A STUDY OF *ANAPLASMA MARGINALE* THEILER IN SELECTED IXODID TICKS

By

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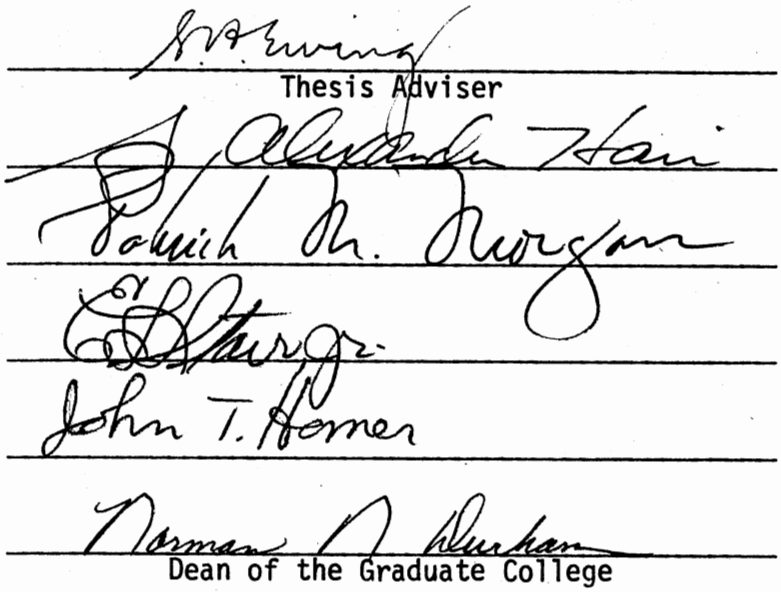

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INTRODUCTION

Bovine Anaplasmosis

Anaplasma marginale Theiler is an intraerythrocytic parasite that is the causative agent of anaplasmosis, a disease characterized by severe anemia. It occurs in cattle and a few other ungulates. The scientific name is based on staining characteristics and location within the host cell: "anaplasma" referring to the lack of stained cytoplasm and "marginale" denoting the peripheral location of the organism in the erythrocyte (1) (Figure 1). *A. marginale* was first thought to be a small erythrocytic stage of *Babesia bigemina* and was described as such by Smith and Kilborne (2). Theiler (3) recognized the disease agent as a separate entity and proceeded to obtain a pure infection of the organism and demonstrate the disease in cattle free of babesiosis (4). *A. marginale* is classified in the Family Anaplasmataceae, Order Rickettsiales (5) (6) according to the 8th edition of Bergey's Manual (Table I). A separate genus, *Paranaplasma* sp. was established more recently to distinguish those infections of anaplasmosis where an appendage-like structure is associated with the marginal body.

The occurrence of anaplasmosis is world-wide (7) and the disease has been reported to be endemic in several areas of the United States. The major endemic regions of the United States are the northwestern states and California and the southeastern Gulf states, including the

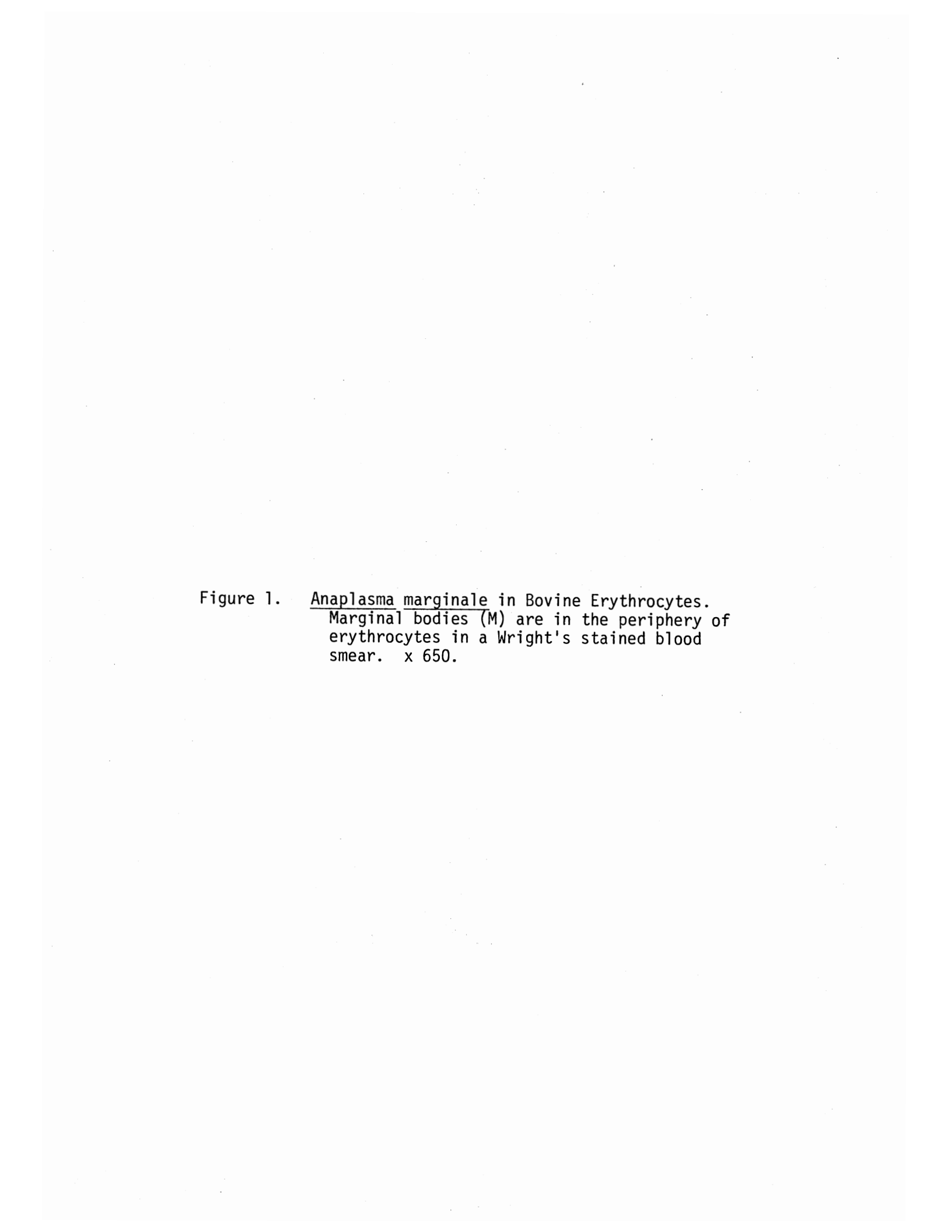
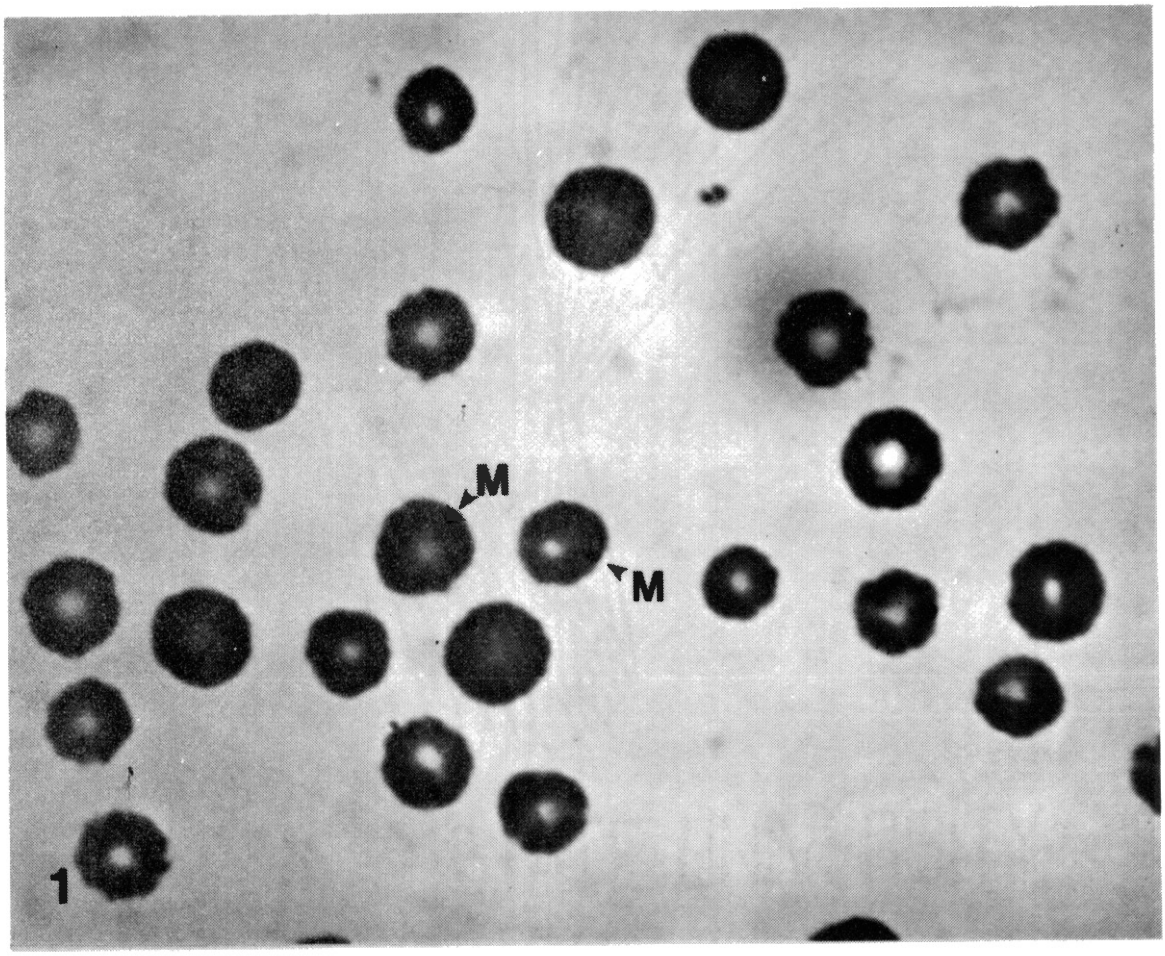


Figure 1. *Anaplasma marginale* in Bovine Erythrocytes. Marginal bodies (M) are in the periphery of erythrocytes in a Wright's stained blood smear. x 650.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M
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TABLE I

CLASSIFICATION OF THE ORDER RICKETTSIALES ACCORDING TO THE 8TH EDITION OF BERGEY'S MANUAL

ORDER: Rickettsiales

FAMILY I: Rickettsiaceae

Tribe 1: Rickettsieae

Genus	I:	Rickettsia
Genus	II:	Rochalimaea
Genus	III:	Coxiella

Tribe 2: Ehrlichieae

Genus	IV:	Ehrlichia
Genus	V:	Cowdria
Genus	VI:	Neorickettsia

Tribe 3: Wolbachieae

Genus	VII:	Wolbachia
Genus	VIII:	Symbiotes
Genus	IX:	Blattabacterium
Genus	X:	Rickettsiella

FAMILY II: Bartonellaceae

Genus	I:	Bartonella
Genus	II:	Grahamella

FAMILY III: Anaplasmataceae

Genus	I:	Anaplasma
Genus	II:	Paranaplasma
Genus	III:	Aegyptianella
Genus	IV:	Haemobartonella
Genus	V:	Eperythrozoon

Source: J. W. Moulder, "The Rickettsias," Bergey's Manual of Determinative Bacteriology (1974).

eastern portion of Oklahoma (8). The disease in cattle, which is characterized by a severe anemia, weakness, anoxia, fever, and jaundice, is important to the cattle industry and results in large economic losses annually (9) (10). Cattle that recover from an initial attack of anaplasmosis are resistant to further clinical episodes of the disease because very low levels of the organisms persist in a carrier state. The carrier state confers immunity to the individual and in most cases these animals can be identified by complement-fixation serology (11).

