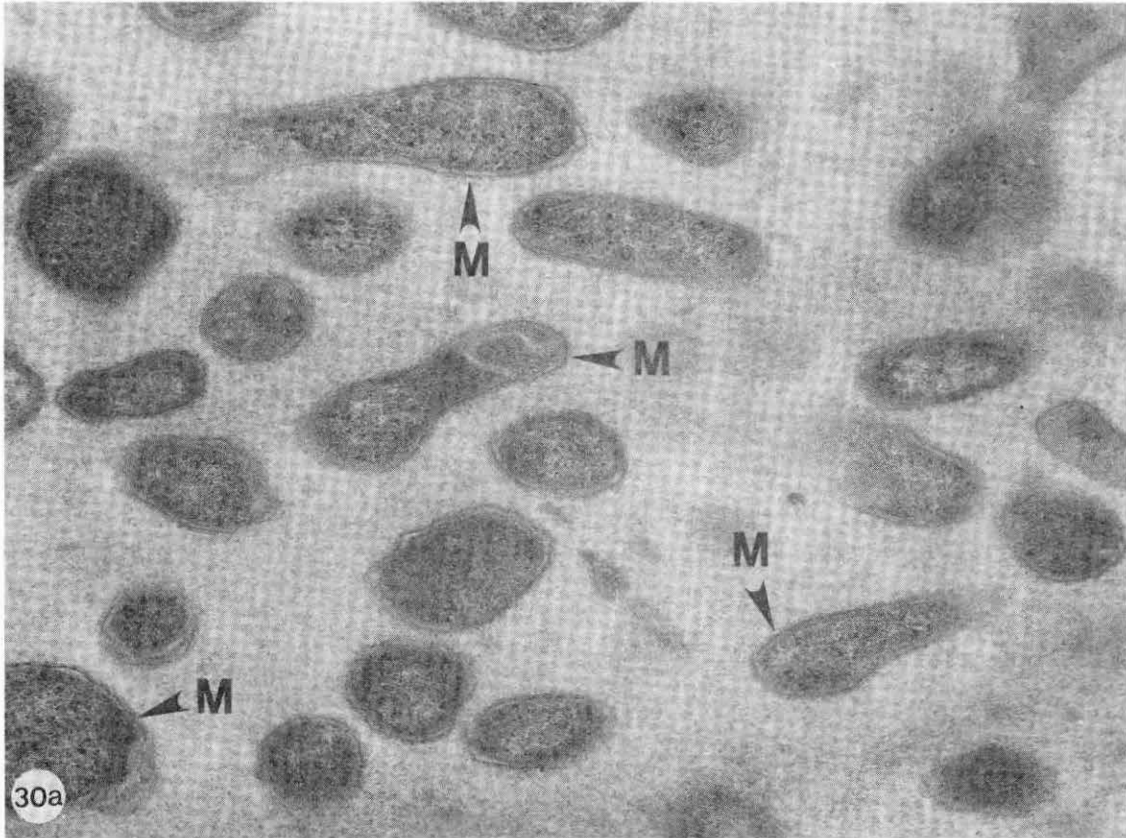

M
M
29b

Figure 30. Electron micrographs of *A. marginale* organisms within colonies from nymphal *D. andersoni* at 20 days post-repletion.

(a) Most rod-shaped organisms are surrounded by two cell membranes (M). x 38,700.

(b) Some organisms are surrounded by more than two cell membranes (D). x 39,700.


M
M
M
M
30a

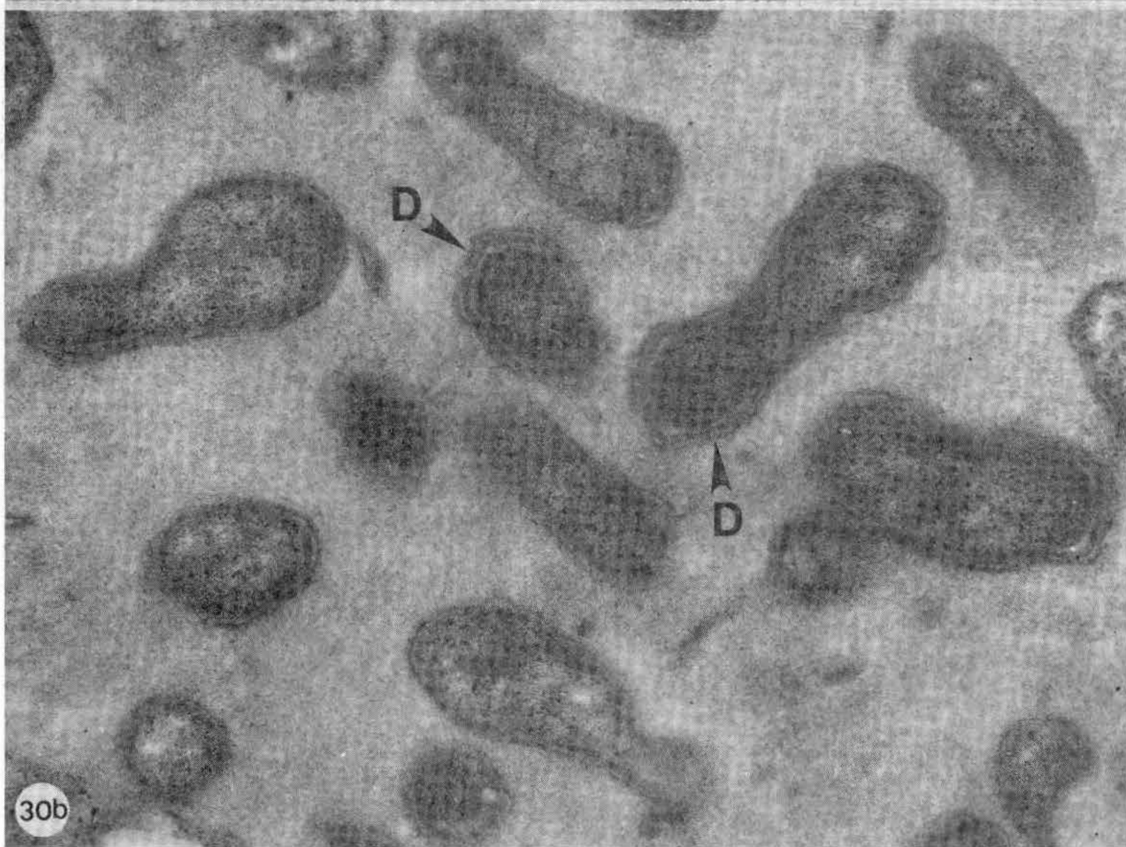
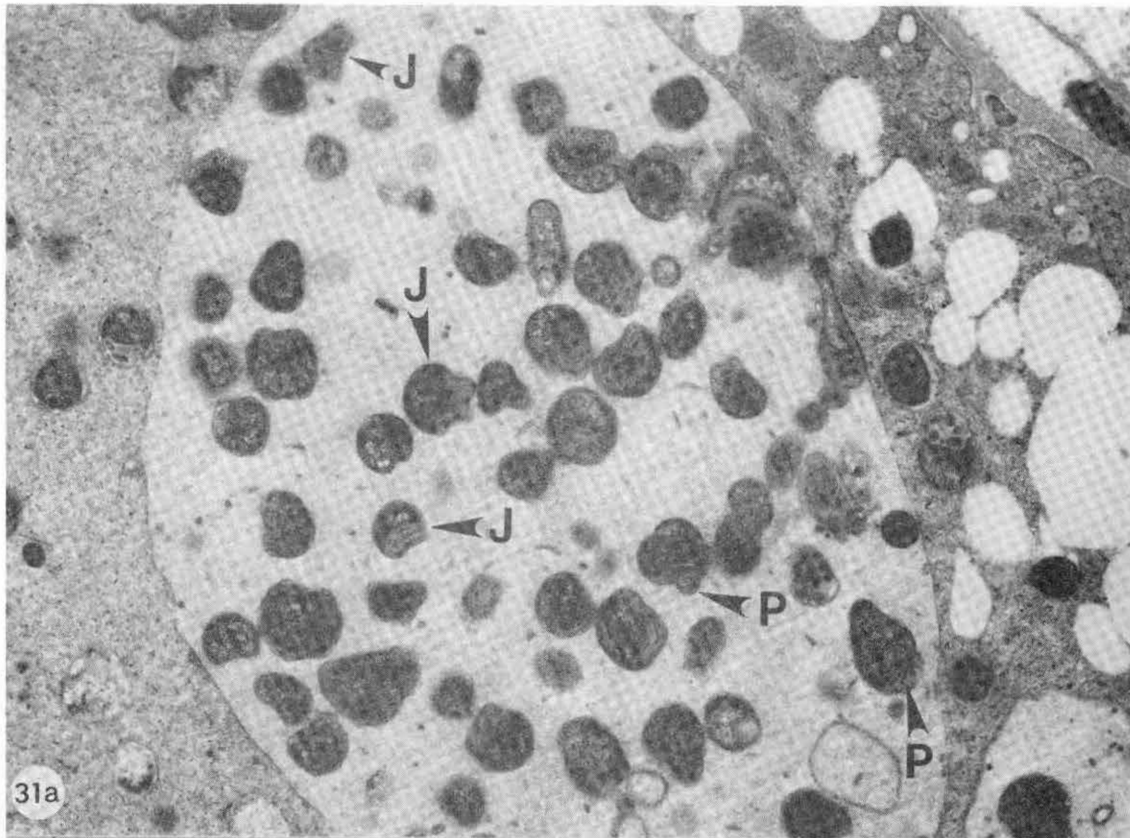