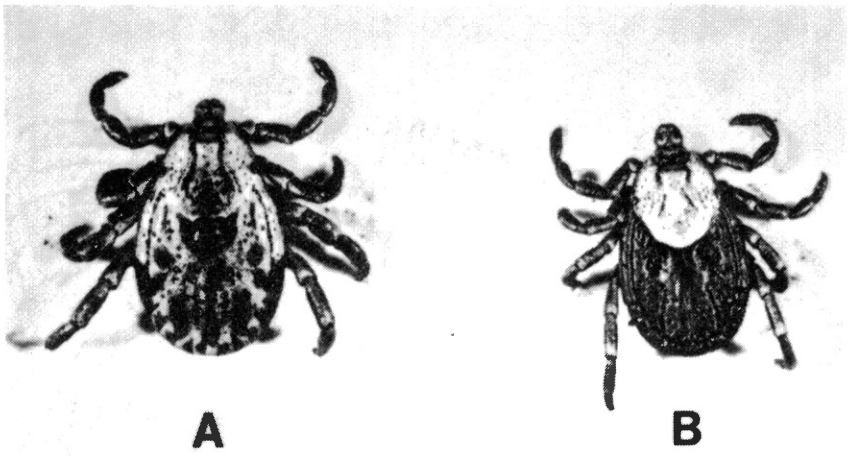
Ticks as Vectors of Disease

Ticks (Figure 2) transmit protozoal, rickettsial, viral, and bacterial diseases to various vertebrate hosts throughout most of the world. Some of these diseases result in large economic losses to the livestock industry. Those tickborne diseases of main economic importance in cattle are babesiosis (Babesia bigemina), east coast fever (Theileria parva), heartwater disease (Cowdria ruminantium), and anaplasmosis (Anaplasma marginale) (12). None of these agents is known to cause harm to the arthropod host, but all are pathogenic in the associated vertebrate host(13).

Ticks are very suitable as vectors of disease for a variety of reasons. They are resistant to changes in the environment, long-lived and can overwinter. Many species of ticks attack more than one type of vertebrate host in a given generation and have long attachment times which may be a factor in dispersal of disease-carrying vectors (14) (15). In some instances, disease agents persist through several stages; in others, agents are passed to progeny via the egg. Cyclic propagation of disease agents may occur in ticks (14).

Figure 2. Male and Female Dermacentor andersoni Ticks in the Flat Adult Stage. The male (A) is on the left and the female (B) is on the right. x 12.

Figure 3. Scanning Electron Micrograph of the Mouthparts of an Adult Ixodid Tick. The ventral part of the spined hypostome (H) is central to two palps (P). x 175.



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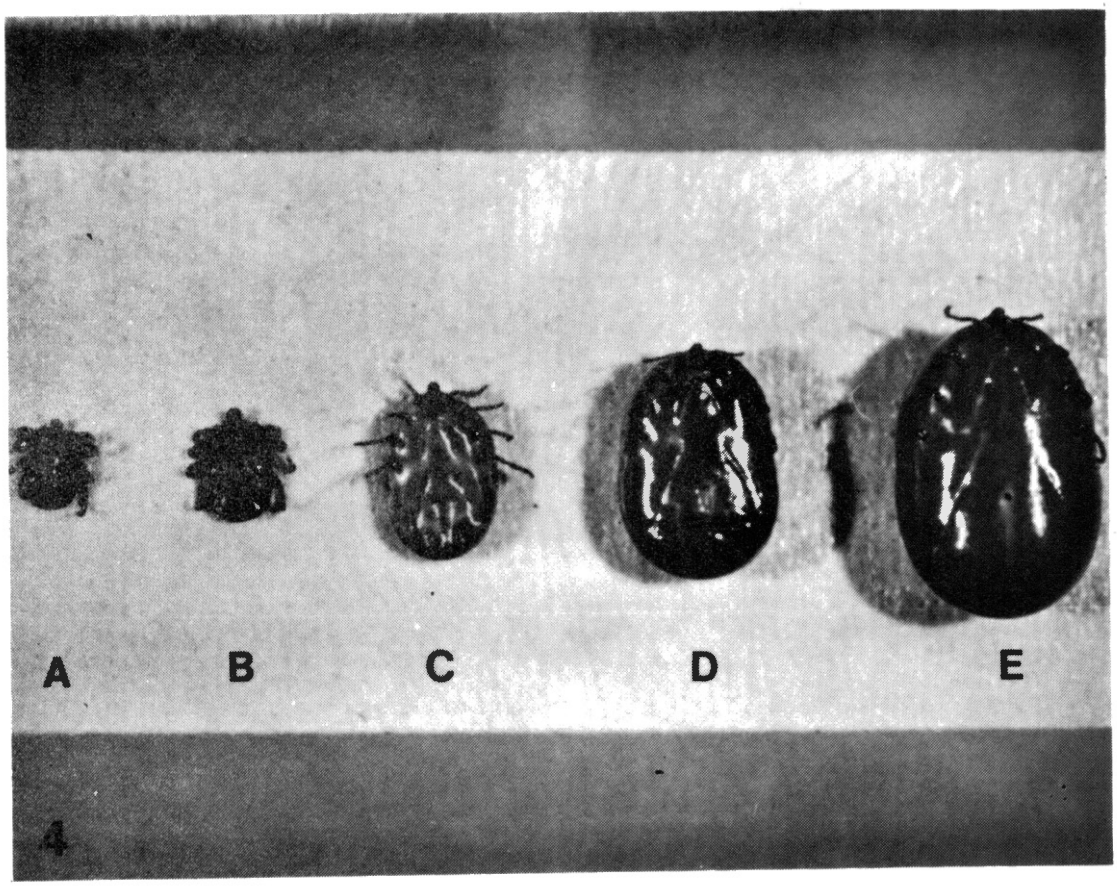
Rickettsial disease in ticks is believed to represent a primitive symbiotic association with the invertebrate host because they are non-pathogenic (16). Vertebrate rickettsial diseases have probably been secondarily derived from symbiotic tick infections by transmission during specialized arthropod feeding habits (13). This hypothesis is supported by many reports of rickettsial symbionts in ticks that are transferred from generation to generation via the ovaries and do not appear to occur in hosts other than the invertebrate, i.e., they have not been found to be pathogenic for any vertebrate host (17 through 23).

This study involves disease transmission by two ixodid ticks, Dermacentor andersoni Stiles and D. variabilis (Say). Dermacentor sp. are almost exclusively blood feeders and take one blood meal per feeding stage which includes larval, nymphal, and adult stages. Feeding is characterized by attachment, embedment, and cementing-in of the spined hypostome (Figure 3) in the dermis of the host (24). Ingestion of a blood meal may require several days and the terminal stage is characterized by rapid engorgement with concurrent great increase in size (Figure 4). After completion of feeding, the tick will fall off the host and proceed to molt to the next developmental stage and, if an adult, the female will lay eggs, and die. Most Dermacentor sp. are three-host ticks, but some other ixodids complete their life cycle attached to one vertebrate host.

The transmission of disease agents by ticks to the vertebrate host usually occurs during the blood meal and agents could be passed by contamination of body fluids via the bite or by fecal contamination (23). Disease agents may be transmitted among arthropod vectors by either transstadial transmission (from stage to stage) or by transovarial transmission (via the egg to successive generations). Experimental

Figure 4. Adult Ticks During Various Stages of Feeding. Stages include an unfed male (A), and female ticks ranging from unfed (B); five days after attachment (C); eight days after attachment (D); and nine days after attachment (replete) (E). x 3.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transmission of a disease agent by ticks may indicate that the type of tick can become infected but does not prove that it is a vector in nature. Enzootic transmission is dependent on several factors in addition to the presence of a susceptible tick population. Sufficient populations of susceptible cattle are necessary, as well as populations of reservoir hosts (30).

Transmission of *Anaplasma marginale* by Ticks

Anaplasmosis can be transmitted to cattle by arthropod vectors, both mechanically and biologically. Mechanical transmission has been shown to be most frequently caused by horseflies (*Tabanus sp.*) (26) (27) (28), although stable flies (*Stomoxys sp.*), deer flies (*Chrysops sp.*) and horn flies (*Siphona sp.*) may also be potential mechanical vectors (26). A common method of mechanical transmission is also via blood-contaminated surgical instruments and syringes. When such instruments are used for multiple dehorning, inoculation, or castration procedures, associated outbreaks of anaplasmosis have occurred (29) (30) (31). Ticks appear to be the only arthoropod responsible for biological transmission of bovine anaplasmosis (25) (27).

Although ticks have long been known to transmit anaplasmosis, the life cycle and morphologic stages in the vector are not known. Studies have shown that *A. marginale* may be harbored in the tick for long periods (32). Morphologic and fluorescent antibody studies reported previously have been limited in scope and have not demonstrated the specific location of the organism in the tick (33).

The Research Problem

The ability to control any infectious disease is greatly improved by knowledge of the life cycle of the etiologic agent in all its hosts. Demonstration of A. marginale in ticks and determination of its life cycle would be a fundamental contribution and would influence the direction of future anaplasmosis research. A more appropriate classification of this organism could be made if the morphologic stages that exist outside the bovine erythrocyte were known. Furthermore, it would be much easier to study A. marginale in cell culture systems if one knew what morphologic stage was present. It is also hoped that tick tissues infected with A. marginale may provide an alternate inoculum for cell culture from the previously used inoculum of infected bovine erythrocytes.

The objective of the present study was to confirm the presence of A. marginale in two species of ticks and to study its morphology. The experiments were designed to coordinate tick transmission of the disease with morphologic and antigenic studies of the parasite in these ticks in order to demonstrate that the tissues studied were from populations of ticks known to be infective. Light and electron microscopy were used to demonstrate the location of the parasite and its morphology in ticks. Labeled antibody studies (both fluorescein and ferritin) were used to confirm that the organisms observed were A. marginale.

CHAPTER II

TICK TRANSMISSION AND CATTLE INOCULATION STUDIES

Introduction

Ticks appear to be the only arthropod responsible for biological transmission of bovine anaplasmosis (25) (27). Tick transmission has been documented in several states of western United States including Wyoming (34), Idaho (35), California (36) (37), and Oregon (38). The role of tick transmission has not been studied extensively in the Gulf State region, even though anaplasmosis is enzootic. A number of ticks that have been found associated with cattle during outbreaks of anaplasmosis have been shown experimentally to be potential vectors of the disease.

Two ticks, Dermacentor andersoni and D. occidentalis, have been shown by controlled laboratory and field studies to be important natural vectors of anaplasmosis in the United States (31 through 35). D. variabilis, D. albipictus and Ixodes scapularis are considered vectors but additional studies are needed to define their role in transmission more clearly (25). Experimentally, 20 species of ticks have been shown capable of transmitting anaplasmosis (27).

Transstadial transmission of anaplasmosis has been demonstrated for many species of ticks. An extensive review of these studies, including brief descriptions of experimental designs, was reported by Dikmans (27). Some workers have reported transovarial transmission of anaplasmosis (27)

(39), but approximately 50 other attempts to demonstrate this phenomenon have been reported unsuccessful. In other studies, transstadial transmission was demonstrated by injecting supernatant from adult feeding ticks that were macerated in Eagle's minimal essential medium (MEM). However, oral secretions collected from the same group of ticks did not cause infection when injected into a susceptible cow (40). Long-term infectivity in naturally infected and laboratory-reared ticks was demonstrated by hibernation studies of male ticks (41).

Studies of other disease agents transmitted by ticks were helpful in determining tick tissues that were infected. Smith et al. injected tissue homogenates of gut and salivary gland of Rhipicephalus sanguineus into susceptible dogs in order to determine tissues that were infected with Ehrlichia canis (42). Purnell et al. collected saliva from ticks infected with Theileria parva and by inoculation into a susceptible animal, proved that it contained infective organisms (43).

Recent studies on protozoans transmitted by ticks have suggested that development and transmission of the disease agent is coordinated with the complex feeding physiology of these arthropods. Purnell and Joyner demonstrated that development of Theileria parva in the salivary gland of Rhipicephalus appendiculatus coincided with the feeding process and that mature forms of the parasite were not observed in the salivary glands until 4 to 8 days after feeding began (44). Other investigations have demonstrated that incubation of flat (unfed) ticks at 37°C that were infected in a previous stage also initiated development of infective organisms.

Dalgleish and Stewart reported that incubation of flat adult ticks would cause development of infective particles of Babesia bovis in the

tick, Boophilus microplus, and that infective stages could not be detected in flat, unincubated ticks (45) (46). Temperature (37°C for two days) was found to be important in causing increased virulence of Rickettsia rickettsii in Dermacentor andersoni (47) (48). Recent studies have demonstrated that homogenates of incubated, flat adult ticks infected with A. marginale as nymphs produced infection with shorter prepatent periods than those produced by unincubated controls (49).