D
D
30b

Figure 31. Electron micrographs of colonies of _A_. _marginale_ in the midgut epithelial cells of nymphal _D_. _andersoni_ at 20 days post-repletion. Some colonies contain more compact and irregularly-shaped organisms (J) than usually seen. Small electron-dense particles (P) are present within the limiting membrane of some organisms. (a) x 13,300. (b) x 9900.


J
J
J
P
P
31a

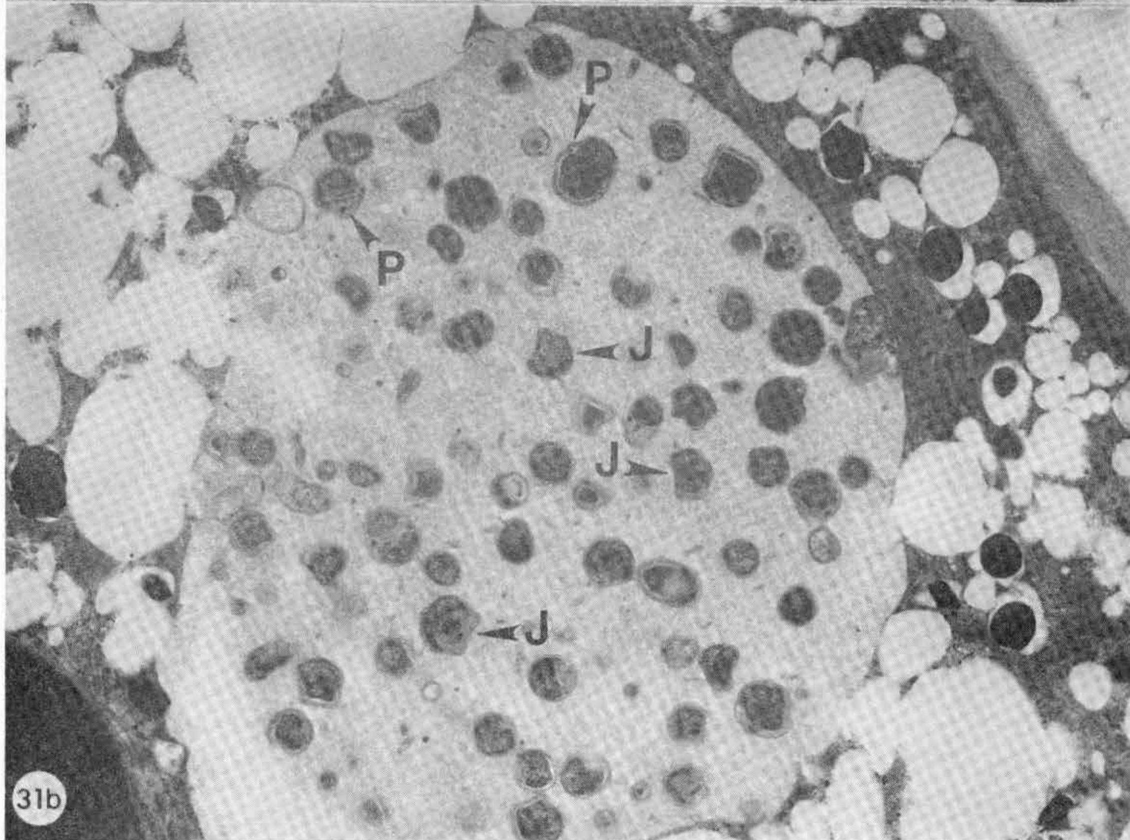
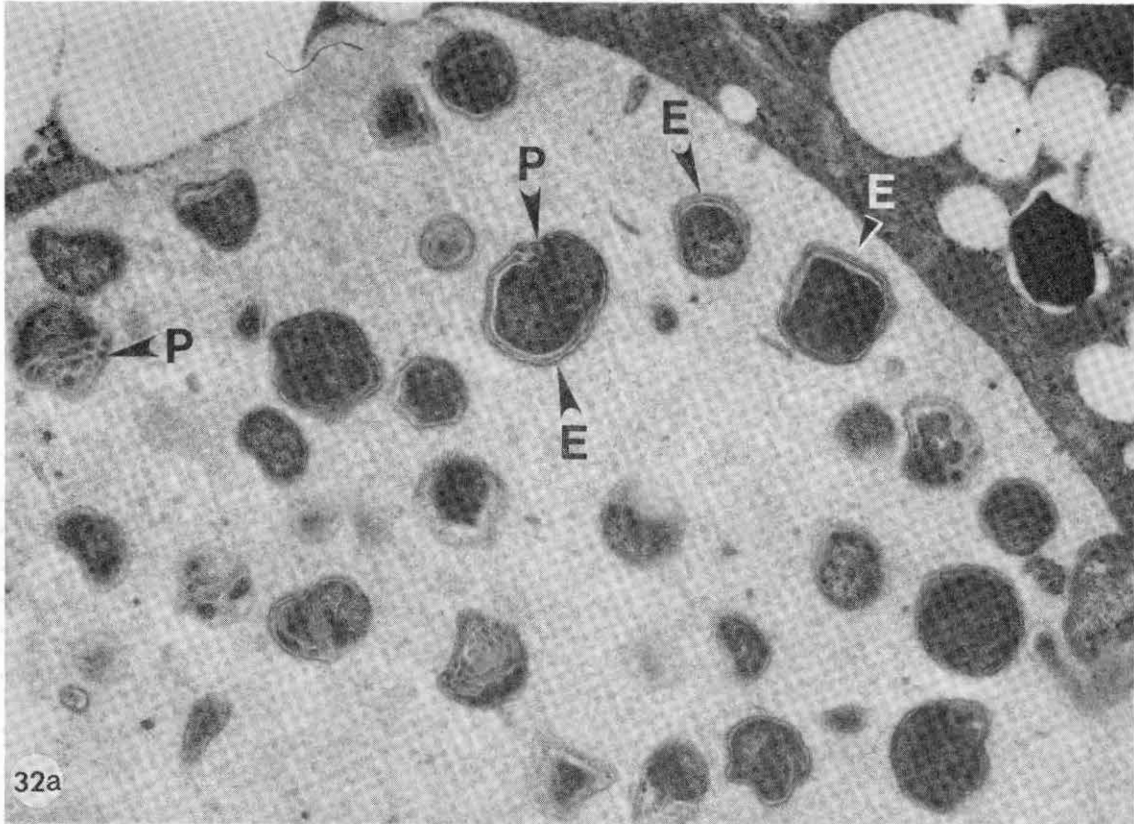

P
P
J
J
J
31b

Figure 32. Electron micrographs of _A. marginale_ organisms within colonies from nymphal _D. andersoni_ at 20 days post-repletion. Small electron-dense particles (P) are present within the limiting membrane of some organisms. Organisms are subdivided on their margin by alternating light and dark bands (E). (a) x 19,100. (b) x 18,900.


P
E
E
P
E
32a

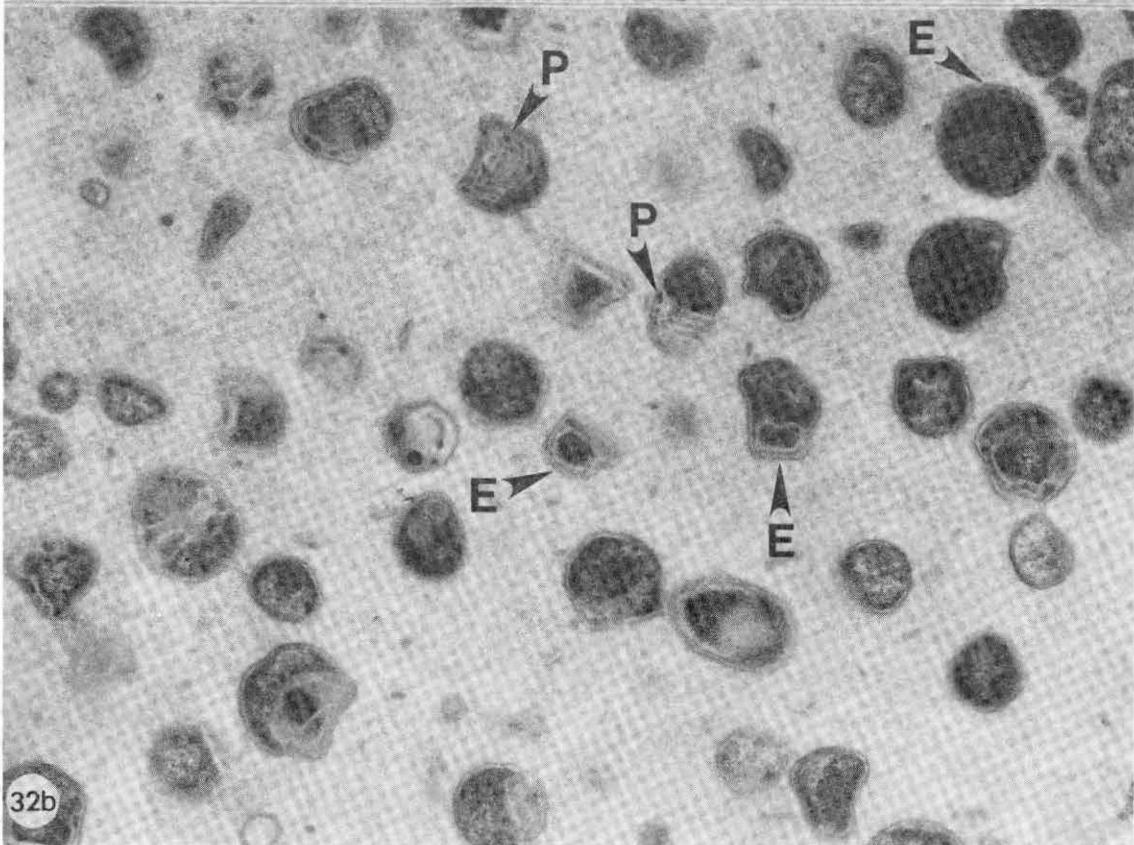
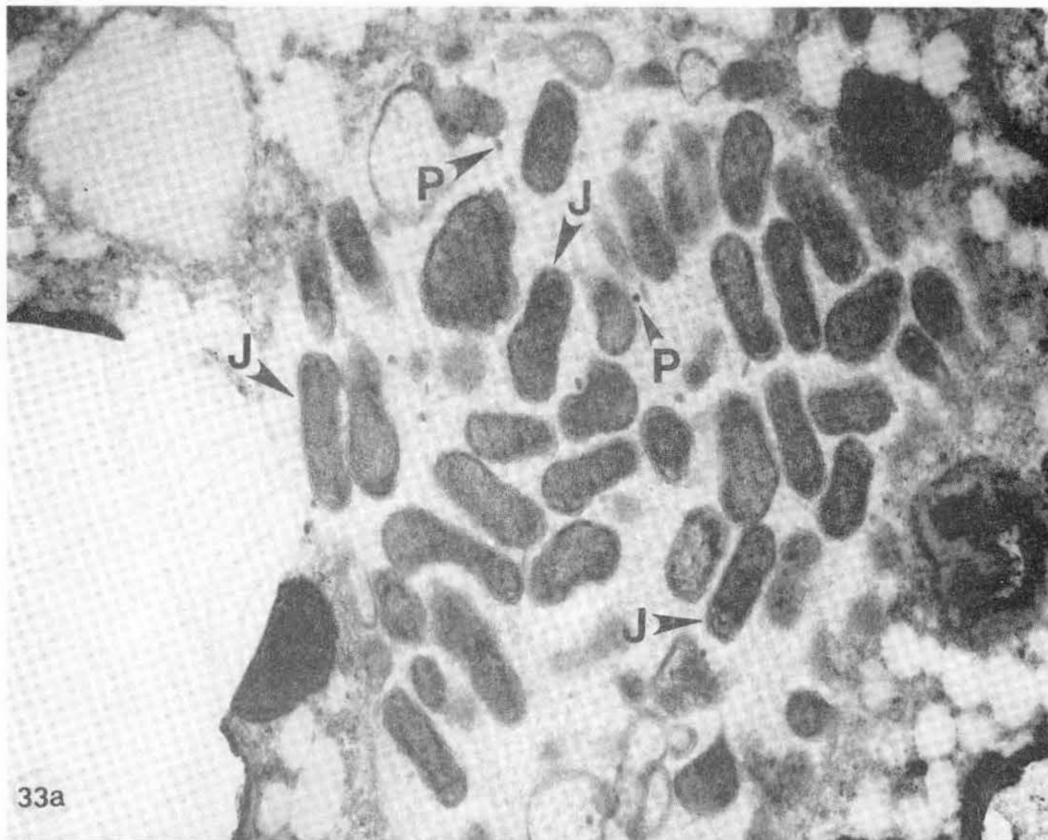
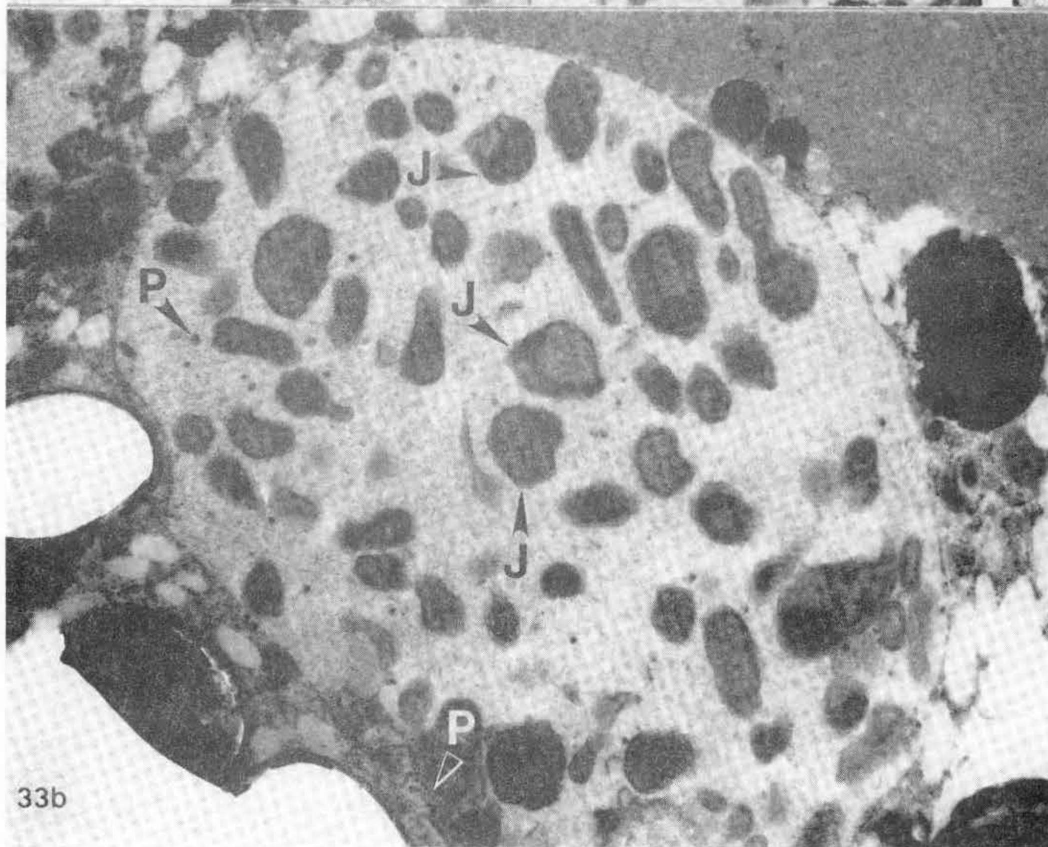