Little information is available on what parasitemia levels of infected bovine blood are needed to infect ticks. Howell and coworkers reported mechanical transfer of blood from a carrier animal by horseflies (50). Transfer in this case was immediate and infection did not occur when the interval between feeding was more than five minutes. The ability of ticks to become infected by feeding on a carrier cow has not been reported.

The objectives of the tick transmission studies were to confirm that Dermacentor andersoni and D. variabilis were experimental vectors of A. marginale. The inoculation of gut and salivary gland homogenates into susceptible cows was done to detect if these organs were infective. Tissues collected from ticks known from transmission studies to be infected were used for morphologic and antigenic studies that are reported in Chapter III and Chapter IV. Additional studies on transmission were designed to test for transovarial passage of A. marginale and to compare transstadial transmission of gut homogenates from flat adult, incubated flat adult, and feeding adult ticks.

Materials and Methods

Animals

Intact dairy cows (4 to 10 years old), found to be negative by the anaplasmosis complement-fixation test, were used for feeding and inoculation studies. A Virginia isolate[1] of A. marginale was inoculated intravenously (IV) into a susceptible animal which developed anaplasmosis and was then used for feeding large numbers of laboratory-reared nymphal ticks. Subsequent infections of susceptible cows were produced by allowing some of these ticks, which had molted to become adults, to feed. Once exposed to ticks, cows were monitored biweekly for patent infection by three methods, viz., microscopic examination of Wright-stained blood smears, determination of the packed cell volume (PCV), and complement-fixation (CF) serology. Once marginal bodies were detected in the peripheral blood, the animals were monitored daily. Infected animals were treated with 11 mg/kg oxytetracycline for three consecutive days when the PCV dropped to between 15 to 19 percent.

Animals used for tick feeding experiments were confined to individual platform stalls equipped with head stanchions (Figure 5). Stalls were housed in a temperature-controlled building which provided for some environmental control and easy access for daily observations of host animals and ticks.

Laboratory Propagation and Infection of Ticks

D. andersoni and D. variabilis were reared and maintained at the

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[1]United States Department of Agriculture, Animal Parasitology Institute, Beltsville, Maryland.

Figure 5. An Aged Dairy Cow Confined in an Elevated Stanchion for Tick Feeding.

Figure 6. An Orthopedic Stockinette Glued to the Side of a Cow to Confine Feeding Adult Ticks.

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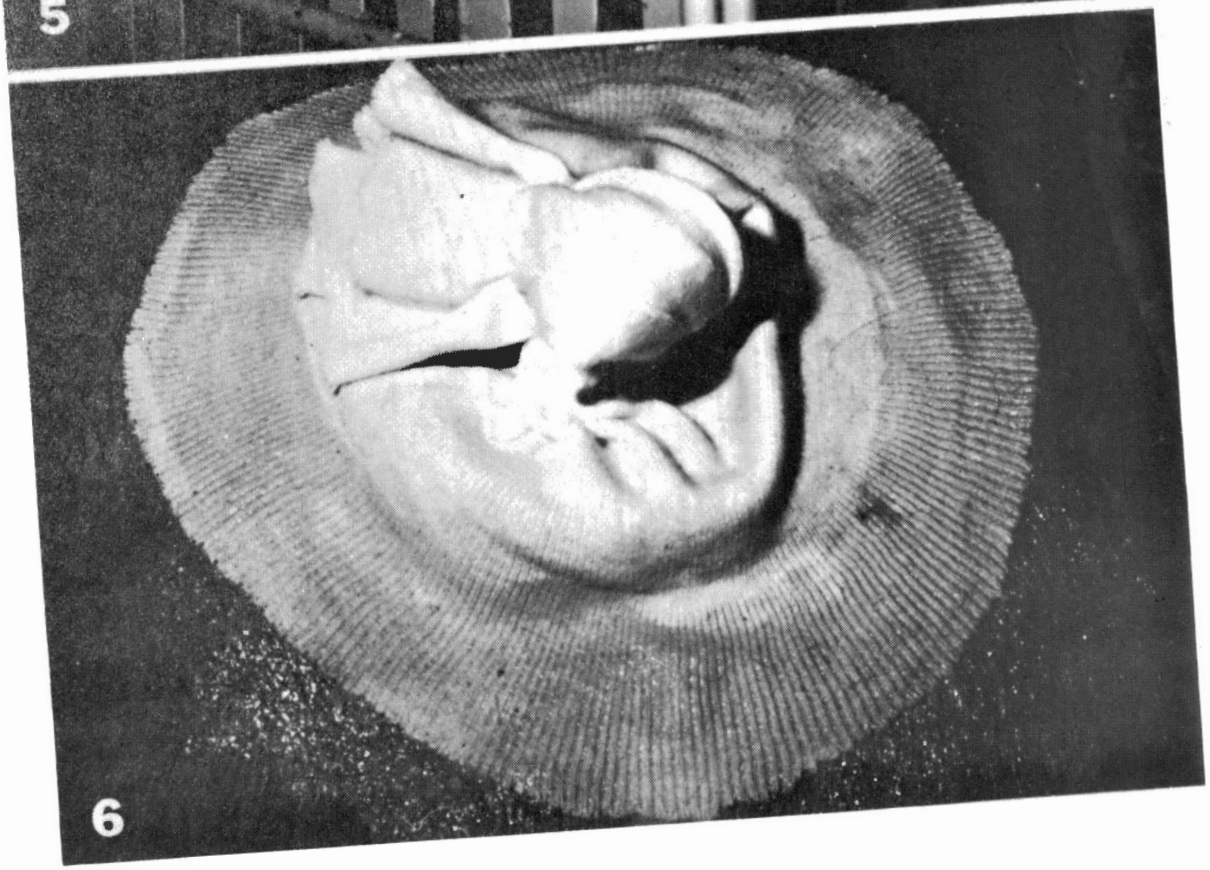

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Oklahoma State University, Department of Entomology, Tick Laboratory (51). The larvae were fed on rabbits and allowed to develop to the nymphal stage. Approximately 1,000 to 3,000 nymphs of each species were placed in muslin cells attached to infected cows when the A. marginale parasitemia was approximately 1 percent; the rapid phase of tick feeding (6 to 10 days after attachment) coincided, then, with the more elevated parasitemias. Only fully engorged ticks were retained for further studies. Nymphs randomly selected from the laboratory colony were fed on a CF-negative cow and served as controls. Engorged nymphs were placed in a humidity chamber (90 to 98% relative humidity) at 25°C with a 14-hour photophase and allowed to molt (Figure 7). Adult ticks were maintained under these conditions until used to determine their ability to transmit A. marginale by feeding on susceptible cows.

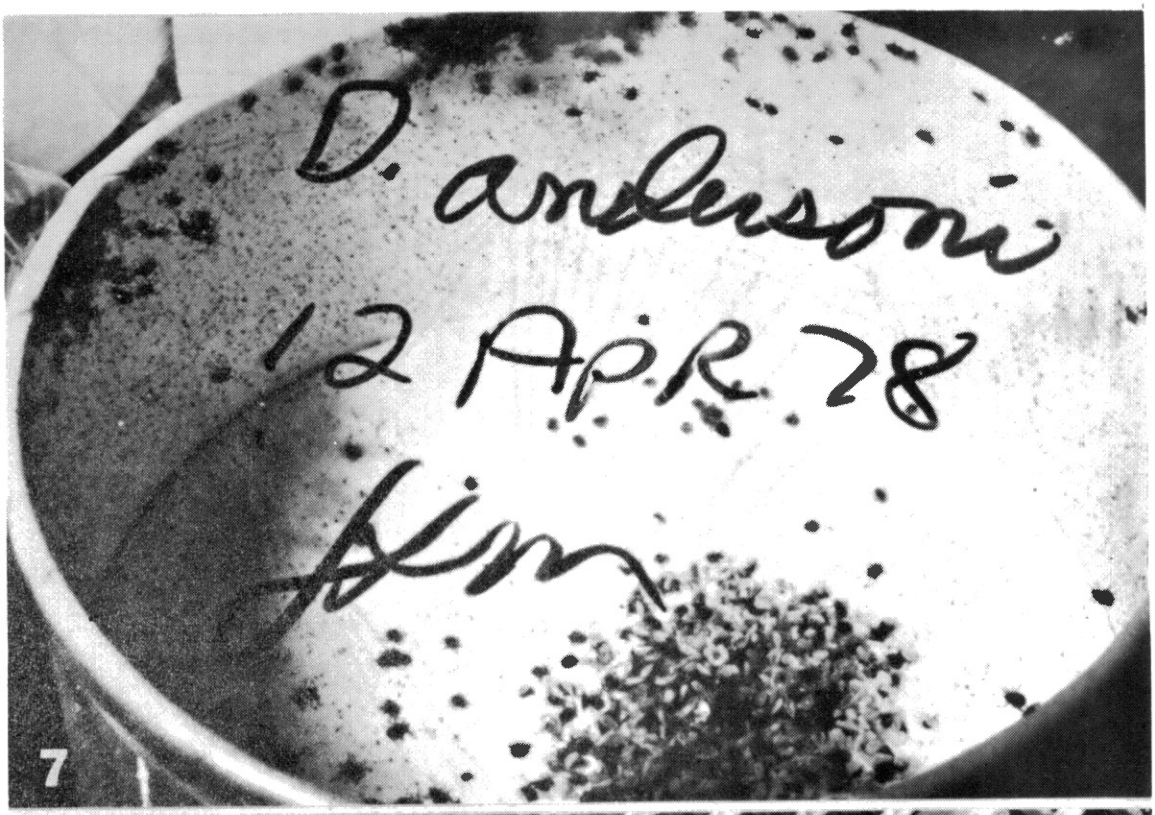
Transmission Studies With Adult Ticks Infected as Nymphs

Infectivity of adult ticks was tested by five methods: (1) ticks were allowed to engorge on CF-negative cows, (2) homogenates of tick salivary gland and gut tissue collected on days 5 and 7 post attachment were injected into susceptible cows, (3) oral secretions collected from ticks on day 6 post attachment were injected into a susceptible cow, (4) eggs were collected from known infective adult ticks and the hatched larvae were fed on a susceptible cow (test for transovarial transmission), and (5) homogenates of (a) unfed, unincubated and (b) unfed, incubated adult ticks known to be infected were inoculated into susceptible cows.

Feeding Studies of Adult Ticks. Approximately 600 to 1,200 exposed

Figure 7. A Carton of Nymphal Ticks Maintained at Constant Temperature and Relative Humidity in the Laboratory.

Figure 8. Adult Female Ticks, Replete, Placed in Vials for Ovipositing and Collection of Eggs.



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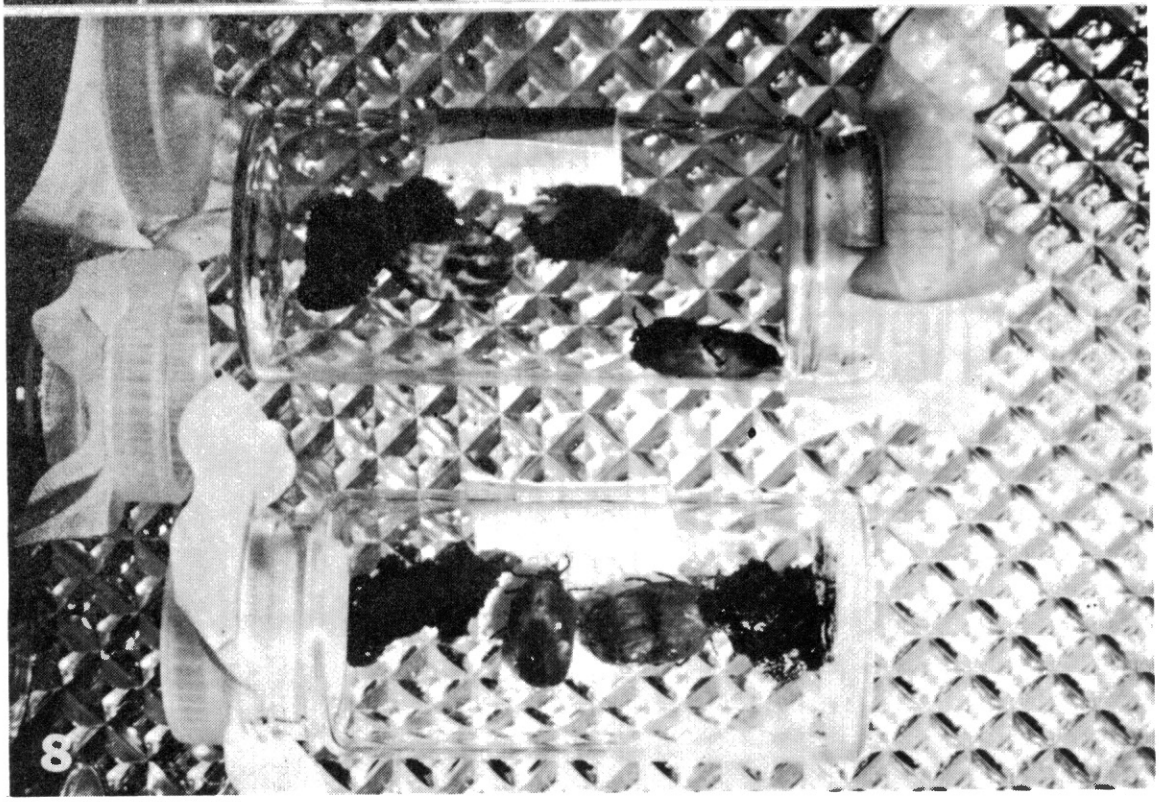

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adult ticks were placed into orthopedic stockinette cells glued on the side of a cow (Figures 6, 9, and 10). These ticks had been exposed to A. marginale as nymphs. Ticks that did not attach within 24 hours were removed from the stockinette by aspiration. For determination of the level of parasitized bovine blood needed to infect ticks, nymphs were exposed on cows in which the parasitemia varied from a high of 27 percent to the unobservable parasitemia of a carrier cow.