P
E
P
E
E
32b

Figure 33. Electron micrographs of colonies of *A. marginale* in the midgut epithelial cells of nymphal *D. andersoni* at 25 days post-repletion. Small electron-dense particles (P) are seen in the spaces between organisms.

(a) Most of the organisms (J) in this colony are elongated. x 18,200.

(b) Some organisms (J) in this colony are more compact and irregularly shaped. x 15,500.


P
J
J
P
J
33a


J
P
J
J
P
33b

(Figure 33b). Organisms appeared to be similar in size to those seen in the 20 day group. Some colonies contained clumps of organisms in which individual organisms were surrounded by membrane whorls (Figures 34a and b). Internal subdivision of organisms was occasionally seen (Figure 34b). Small electron-dense particles occurred within the limiting membrane of organisms (Figure 35) as well as in spaces between organisms (Figures 33a and b) as was previously described.

Colony Density and Distribution

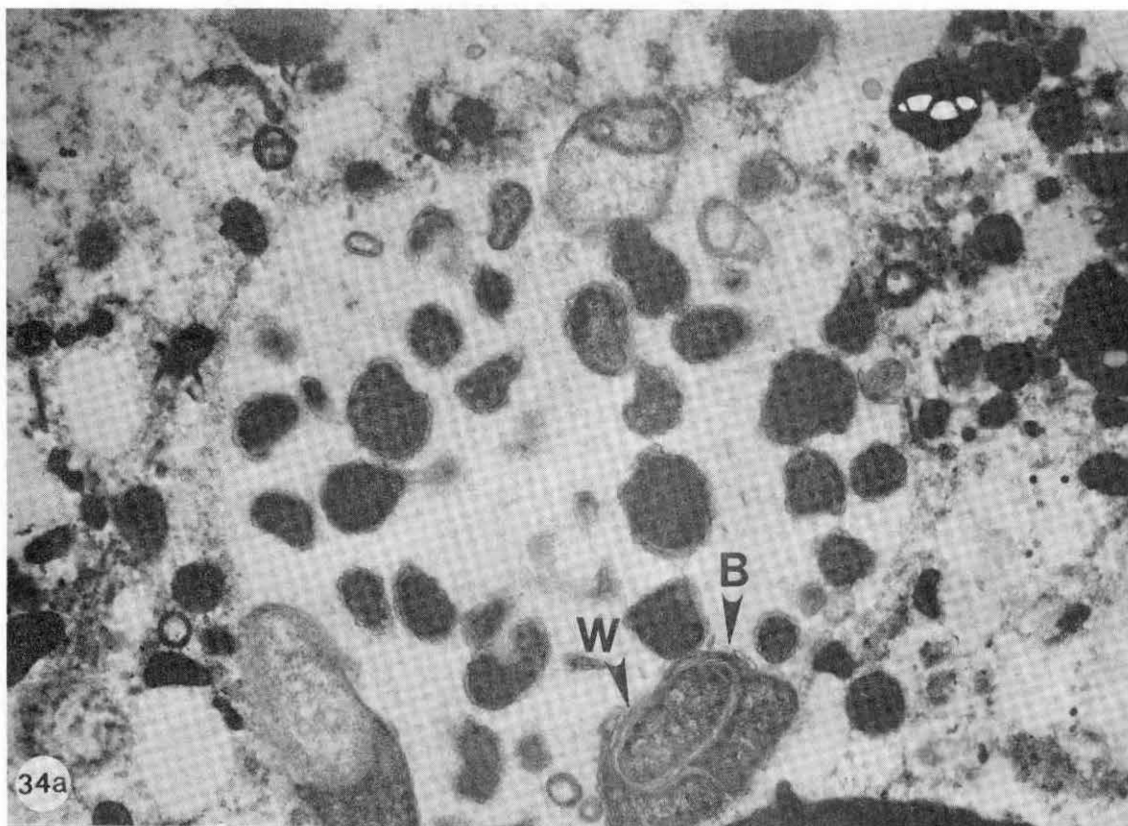
Mean colony densities (number of colonies per 0.1 mm^2 gut tissue examined) found in gut cross-sections from nymphal ticks at various repletion times are listed in Table VI. Colonies were present in ticks collected on each repletion day (5 to 25 days); colonies were not observed in gut tissues from control ticks. Mean colony densities increased to a peak of 2.25 at 15 days post-repletion, decreased slightly at 20 days, and then dropped sharply at 25 days (Figure 36). The standard error of the mean (SEM) was low for most repletion days but was higher at 15 and 20 days post-repletion, the time at which the highest mean colony densities were observed.

Table VII shows the analysis of variance for mean colony densities in replete nymphal ticks. The differences in colony density on the various days of repletion were not statistically significant. Even though statistically significant differences were not demonstrated with analysis of variance, the mean colony densities in gut tissue from ticks of the different repletion groups reflected a significant quadratic trend ($P = 0.03$). This could be illustrated in Figure 36 by hypothetically connecting the midpoints of the peaks for each repletion group with a line. A

Figure 34. Electron micrographs of a colony of _A. marginale_ in the midgut epithelial cells of nymphal _D. andersoni_ at 25 days post-repletion.

(a) A clump of organisms (B) contains an individual organism surrounded by membrane whorls (W). x 16,000.

(b) A clump of organisms (B) contains an individual organism surrounded by membrane whorls (W). A few organisms are subdivided on their margin by alternating light and dark bands (E). x 19,900.


B
W
34a

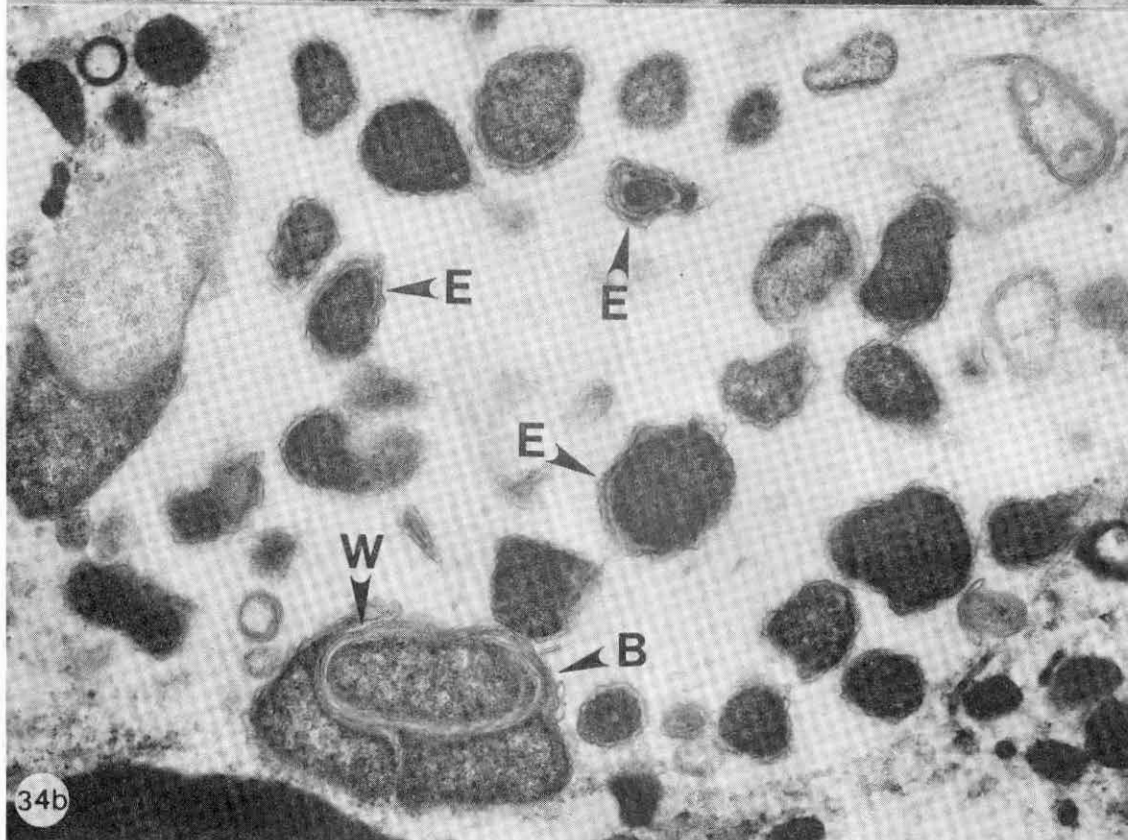

E
E
E
W
B
34b

Figure 35. Electron micrograph of *A. marginale* organisms within a colony from nymphal *D. andersoni* at 25 days post-repletion. A small electron-dense particle (P) is seen within the limiting membrane of an organism. x 37,200.