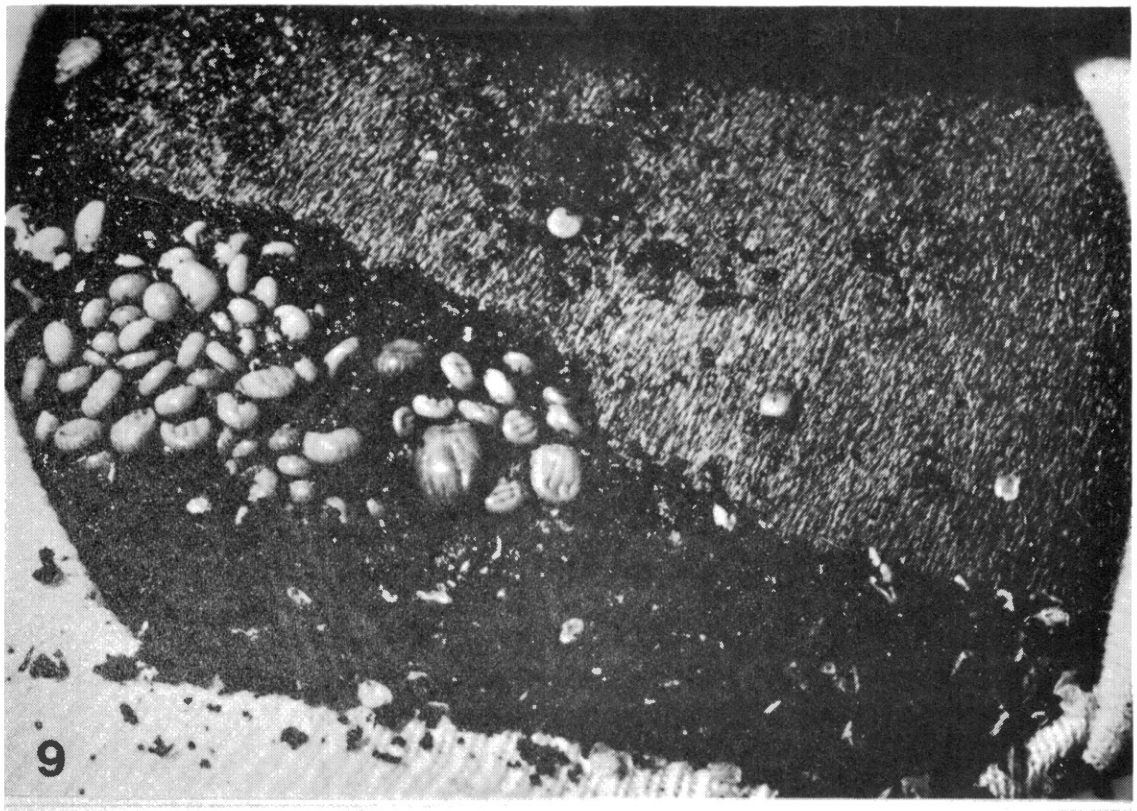
Animal Inoculation Studies. Approximately 30 ticks were removed from the cow on days 5 and 7 post attachment and immediately dissected. The specified tissues (salivary gland and gut) were collected, placed into tick culture medium (52), homogenized in a tissue grinder, and each homogenate immediately injected IV into separate, susceptible cows.

The transmission and animal inoculation studies were done in two trials. Trial 1 consisted of transstadial transmission, tissue homogenates, and oral secretion (see next section) with D. andersoni and transstadial transmission with D. variabilis. Trial 2 consisted of transstadial transmission and tissue inoculation studies with both D. andersoni and D. variabilis. All cows used for these experiments were monitored in the three ways specified previously.

Oral Secretion Studies. Oral secretions were collected from 100 D. andersoni adults removed at day 6 of feeding. To induce secretion, 3 to 4 $\mu\ell$ 10^{-2} dopamine suspended in tick culture medium (52) was injected into the anus of the tick using a 10 $\mu\ell$ syringe equipped with a 26 x 7.6 cm needle (Figure 11) (43) (53). The oral secretion was collected with a finely drawn capillary tube (Figure 12), pooled, placed in an equal volume of tick culture medium, and immediately injected into a susceptible cow.

Figure 9. Ticks, Confined by a Stockinette, Actively Feeding on a Cow; Six Days Post Attachment

Figure 10. Ticks, Confined by a Stockinette, Actively Feeding to Repletion on a Cow; Nine Days Post Attachment



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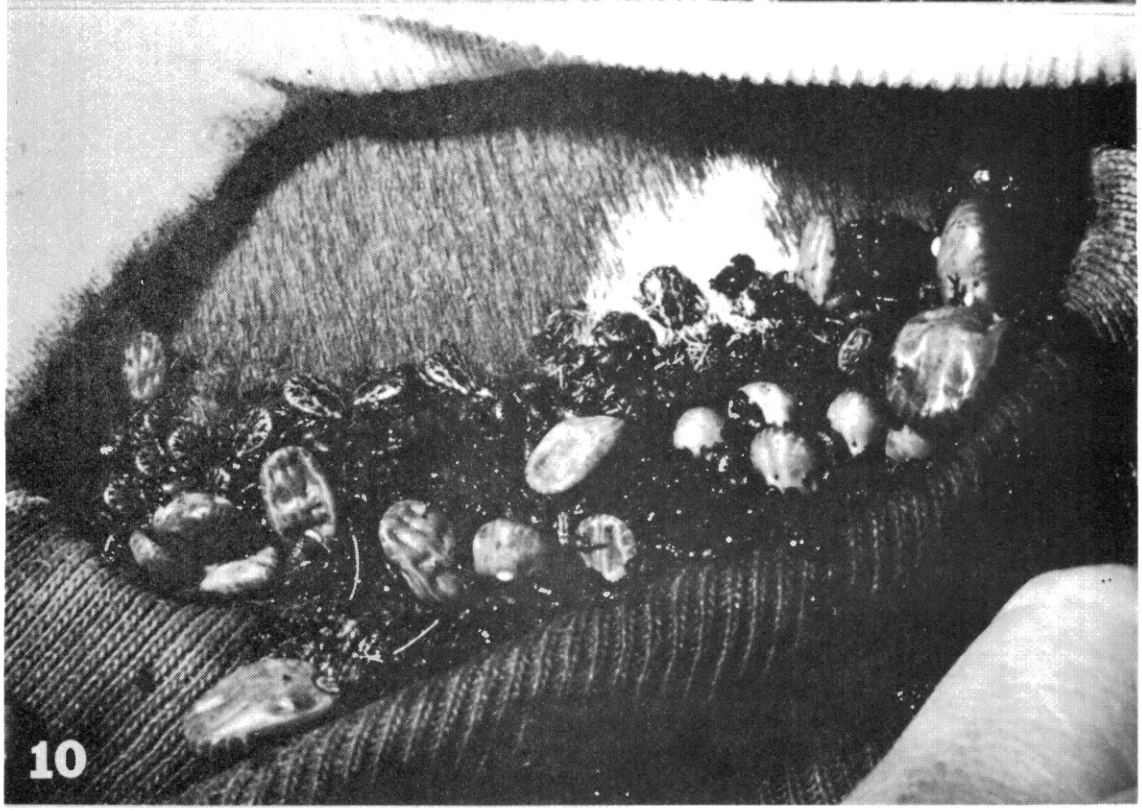

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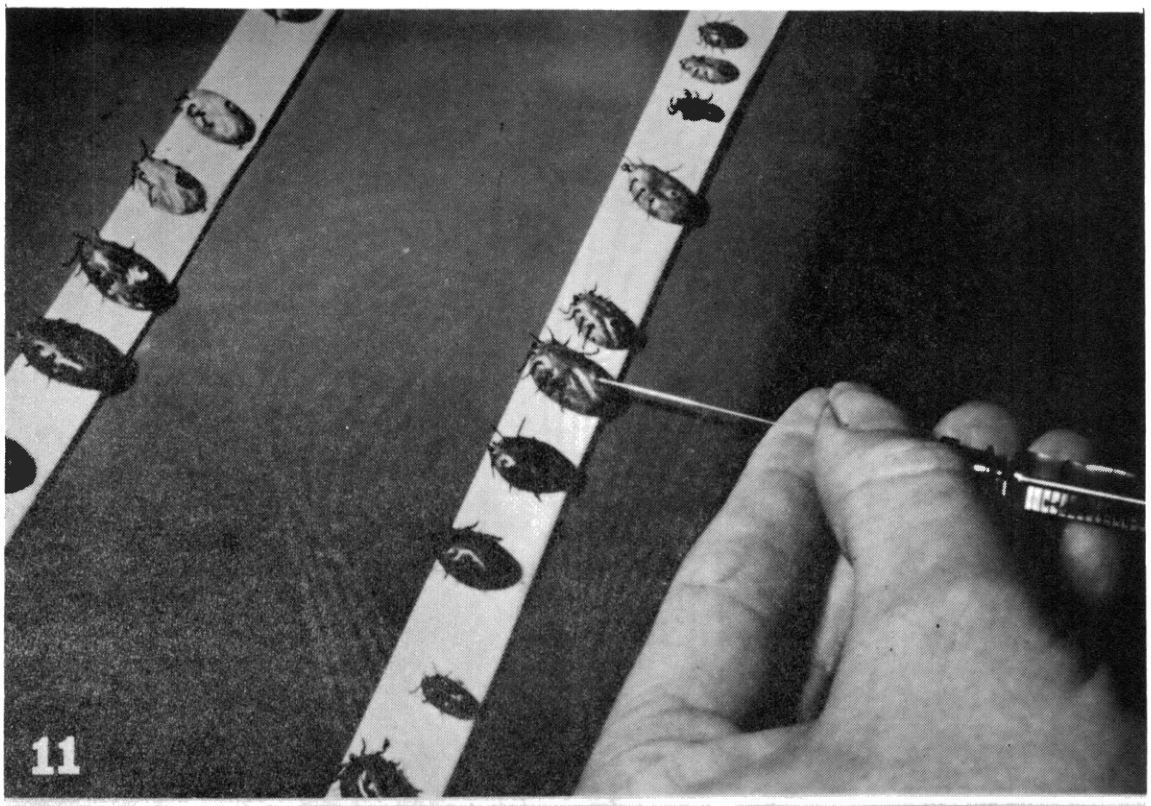
Figure 11. Anal Injection of Ticks With Dopamine for Induction of Salivation.