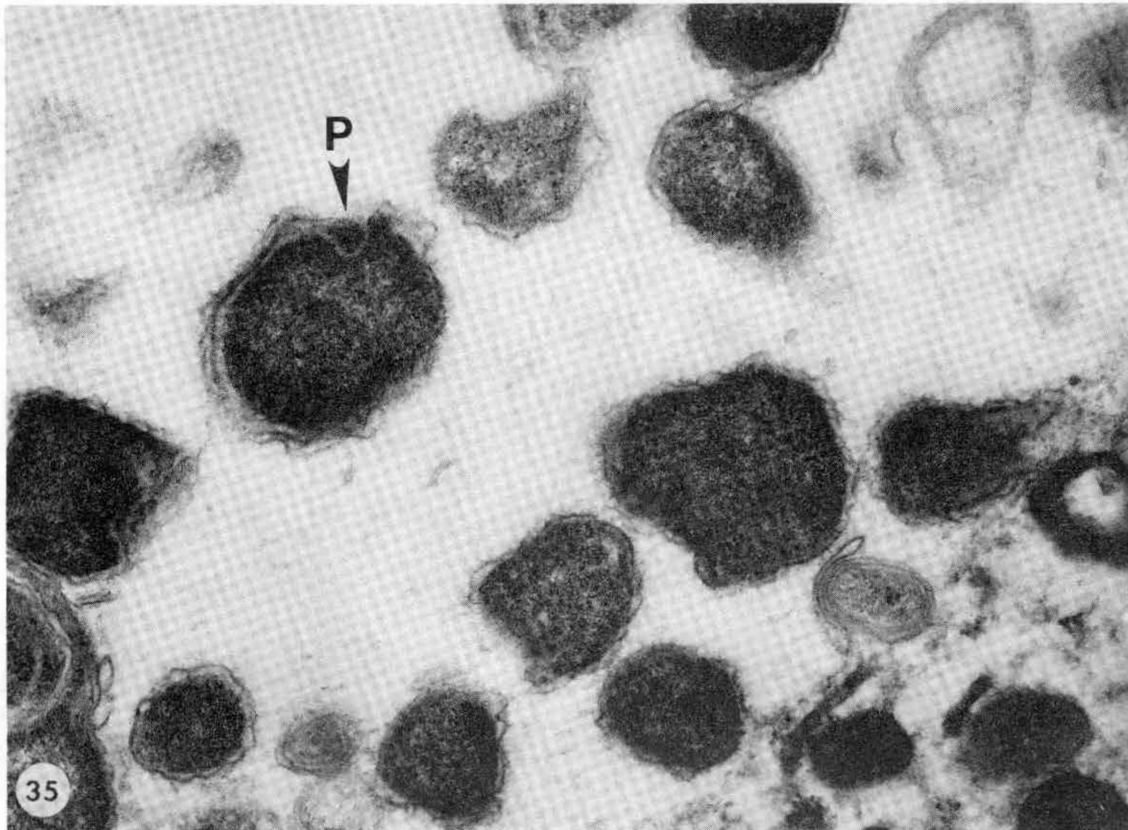

P
35

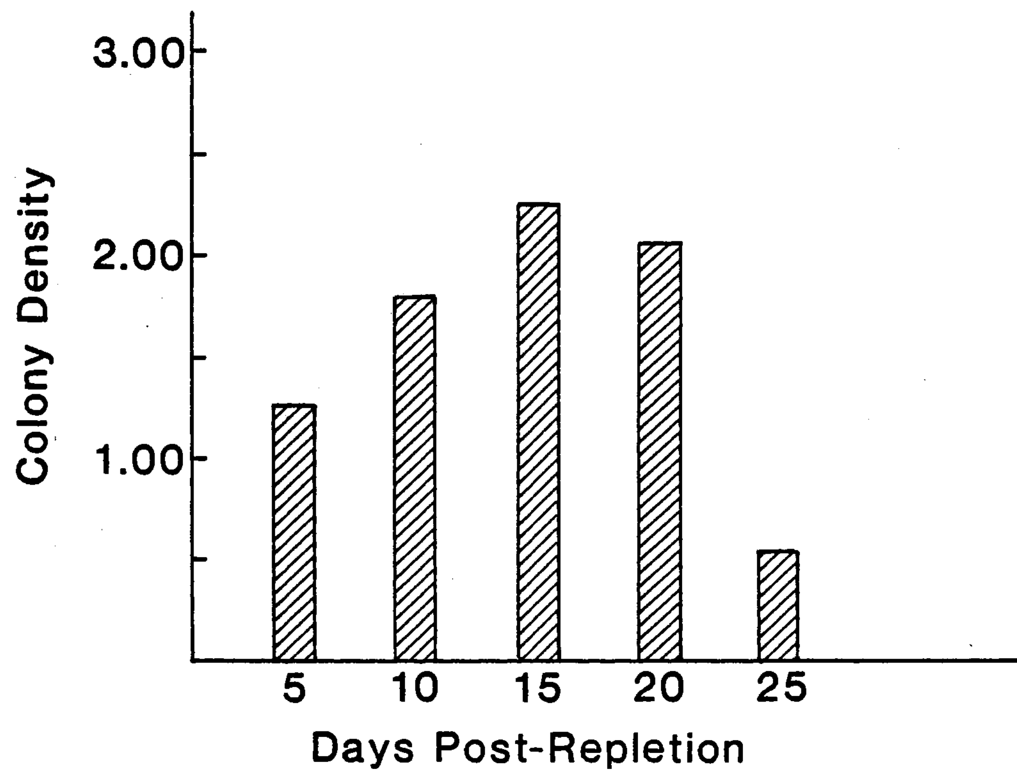
TABLE VI

MEAN COLONY DENSITY* IN NYMPHAL DERMACENTOR ANDERSONI THAT WERE REPLETE FOR 5 TO 25 DAYS AFTER FEEDING ON A CALF INFECTED WITH ANAPLASMA MARGINALE

Days Post-Repletion	No. of Ticks Per Group	Mean Colony Density	Range	SEM
5	10	1.33	0.29-4.22	0.41
10	10	1.79	0.49-3.62	0.27
15	10	2.25	0.08-7.82	0.76
20	10	2.07	0.27-8.06	0.79
25	10	0.56	0.00-1.27	0.11

*Colony density (mean number of colonies per 0.1 mm^2 of gut tissue examined).

Figure 36. Mean colony density (number of colonies per 0.1 mm^2 gut tissue examined) in nymphal *D. andersoni* that were replete for 5 to 25 days after feeding on a calf infected with *A. marginale*.



3.00
2.00
1.00
Colony Density
5
10
15
20
25
Days Post-Repletion

TABLE VII

ANALYSIS OF VARIANCE OF THE NUMBER OF COLONIES PER 0.1 MM^2 GUT TISSUE EXAMINED FROM NYMPHAL *DERMACENTOR ANDERSONI* THAT WERE REPLETE FOR 5 TO 25 DAYS AFTER FEEDING ON A CALF INFECTED WITH *ANAPLASMA MARGINALE*

Source of Variation	DF	Sum of Squares	Mean Square	F	P Value
Total	49	1.49	---	---	---
Days Post-Repletion	4	0.19	0.05	1.6	0.189
Error	45	1.30	0.03	---	---

curve would be produced that indicated a quadratic trend. The existence of this trend was supported by a pairwise comparison of the different repletion groups. The P values suggested that ticks from the 15 and 20 day post-repletion groups had significantly greater mean colony densities than did those from the 5, 10, and 25 day groups.

Mean colony areas for each repletion group are listed in Table VIII. Slight differences were observed in the colony areas between the five repletion groups, but the means were not statistically different. Values for the standard error of the mean (SEM) were high due to the wide variation in colony areas observed within each repletion group.

TABLE VIII

MEAN COLONY AREA IN NYMPHAL *DERMACENTOR ANDERSONI* THAT WERE REPLETE FOR 5 TO 25 DAYS AFTER FEEDING ON A CALF INFECTED WITH *ANAPLASMA MARGINALE*

Days Post-Repletion	No. of Colonies Measured	Colony Area (μm^2)*		
		Mean	Range	SEM
5	310	100.5	78.7-195.8	10.8
10	420	87.6	52.8-140.5	8.4
15	412	88.9	36.0-119.0	7.9
20	473	101.9	30.4-253.4	19.1
25	115	99.6	53.2-144.0	10.4

*Colony area measured as a rectangle using longest and shortest diameters.

CHAPTER IV

DISCUSSION

Colonies of *Anaplasma marginale* have been described previously in the midgut epithelial cells of adult *Dermacentor andersoni* infected as nymphs.[90,91] In the present study, colonies were found in adults that fed for 3 to 6 days as nymphs on a calf with anaplasmosis. These colonies were similar to those described by Kocan et al.[91] and included all five morphologic colony types. Apparently, three days of feeding were required for nymphs to acquire sufficient numbers of organisms to give rise to colony levels detectable by light microscopy in subsequently-molted adults. In a recent study on effects of elevated temperature on colony formation in adult *D. andersoni* that were infected as nymphs, type 3 colonies were most prevalent in ticks following incubation for 2.5 days at 37 C.[92] In the present study in which ticks were also incubated for 2.5 days at 37 C, 89% of the colonies observed were type 3. Electron microscopy examination of type 3 colonies revealed the presence of electron-dense forms, reticulated forms, and small particles, all typical of type 3 colonies described previously.[91]

The process of feeding ticks on calves with high *A. marginale* parasitemia levels has been shown to result in larger numbers of colonies than when ticks are fed on calves with low levels of parasitemia.[82] The donor calf used in this study had its highest parasitemia levels of 48.2% and 45.6% on the last two days of nymphal feeding (days 5 and 6) just

prior to the transfer of ticks to an uninfected calf. The parasitemia levels on days 5 and 6 were comparable to the high parasitemia levels reported previously to result in maximal colony development.[82] A susceptible calf on which nymphs from the last two transfer days were fed developed anaplasmosis. This suggests that high parasitemia levels are necessary for ticks feeding on an infected calf to acquire sufficient numbers of organisms to transfer the infection to another susceptible animal. However, in this case transfer of parasites to an uninfected calf may have been due to mechanical transmission of organisms via blood-contaminated mouthparts, rather than through normal biological development of the organisms in ticks.

Gut homogenates prepared from adult D. andersoni infected with A. marginale as nymphs have been shown to produce typical anaplasmosis in susceptible calves.[78,79,81] Gut homogenates prepared in this study from unfed, incubated adult ticks that fed 2 to 6 days as nymphs on an infected calf produced anaplasmosis when inoculated into susceptible calves. A minimum of two days of feeding was thus necessary for the nymphs to acquire enough A. marginale organisms to transmit infections to susceptible calves. With the exception of the transfer day 4 group, the prepatent periods in inoculated calves decreased from the day 2 group to the day 6 one. These data suggest that nymphs ingest more infective organisms with increased feeding time, especially when feeding on hosts with high parasitemia levels. Prepatent periods have been shown to be shortened using gut homogenates from infected ticks that were incubated for 2.5 days[81] and from ticks that fed on calves with high parasitemia levels.[82] The incubation times and parasitemia levels observed in the present study are consistent with the earlier studies in which shortened

prepatent periods occurred. Anaplasmosis infection did not occur with the gut homogenate from transfer day 1 ticks. If the parasitemia level had been higher on the first day of nymphal feeding, perhaps this gut homogenate might have been infective because more infective organisms would have been ingested by these ticks. The gut homogenate from transfer day 4 produced the longest prepatent period for unknown reasons; this may reflect the individual susceptibility of the calf used in this experiment.

The mean colony density (number of colonies per 0.1 mm^2 gut tissue examined) has been reported for gut tissues from unfed, incubated D. andersoni adults infected as nymphs.[82,92] The greatest density of colonies occurred after incubation at 37 C for 2.5 days[92] and when the donor calf had a high parasitemia level.[82] In the present study there was an increase in the mean colony density for the combined groups of male and female ticks from each of the transfer days 3 through 6. Ticks from the transfer day 6 group had a significantly higher mean colony density than did ticks from the other transfer day groups. Colonies were not observed in day 2 ticks, even though gut homogenates from this group produced infection in a susceptible calf. If additional gut cross-sections had been examined, perhaps colonies might have been detected in this group. It is possible that an insufficient number of infective organisms entered the nymphal gut tissue to result in colony formation in the day 2 group of adult ticks. It appears from the present study that increased feeding time and the increasing parasitemia level in the donor calf both influenced colony densities, making them greater.

Even though nymphs contained infective A. marginale organisms as early as the second day of feeding, as verified by the animal inoculation study, colonies were not seen in their gut tissue. However, colonies

were observed in the same group of nymphs five days after repletion. Digestion of the bloodmeal by nymphs may not have been adequate during the time of their feeding for infective organisms to enter the gut tissue or insufficient time had elapsed for colony development to take place in nymphs while they were feeding. Nymphal gut tissues from the 5 and 6 day feeding groups were also difficult to infiltrate with plastic and to section for light microscopy; these technical difficulties may account for the inability to locate colonies in this group of ticks.

The morphology of colonies of A. marginale has been described in gut tissue from adult ticks infected as nymphs.[91] Colonies have not been reported for replete nymphal ticks previously, even though infected nymphs were examined at 10 days after repletion.[90] In the present investigation, colonies were found in nymphal ticks from 5 to 25 days post-repletion. The colonies were generally oblong in shape, whereas the colonies described in adult ticks were usually round. The Anaplasma organisms seen within colonies from nymphal ticks appeared to decrease in size, and they also changed in shape from round to rod-shaped between 5 and 20 days post-repletion. No comparable change in size or shape of organisms was reported for colonies from adult ticks.[91] Colonies in nymphal ticks contained individual organisms that were separate from each other, whereas organisms from certain colonies in adult ticks occurred in clumps.[91] The organisms seen in nymphal tick colonies were all reticulated forms; electron-dense forms were not observed. Colonies from adult ticks contained either electron-dense forms or reticulated forms or both, depending on the morphologic colony type.[91] Small electron-dense particles that have been described in colonies from adult ticks[91] were also seen in colonies from nymphal ticks. Such particles were found within the

limiting membrane of reticulated forms and in the spaces between organisms in colonies from all repletion groups.

Membrane whorls have been reported previously in colonies from adult ticks and were associated with reticulated forms in the type 4 and type 5 colonies.[91] Membrane whorls were also seen in colonies from nymphal ticks in all of the post-repletion groups. The whorls were generally associated with clumps of reticulated forms. It has been suggested that membrane whorls may be a sign of organismal degeneration.[91] The membrane whorls seen in nymphal tick colonies may likewise indicate degeneration of organisms, as has been hypothesized for colonies from adult ticks. However, they may also be a result of normal colony development. Because the membrane whorls were generally seen in association with clumps of organisms, perhaps these clumps expand and become the separated organisms that populate the nymphal tick colonies.

Small electron-dense particles seen in adult tick colonies of _A. marginale_ were postulated to be similar to the miniature reticulate bodies described in _Chlamydia_ inclusions.[91] Miniature reticulate bodies were formed within larger reticulate bodies and then released either by a mechanism similar to budding or by rupture of the parent reticulate body cell wall.[98] Many small particles were observed within the limiting membranes of the reticulated forms in colonies from nymphal ticks. These small particles may be released into the colony from reticulated forms in a fashion similar to that reported for _Chlamydia_, but this process has not been observed in _A. marginale_. Small particles have also been described in the bovine erythrocyte in association with _A. marginale_ initial bodies,[10] but the role of these particles is not known at present.

Reticulated forms in nymphal tick colonies were frequently subdivided on the margin of their limiting membrane by alternating light and dark bands. These subdivisions may be a preliminary process in the formation of the small electron-dense particles. It has been suggested in Chlamydia development that the formation of miniature reticulate bodies might result from unequal synthesis of the reticulate body cell membranes.[99] Membrane associated subdivisions seen in A. marginale reticulated forms may result in a similar manner.

Tail-like inclusion appendages have been described previously in association with initial bodies in the bovine erythrocyte.[10,18-23] These inclusion appendages were visible only in lysed erythrocytes from which the electron-dense hemoglobin was removed.[10] Inclusion appendages have also been demonstrated in the gut lumen of feeding adult D. andersoni using the fluorescent-antibody technique.[83] Inclusion appendages were seen with electron microscopy in one gut cross-section near a group of colonies in nymphal ticks at 5 days post-repletion. This appears to be the first observation and report of inclusion appendages in tick gut tissue using electron microscopy. The appendages were attached to or interdigitated with the luminal surface of the host cell membrane, and some were free in the gut lumen. These appendages may aid the parasite in its entry into the tick gut tissue; however, no initial bodies were seen in association with them. Inclusion appendages may also have been present in the gut lumen of feeding nymphs, but the electron-dense hemoglobin in the lumen at this time would have obscured their presence. In later post-repletion groups, where the bloodmeal would have been more fully digested, appendages were not seen at all. These initial observations have been confirmed by Kocan et al.[100]

Some reticulated forms were observed that appeared to be dividing by binary fission in nymphal tick colonies at 5 days post-repletion but were rarely seen in the 10 day group. In this respect they resemble rickettsial organisms such as Rickettsia rickettsii which also divides by binary fission.[3] Rickettsia rickettsii has a relatively simple cycle of development in its tick vector. Rickettsiae are ingested with a bloodmeal and enter the midgut cells where they multiply and migrate into the hemolymph. From there they are disseminated to other body tissues such as salivary glands, ovaries, and Malpighian tubules.[28] Binary fission was not observed in nymphal tick colonies of A. marginale in the later repletion groups, suggesting that this parasite undergoes a more complex cycle of development than do typical rickettsiae.