Figure 12. Collection of Oral Secretion From Dopamine-Injected Ticks.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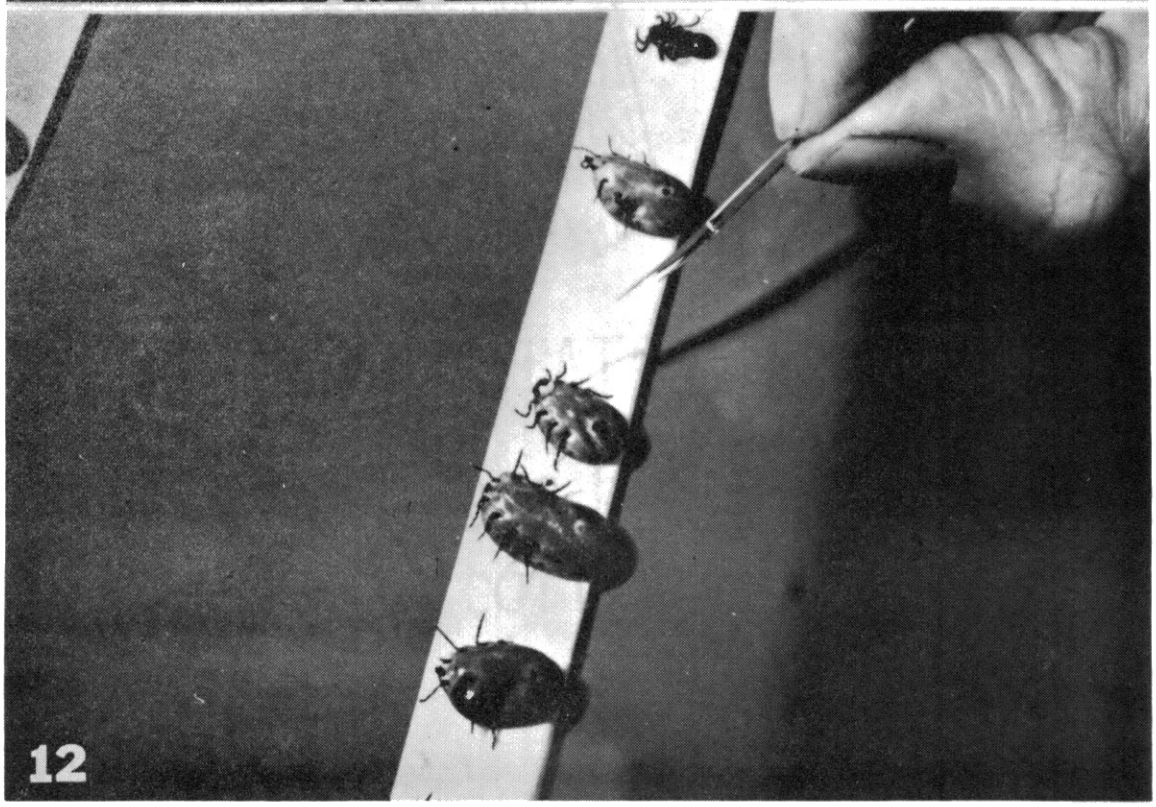
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Transovarial Transmission Studies. Replete D. andersoni adults were collected and placed in humidity chambers where they laid eggs (Figure 8). Larvae that hatched from these eggs were fed on a susceptible cow in two trials. The cow was monitored in the aforementioned ways for 60 days in Trial 1, confirmed negative and utilized for Trial 2. The cow was once again monitored for 60 days and then challenged with 50 mℓ of carrier blood to demonstrate susceptibility.

Cattle Inoculation Studies of Gut Homogenates From Flat Adult Ticks Infected as Nymphs. D. andersoni ticks were used from a laboratory-reared population known to harbor A. marginale. The population had been shown to be infective through adult feeding studies. A homogenate of gut tissues collected from 50 flat (unfed) ticks was inoculated into a susceptible cow. A group of 50 similar ticks was placed in an incubator at 37°C for three days and a gut homogenate was prepared from these ticks and injected into a susceptible cow. Similar groups of ticks from groups (a) unfed, infected, and (b) unfed, incubated, infected were collected at this time, dissected and prepared for morphologic studies (see Chapter III).

Results

Adult Tick Feeding and Animal Inoculation Studies

The data obtained in Trial 1 are presented in Table II. Transmission to susceptible animals was observed in those cows on which ticks fed and in those inoculated with gut and salivary gland homogenates but was not observed in the cow inoculated with the oral secretion. All animals were checked for antibody titers to the anaplasmosis complement-fixation

TABLE II

TRIAL 1: ATTEMPTS TO TRANSMIT BOVINE ANAPLASMOSIS BY *DERMACENTOR ANDERSONI* AND *D. VARIABILIS* ADULTS AND BY INOCULATION OF *D. ANDERSONI* GUT AND SALIVARY GLAND HOMOGENATES AND ORAL SECRETION COLLECTION

	Treatment	Cow No.	Age (yrs)	Prepatent[a] Period (days)	Peak Parasitemia (%)
I	*D. variabilis*[b]				
	Adult Feeding	277	8	17	12.0*[c]
II	*D. andersoni*[b]				
	Adult Feeding	478	4	19	8.0*[c]
	Gut Homogenate[d]	368	8	19	41.0*[c]
	Salivary Gland[d] Homogenate	536	6	22	2.3[c]
	Oral Secretion[e] Collection	713[f]	4	None	None

*Cows treated with oxytetracycline (11 mg/kg) for three consecutive days when PCV values were between 15 to 19 percent.

[a]Prepatent period determined from day 5 of feeding to the appearance of marginal bodies in the peripheral blood smear.

[b]Nymphal ticks for these studies were infected on Cow No. 450 with a peak parasitemia of 27 percent.

[c]Cows found to undergo seroconversion and became CF positive.

[d]Gut and salivary gland tissues were collected from ticks on days 5 and 7 of feeding.

[e]Oral secretion collected from ticks on day 6 of feeding.

[f]Cow was subsequently challenged and found to be susceptible.

test and those exhibiting parasitemia were confirmed positive by the CF test.

The data obtained in Trial 2 are presented in Table III and include repeated experiments for both D. andersoni and D. variabilis except for the collection and injection of oral secretion. A. marginale infection developed in all cows on which adult ticks fed and in those into which homogenates of either gut or salivary gland homogenates were injected. All animals were CF tested after developing clinical signs of anaplasmosis and were found positive.

Cows injected with homogenates of tick salivary gland and gut developed signs of anaplasmosis in both trials. The highest parasitemia in Trial 1 developed in a cow injected with gut homogenate but the highest parasitemia observed in Trial 2 was in a cow injected with salivary gland homogenate.

The cow injected with oral secretion did not become infected and was subsequently challenged and found to be susceptible.

Parasitemia Levels of Bovine Blood Infective for Nymphal Ticks

The data obtained in this experiment are presented in Table IV. Ticks which fed on cows with all levels of parasitemia from a high of 27 percent to an unobservable infection of a carrier cow transmitted A. marginale to susceptible cows. The shortest prepatent period (19 days) was observed in a cow fed on by ticks infected as nymphs on a cow with the highest infecting parasitemia (27%); the longest prepatent period (35 days) was observed in a cow fed on by ticks infected as nymphs on a cow with the lowest parasitemia (carrier cow).

TABLE III

TRANSMISSION OF BOVINE ANAPLASMOSIS BY DERMACENTOR ANDERSONI AND D. VARIABILIS ADULTS AND BY INOCULATION OF GUT AND SALIVARY GLAND HOMOGENATES COLLECTED AT DAYS 5 AND 7 OF FEEDING

	Treatment	Cow No.	Age (yrs)	Prepatent[a] Period (days)	Peak Parasitemia (%)
I	D. andersoni[b]				
	Adult Feeding	713	4	21	28.0*[c]
	Gut Homogenate	401	7	20	10.0*[c]
	Salivary Gland Homogenate	755	4	27	34.0*[c]
II	D. variabilis[b]				
	Adult Feeding	588	5	23	9.0[c]
	Gut Homogenate	510	6	20	28.0*[c]
	Salivary Gland Homogenate	695	4	21	19.0*[c]

*Cows treated with oxytetracycline (11 mg/kg) for three consecutive days when PCV values were between 15 to 19 percent.

[a]Prepatent period determined from day 5 of feeding to the appearance of marginal bodies in the peripheral blood smear.

[b]Nymphal ticks for these studies were infected on Cow No. 277 with a peak parasitemia of 12 percent.

[c]Cows found to undergo seroconversion and became CF positive.

TABLE IV

TRANSSTADIAL TRANSMISSION STUDIES OF ADULT *DERMACENTER ANDERSONI* AND *D. VARIABILIS* INFECTED IN THE NYMPHAL STATE BY FEEDING ON COWS WITH VARYING *ANAPLASMA MARGINALE* PARASITEMIAS

Nymphs Fed on Infected Cows		Adults Fed on a Susceptible Cow		
Cow No.	Peak Parasitemia (%)	Cow No.	Peak Parasitemia (%)	Prepatent[a] Period (days)
450[d]	27.0	278	8.0[b,c]	19
277[e]	12.0	713	28.0[b,c]	21
536[d]	2.3	887	7.8[c]	21
36[e]	Carrier	879	2.4[c]	35
36[e]	Carrier	703	15.0[c]	32

[a]Prepatent period determined from day 5 of feeding to appearance of marginal bodies in the peripheral blood smear.

[b]Cows treated with oxytetracycline (11 mg/kg) for three consecutive days when PCV values were between 15 to 19 percent.

[c]Cows found to undergo seroconversion and became CF positive.

[d]*D. andersoni*.

[e]*D. variabilis*.

Transovarial Transmission Studies

The data obtained from the two trials of transovarial transmission by second generation larvae are presented in Table V. The larval ticks did not produce infection in either trial and the cow (No. 925) subsequently was found to be susceptible when challenged with blood from a carrier cow.

Animal Inoculation Studies of Gut Homogenates From Unfed, Adult, Infected Ticks

The data in this experiment are summarized in Table VI. All gut homogenates were found to be infective. The results from injecting homogenates from ticks that had been incubated suggested that this procedure increased infectivity; the prepatent period was 34 days shorter.

Discussion

The results of the tick transmission experiments clearly demonstrate that both D. andersoni and D. variabilis are good experimental vectors of anaplasmosis. Furthermore, the cattle inoculation studies demonstrated that homogenates of both salivary gland and gut were infective, thus suggesting that both of these organs harbor the organism. The possibility does exist, however, that contamination among tissues occurred because tick dissection was very difficult. Chances of contamination of salivary glands from gut tissue were especially great because gut tissue was very fragile and ruptured easily.

Failure to produce infection by injection of oral secretions from known carriers supports the observations of Howarth and Hokama (40). The method used to collect oral secretions has been used successfully to

TABLE V

ATTEMPTED TRANSOVARIAL TRANSMISSION OF *ANAPLASMA MARGINALE* BY SECOND GENERATION *DERMACENTOR VARIABILIS* LARVAE

Treatment	Cow No.	Age (yrs)	Prepatent[a] Period (days)	Peak Parasitemia (%)
Trial 1:				
Adult Feeding	478[b]	4	19	8.0[c]
Second Generation Larvae	925	4	None	None
Trial 2:				
Adult Feeding	277[b]	8	17	12.0[c]
Second Generation Larvae	925[d]	4	None	None

[a]Prepatent period determined from day 5 of feeding to the appearance of marginal bodies in the peripheral blood smear.

[b]Nymphal ticks for these studies were infected on Cow No. 450 which had a peak parasitemia of 27.0 percent.

[c]Cows found to undergo seroconversion and became CF positive.

[d]Cow No. 925 was subsequently challenged with 50 ml carrier blood from Cow No. 36 and was found to be susceptible; the prepatent period on challenge was 15 days and a peak parasitemia of 29.0 percent developed.

TABLE VI

TRANSMISSION OF *ANAPLASMA MARGINALE* BY INJECTION OF GUT HOMOGENATES COLLECTED FROM FLAT, INFECTED, ADULT *DERMACENTOR ANDERSONI* THAT WERE EITHER INCUBATED OR NOT INCUBATED

Treatment	Cow No.	Age (yrs)	Prepatent[a] Period (days)	Peak Parasitemia (%)
Adult Feeding	852[b]	3.5	30	13.0[c]
Unincubated Flat Adult Gut Homogenate	851[b]	3.5	67	25.0[c]
Incubated Flat Adult Gut Homogenate[d]	837[b]	3.5	33	3.6[c]

[a]Prepatent period determined from day 5 of feeding to the appearance of marginal bodies in the peripheral blood smear.

[b]Nymphal ticks for these studies were infected on Cow No. 925 with a peak parasitemia of 29.0 percent.

[c]Cows found to undergo seroconversion and became CF positive.