The occurrence of more than two cell membranes in some of the reticulated forms seen at 20 days post-repletion has not been reported previously in colonies from adult ticks. The presence of rod-shaped organisms in nymphs at this time was also unusual and further substantiates the probable complexity of A. marginale development. The meaning of additional cell membrane formation and elongation of organisms is not known, but it may be possible that hormonal changes in the tick just before molting could induce morphologic alterations in the parasites.

The different forms of A. marginale described in colonies from adult ticks have been postulated to resemble stages seen in the developmental cycle of Chlamydia.[91] The electron-dense elementary body, which is the infective stage for Chlamydia, enters the host cell and reorganizes through an intermediate form into larger reticulated bodies. The reticulated bodies then reorganize into elementary bodies through another intermediate form. Reticulated bodies divide by binary fission and are

noninfective.[94] The reticulated forms seen at 5 days post-repletion in nymphal ticks also appeared to divide by binary fission and perhaps are analogous to the reticulated bodies seen in Chlamydia. A developmental sequence from electron-dense forms to reticulated forms was suggested for A. marginale organisms seen in adult ticks.[91] By contrast, in nymphal tick colonies all the organisms observed were reticulated forms. The change of reticulated forms from round to rod-shaped and the apparent decrease in organismal size with increasing repletion time suggested a possible developmental sequence in nymphs. Because the number of colonies decreased considerably between 20 and 25 days post-repletion, nymphal tick colonies may degenerate and later reorganize into the colonies that are present in adult ticks.

The mean colony density (number of colonies per 0.1 mm^2 gut tissue examined) was described in adult ticks infected as nymphs,[92] but colony density has not been analyzed previously in replete nymphs. In the present study, mean colony densities for replete nymphal ticks increased to a peak at 15 days post-repletion, decreased slightly at 20 days and dropped sharply at 25 days. Although the colony densities did not vary significantly among the different repletion groups, a statistically significant quadratic trend was observed. This quadratic trend was consistent with the pairwise comparison which suggested that the 15 and 20 day post-repletion groups had significantly greater mean colony densities than the other repletion groups. The small number of colonies seen at 25 days post-repletion coincided with the time when the nymphs were becoming ready to molt. The cross-sections of gut tissue examined at this time were frequently missing the basement membrane, which is the area in which most A. marginale colonies occur. This apparent absence of basement

membrane may have resulted from the methods by which the nymphs were dissected. Another possibility is that A. marginale colonies degenerate in replete nymphs. Such degeneration, if it occurs, may be due to hormonal or morphologic changes that ticks undergo near the time of molting.

The mean diameter of colonies seen in adult ticks has been described.[91,92] There was a gradual increase in the mean diameter from type 1 colonies to type 5 colonies.[91,92] It was not possible to measure the diameters in nymphal tick colonies due to their irregular shape; therefore, the mean colony area was determined. In contrast to colonies seen in adult ticks where the mean diameter increased with the colony type number, only slight differences in the mean colony area were observed between the different groups of replete nymphs.

CHAPTER V

SUMMARY

Anaplasmosis is a major disease of cattle that contributes significantly to economic losses in the cattle industry. It can be transmitted mechanically by various arthropod vectors. However, ticks appear to be the only proven biological vectors of this disease.[1,59,66,67] *Anaplasma marginale* was first recognized as the causative agent of this disease 75 years ago.[6] In spite of the years of research devoted to it, the life cycle of *A. marginale* in the bovine host and in tick vectors has not yet been fully described.

The first objective of the present study was to determine when feeding nymphal *Dermacentor andersoni* become infected with *A. marginale*. The nymphal transfer studies demonstrated that feeding nymphs acquired enough *A. marginale* organisms by the second day of feeding to become infected and transmit infection as adults to susceptible calves. However, the nymphs did not acquire a sufficient dosage of organisms to initiate colony development until the third day of feeding. Although all five morphologic colony types were present, the type 3 colony was the most prevalent. The mean colony density for the combined groups of male and female ticks increased up to the last day of feeding (day 6); mean colony density was significantly higher in these ticks than in those from other transfer day groups. The prepatent periods for infection decreased in the same time period as the mean colony density increased. The results

from this study suggest that the feeding nymphs ingest more infective organisms with increased feeding time and with the increased parasitemia levels of the donor calf.

The second objective of the present study was to examine the development of colonies of *A. marginale* in feeding and replete nymphal ticks. Although colonies were not observed in nymphs during the six days of feeding, colonies were present in replete nymphs from 5 to 25 days post-repletion. This finding is significant because colonies were not previously observed in replete nymphs. The morphology of nymphal colonies was found to be different in certain respects from the colonies found in adult ticks. The organisms within the colonies appeared to decrease in size and also changed in shape from round to rod-shaped with increasing repletion time. Furthermore, all the organisms seen were reticulated forms. No electron-dense forms were present. Some reticulated forms from ticks dissected at 5 days post-repletion appeared to be dividing by binary fission. However, binary fission was not observed in organisms studied from ticks in later post-repletion groups. Small electron-dense particles, which have been observed in adult tick colonies,[91] were also present in colonies from replete nymphs.

Tail-like inclusion appendages, similar to those seen associated with initial bodies in bovine erythrocytes,[10,18-23] were observed by electron microscopy at 5 days post-repletion. However, no initial bodies were seen associated with these appendages. These inclusion appendages may aid the parasite in its entry into the tick gut tissue. This appears to be the first time that inclusion appendages have been observed in tick gut tissue with electron microscopy; these initial observations have since been confirmed.[100]

The mean colony density for replete nymphs increased up to 15 days post-repletion, decreased slightly at 20 days, and then dropped sharply at 25 days. Ticks from the 15 and 20 day post-repletion groups were thought to have significantly greater mean colony densities than those from other repletion groups. The reduced number of colonies observed at 25 days post-repletion might have resulted from hormonal or morphologic changes that ticks undergo just prior to molting.

A developmental sequence has been postulated for the different forms of *A. marginale* observed in colonies from adult ticks.[91] The change of reticulated forms from round to rod-shaped and the apparent decrease in organismal size with increasing repletion time suggested a possible developmental sequence in nymphs. The considerable decrease in the number of colonies seen at 25 days post-repletion might indicate that nymphal tick colonies degenerate and later reorganize into the colonies seen in adult ticks. More work needs to be done to clarify the exact mechanism of infection of *A. marginale* in nymphal ticks and to determine the mode of transfer of this parasite from the tick vector to the bovine host.

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