[d]Ticks were incubated for 3 days at 37°C.

demonstrate the infective stage of Theileria parva in the salivary gland of actively feeding Rhipicephalus appendiculatus (44) and to determine numbers of ticks within a given population infected with Theileria cervi (53). Failure to transmit anaplasmosis by injection of oral secretion may mean that the organism does not enter the saliva or that it enters at some time other than that used in this study (day 6 of feeding). Furthermore, the dopamine injection may not trigger release of secretion from the cell type potentially infected with the anaplasmal organism. Continued investigation is needed to determine the role of the salivary gland in transmission of anaplasmosis.

Failure to demonstrate transovarial transmission of anaplasmosis supports the findings of earlier workers. Although there was a report in the 1940's of transovarial transmission (39), more recent studies have not demonstrated this phenomenon. The present attempt to demonstrate transovarial transmission was done under presumably optimal conditions: the parental adult ticks were proved infective by adult transmission studies and the feeding of their progeny commenced soon after the larvae had hatched.

Transmission of anaplasmosis from a carrier cow by ticks has not been reported previously (27) (54). In the present study, two different such attempts were successful. Transmission from carrier animals to susceptible cows may be an important factor which influences the potential of maintaining anaplasmosis as an enzootic infection. Additional field studies are needed to determine if transmission from a carrier animal occurs in nature.

Tick transmission from cows having low parasitemias may also be a factor in enzootic anaplasmosis. Cows may have low parasitemias with no

clinical signs and carrier cows have been shown to have occasional mild recrudesences (27). In the present study, prepatent periods were not remarkably different for bovine infections caused by adult feeding ticks that were infected on cows with any detectable level of parasitemia. The average prepatent period for bovine anaplasmosis is 18 to 21 days (11). Prepatent periods of infections caused by ticks that had fed on infected cows with parasitemias of 2.3 percent to 27.0 percent were all within this range.

The infectivity of gut homogenate from unfed, adult ticks which had acquired A. marginale as nymphs appeared to differ from that of similar homogenates collected from adult ticks in the sixth day of feeding. Injection of homogenates from the unfed ticks caused infection but the prepatent period was 67 days; homogenates of tissues collected from ticks which were feeding caused infections, with a prepatent period of 30 days. These data suggest that transmission of the infective agent is coordinated with the complex feeding process of the tick. This mechanism has been described by Purnell et al. and Morszaria et al. in connection with transmission of other tick-borne diseases (45) (55) (56). Results of experiments with unfed, incubated adult ticks further suggest that a major stimulus derived from feeding is an elevation of the body temperature of the tick. In our experiments, similar prepatent periods were observed in cows exposed to homogenates of gut collected from ticks that had been incubated and those that had been fed, i.e., 33 days and 30 days, respectively. This does not prove, of course, that temperature is the only factor; rather, increased temperature may cause either physiological or developmental changes in ticks that facilitate development of the organism. Spencer and Parker found that ticks infected with spotted

fever rickettsiae and subsequently stored for long periods caused no clinical reaction when guinea pigs were exposed but did cause development of immunity; and further, that avirulent organisms became virulent when ticks took a blood meal or were incubated at 37°C for 27 to 48 hours (57). Price found in experiments with Rickettsia rickettsii that virulence for vertebrates was induced by tick molting hormones and by elevated environmental temperature to which ticks were exposed rather than by increased concentrations of organisms (48). Gilford and Price further suggested that the elevation of temperature caused a modification of metabolites which may be directed by tick hormones and that this influenced the virulence of the organism (58). Additional studies are needed to determine the factors that are immediately responsible for the increase in virulence of rickettsial organisms at the time of tick feeding.

The results of the tick transmission and cattle inoculation studies suggest several interesting relationships in the transmission of anaplasmosis by arthropods. The availability of carrier animals may likely contribute significantly to levels of enzootic anaplasmosis. Furthermore, organisms may be able to survive in ticks for a long time and become activated when the tick body temperature is elevated as a result of feeding on a vertebrate host or from exposure to elevated environmental temperature. Further investigations are needed to substantiate the findings and to determine their influence on enzootic transmission of the disease.

CHAPTER III

ULTRASTRUCTURE OF *ANAPLASMA MARGINALE* IN SELECTED IXODID TICKS

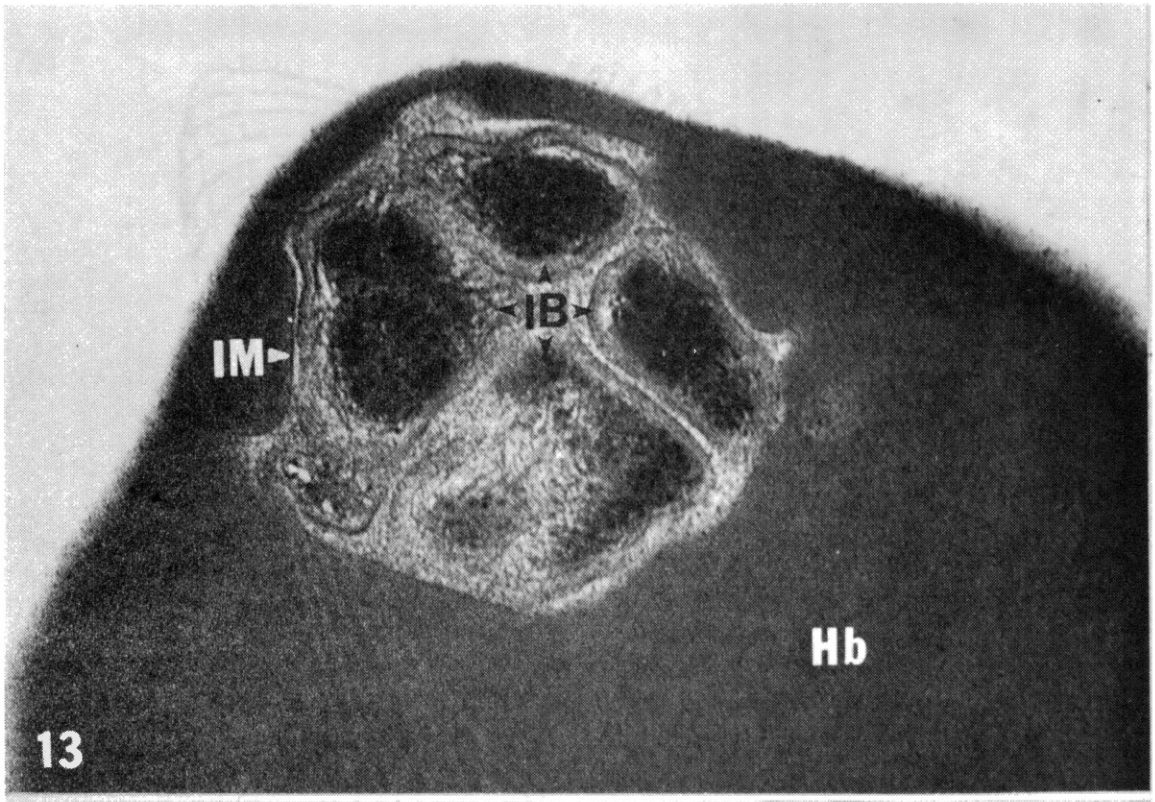
Introduction

Intraerythrocytic inclusion bodies are the only morphologic form of *Anaplasma marginale* described thus far. They have been described by light and electron microscopy (59-72). The ultrastructure of the anaplasmal inclusion body has been redescribed recently in several types of preparations (73). The inclusion body consists of one to several initial bodies (0.55 to 0.85 μm in diameter) enclosed by an inclusion membrane and is located near the erythrocytic plasmalemma (Figures 13 and 14). The initial bodies are enclosed by two membranes termed the pellicle and the organismic membrane with an intermembranous matrix between (Figure 15). The general ultrastructure of anaplasmal initial bodies appears to be most similar to that of rickettsial organisms. The organisms contain a protoplasmic filamentous network that is attached to the organismic membrane and is continuous with dense chromatin clumps and aggregations (73) (Figure 15).

Although the exact mechanism of pathogenesis in the bovine host has not been described, a hypothesis of cyclic reproduction has been proposed by Ristic and Watrach (74) in which the infective agent (initial body) enters the erythrocyte by endocytosis, with the plasmalemma forming an inclusion vacuole. Ferritin-conjugated antibody studies have supported

Figure 13. Electron Micrograph of an Anaplasmal Inclusion Body in an Intact Bovine Erythrocyte. The inclusion membrane (IM) surrounds four initial bodies (IB). The erythrocyte contains electron-dense hemoglobin (Hb). x 70,000.

Figure 14. Electron Micrograph of an Anaplasmal Inclusion Body and Inclusion Appendage in a Hemolyzed Bovine Erythrocyte. The inclusion appendage (IA) is attached to the inclusion membrane (IM) that contains three initial bodies (IB). The inclusion is inside the erythrocyte plasmalemma (EP). x 45,000.


IB
IM
Hb
13

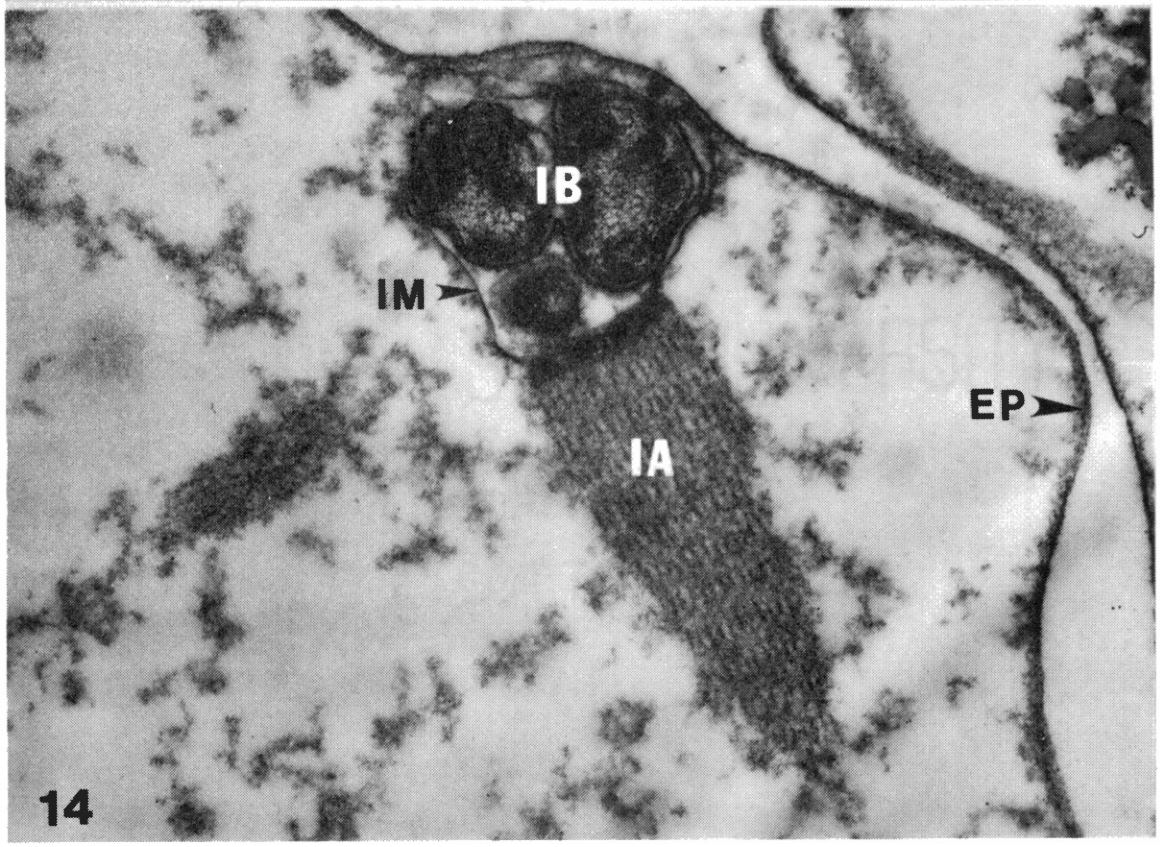
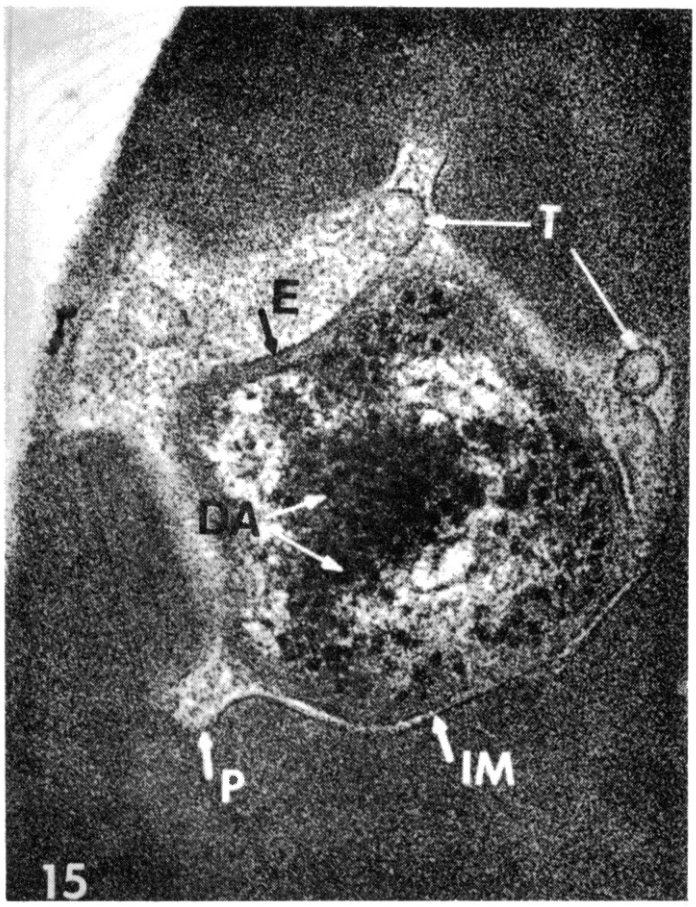

IB
IM
EP
IA
14

Figure 15. Ultrastructure of the Anaplasmal Initial Body in an Intact Bovine Erythrocyte. The inclusion membrane (IM) sometimes has protrusions (P). A bilaminar organismic envelope (E) is constructed of an outer pellicle, inner organismic membrane with an intermembranous matrix between. Tubular extensions (T) of the pellicle are present. Dense aggregates (DA) are in chromatin clumps of the initial body. x 116,000.


T
E
DA
P
IM
15

this hypothesis by determining that the inclusion membrane is of erythrocytic origin (75) (76). However, transfer of the initial body from one erythrocyte to another has not been demonstrated clearly.

The ultrastructure of A. marginale in the invertebrate host has not been reported. Several species of ticks have been demonstrated to serve as biological vectors capable of remaining infective for long periods (27) (32) (40), but attempts to elucidate the morphology of A. marginale in them have not been successful. Studies have been complicated by the common occurrence in ticks of symbiotic rickettsiae which have no demonstrated pathogenecity for vertebrates (17-23), but which are commonly found in ixodids.

The gross anatomy and histology of certain internal organs of a few species of ticks have been described at the light microscope level. Information regarding ultrastructure is limited both in the number of tissues studied and species described. This lack of information is compounded by the growing realization that major morphologic changes occur during the feeding process (77) (78).

The two tick tissues examined for the presence of A. marginale in this study were salivary gland and gut. Both tissues undergo extensive change during feeding, the most obvious of which is hypertrophy, a change necessary for accommodation and digestion of the blood meal.

The main route for ticks to acquire infection is through ingestion of the blood meal and thus would logically involve the gut. Most rickett- organs or cells where they have been observed include hemocytes, salivary glands, ovaries, and malpighian tubules (13). It has been hypothesized that hemocytes are responsible for spreading rickettsial organisms within

the tick. The organs infected may vary with the specific etiologic agent (13).

Salivary glands are paired, glandular structures situated laterally in the body cavity. They are composed of numerous alveoli of two distinct types: Type 1 does not contain granules and Type 2 contains granules. It has been suggested that Type 1 alveoli function in water transport and Type 2 alveoli function in feeding (80). Other histochemical studies of salivary glands have identified nine granular cell types and have associated these types with various feeding functions (78). Salivary glands have been shown to be the site of infection and transmission for many tick-borne diseases, including rickettsiosis (13), canine ehrlichiosis (79), babesiosis (81), and east coast fever (43).

The gut is by far the largest internal organ of the tick. It is histologically diverse and may be divided into foregut, midgut and hindgut. The foregut extends from the orifice of the hypostome to the stomach. The midgut is the portion of the alimentary tract between the esophagus and rectum while the hindgut is posterior to the midgut and includes the rectal sac, anal canal and anus (82). Gut tissues for the present study were collected from the midgut.

Previous ultrastructural studies of midgut epithelium from adult ticks have revealed three cell types: undifferentiated reserve, secretory and digestive cells (83). It is thought that reserve cells can differentiate into either secretory or digestive cells (83). The gut of the unfed, adult ixodid tick contains predominantly reserve and secretory cells. Digestive cells present in unfed ticks are believed to have been retained from the nymphal stage, serving in storage of food but unable to participate in the new digestive cycle. Secretory cells grow

rapidly after the tick attaches to the host and the apical portions bud off as food enters the gut. These sloughed cells disintegrate in the lumen, and some investigators (84) (85) believe they contain a hemolysin and participate in extracellular digestion by lysing erythrocytes. During feeding, digestive cells arise from undifferentiated reserve cells and are capable of two forms of blood ingestion (phagocytosis and endocytosis). The digestive cell is the predominant cell type by the end of feeding (77). The major food of ixodid ticks is hemoglobin and the final end-product of digestion is hematin which is released with the apical portions of cells and is passed in the feces (83). The causative agents of rickettsiosis (13), canine ehrlichiosis (79), babesiosis (86), and east coast fever (87) have all been demonstrated in midgut epithelial cells.

The objectives of the morphologic studies reported herein were to: (1) demonstrate the anaplasmal organisms in tick tissues known by previous transmission studies to be infective, and (2) to describe the structure of the organism in replete nymphs, and in flat adults, incubated adults, and feeding adults. Tissues were studied with light and electron microscopes.

Materials and Methods

Collection of Tissues

Dermacentor andersoni was used for these studies. Gut and salivary gland tissues were collected from nymphal ticks (control and infected) at days 5 and 7 of feeding. Tissues were also collected from control and infected nymphs 10 days after repletion. Tissues from approximately 30 to 50 ticks were used in each experimental group. Part of the tissues

was processed and embedded for standard paraffin sectioning and the remaining tissues were processed for electron microscopy. Paraffin sections were prepared and stained with either hematoxylin and eosin or with Giemsa stain. Tissues collected from adult, feeding ticks were from the tick transmission experiments reported in Chapter II, Tables II and III.

For morphologic studies of flat adult ticks, both incubated (37°C for three days) and unincubated, salivary gland and gut tissues were collected from both infected and uninfected ticks of both groups and processed for electron microscopy. Similar groups of ticks were used as donors of gut tissue which was homogenized and used for animal inoculation studies; the data from this experiment are presented in Chapter II, Table VI.

Electron Microscopy

Tissues were placed immediately in cold 2 percent glutaraldehyde in a 0.25 M sodium cacodylate buffer and allowed to fix for two hours. After several washes in the same buffer, the tissues were post-fixed in 2 percent osmium tetroxide in 0.25 M cacodylate buffer, washed several times, dehydrated through a graded series of ethanol and infiltrated in Dow Epoxy Resin 732 using propylene oxide as the intermediate solvent. Thick sections (1.5 μ) were stained with Richardson's blue (88) and used for orientation of the tissue in the light microscope. Ultrathin (silver-reflective) sections were cut with a Sorval MT-2 ultramicrotome and a DuPont diamond knife. The sections were collected on 300 mesh copper grids, stained with uranyl acetate and lead citrate (89), and observed and photographed in a Philips 200 electron microscope operated at 60 kV.

Results

Morphologic Studies on Nymphal Tick Gut

Light Microscopy Studies. A cross section (paraffin-embedded tissue, 6 μ) from the midgut region of an infected tick collected 10 days after repletion is presented in Figure 16. The newly forming exoskeleton and cross sections of the gut can be seen. The gut lumen of these ticks is not well defined. No anaplasma-like organisms were seen in the sections and no difference was observed in sections of infected and uninfected nymphal ticks. A similar cross section from plastic embedded tissue (1.5 μ) was prepared and stained with Richardson's blue (Figure 17). The improved resolution showed more clearly the gut morphology inducing the distribution of granules within the gut cells. No anaplasma-like organisms were observed and no differences were seen between the sections of infected and uninfected nymphs.

Electron Microscopy Studies. An electron micrograph of nymphal gut cells is presented in Figure 18. The epithelial cells of both infected and uninfected ticks contained large numbers of small circular structures. These structures were seen in both control and infected ticks, and it is thought that they represent cellular structures which are associated with the normal metabolism of nymphal ticks. Anaplasma-like organisms were observed in the gut epithelial cells of infected nymphal ticks (Figures 19 and 20). These organisms were somewhat pleomorphic and had two cell membranes which were not always closely adhered to one another (Figure 19). The inner membrane appeared to be continuous with the internal components of the organism (Figures 19 and 20). Small electron-dense

Figure 16. Cross Section Through an Infected Nymphal Tick 10 Days After Repletion. Three cross sections of gut (G) are within the newly developing exoskeleton (Ex). Paraffin embedment, Giemsa stain. x 200.

Figure 17. Cross Section Through an Infected Nymphal Tick 10 Days After Repletion. A one micron section of two cross sections of gut (G) are within the newly developing exoskeleton (Ex) and provide better resolution of tissue components. Plastic embedment, Richardson's blue stain. x 220.

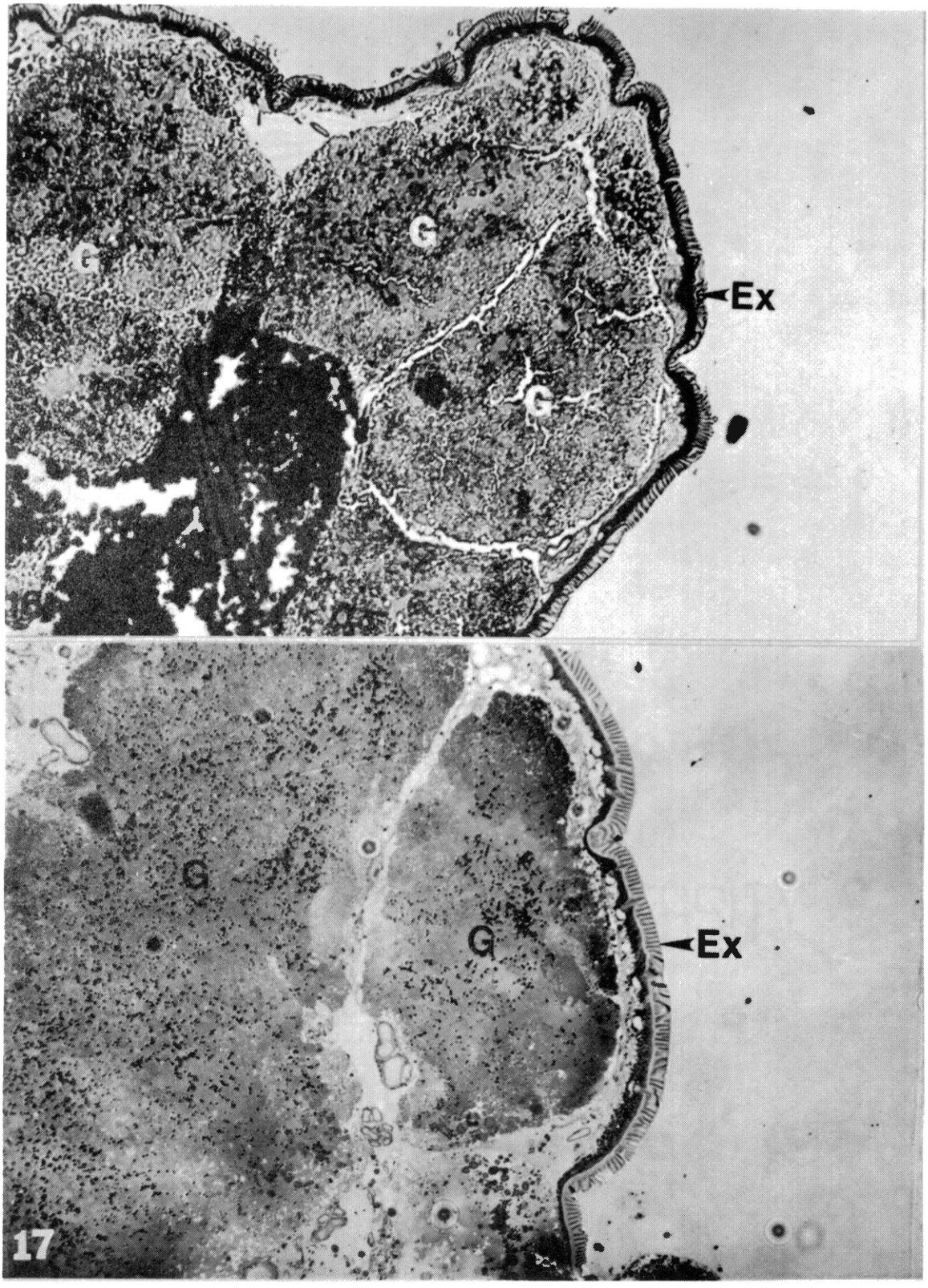

G
G
G
Ex
16
G
G
Ex
17

Figure 18. Electron Micrograph of the Junction of Three Epithelial Cells in the Midgut of a Nymphal Tick. The cells form a junction (CJ) and contain many small circular structures (CS). One cell shows a portion of the nucleus (Nu). x 25,000.

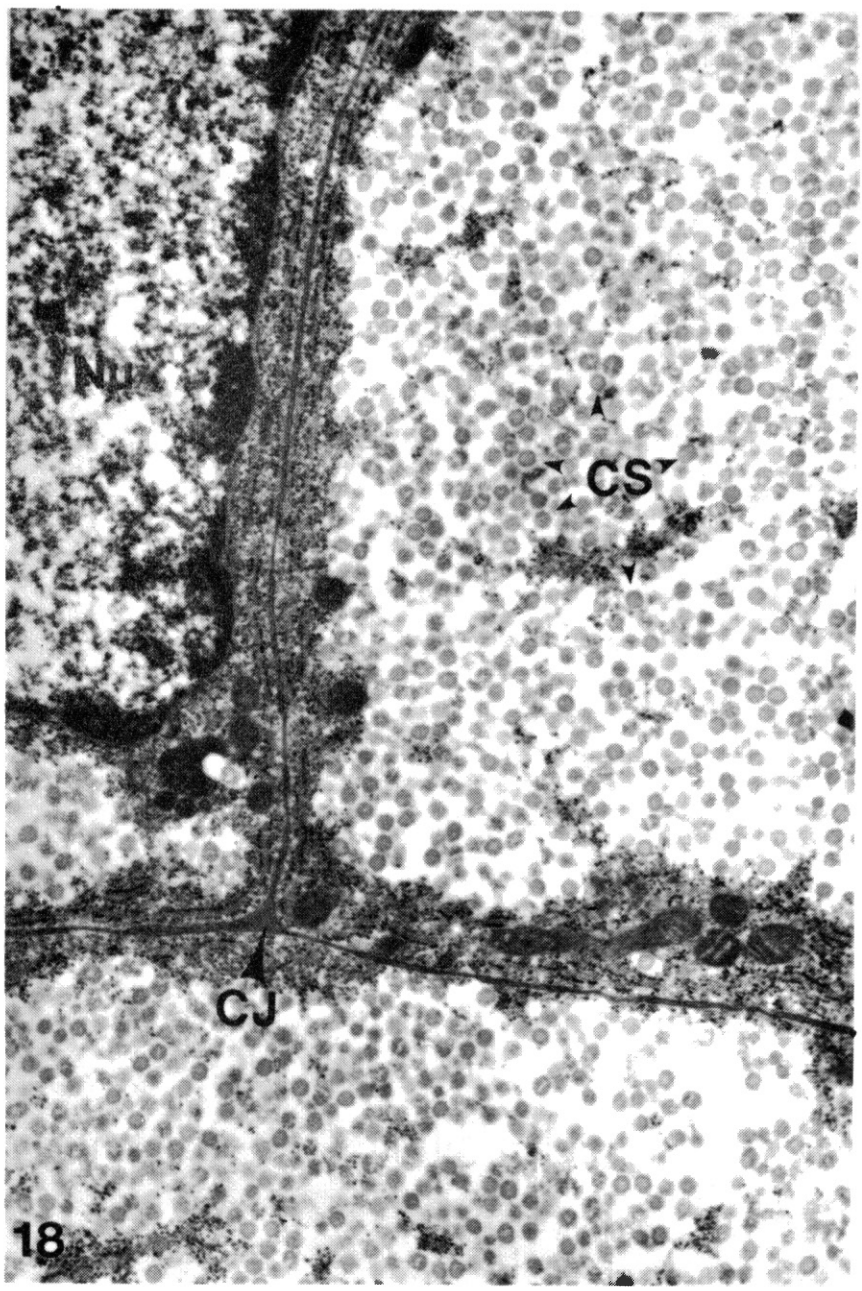
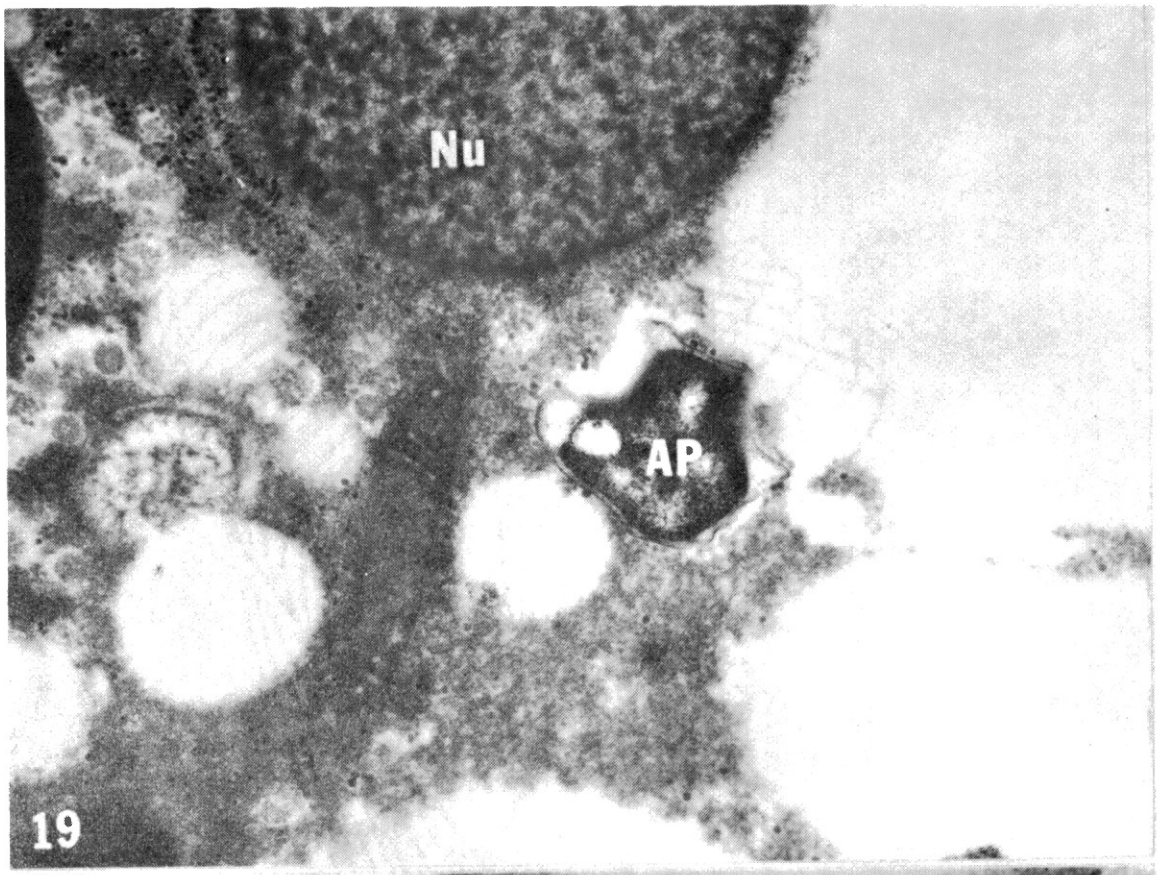
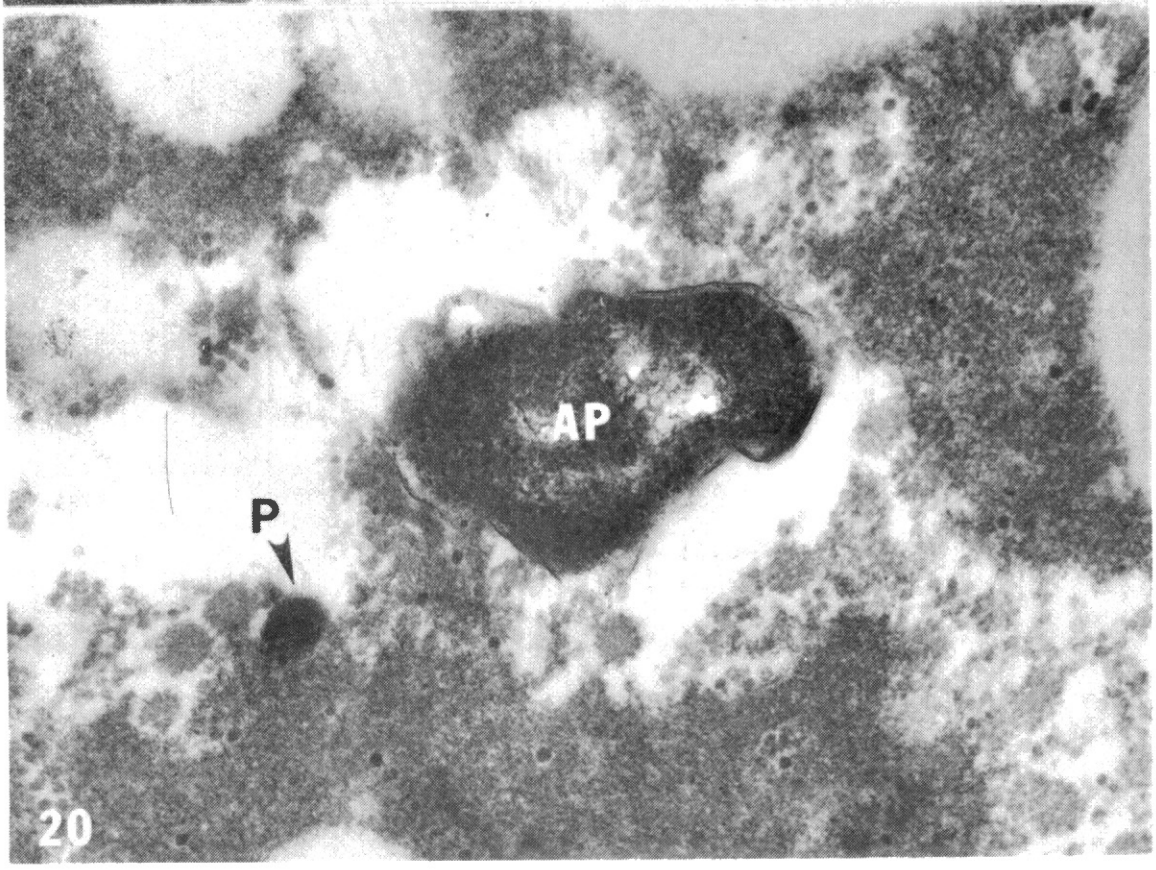

Nu
CS
CJ
18

Figure 19. Electron Micrograph of an Anaplasma-Like Organism in the Cytoplasm of a Gut Epithelial Cell From an Infected Nymphal Tick. The Anaplasma-like organism (AP) is in the cytoplasm near the nucleus (Nu). x 38,000.

Figure 20. Electron Micrograph of an Anaplasma-Like Organism in the Cytoplasm of a Gut Epithelial Cell From an Infected Nymphal Tick. An electron-dense particle (P) is near the Anaplasma-like organism (AP). x 80,000.


Nu
AP
19


AP
P
20

particles were often seen in the host cell cytoplasm near the organisms (Figure 20). These Anaplasma-like organisms were not seen in control nymphs.

Morphologic Studies on Gut of Feeding Adult Ticks

Light Microscope Studies. Paraffin sections (6 μ) of gut tissue from ticks collected at days 5 and 7 of feeding are presented in Figures 21 and 22, respectively. The sections of gut have a well-defined lumen which was greatly enlarged at day 7 of feeding (Figure 22). The gut epithelium was one-cell-layer thick; the luminal (or apical) part of the cells was typically enlarged and contained numerous darkly-stained granules. Cells without apparent attachments were observed in the lumen (Figure 21). No differences were seen between the infected and control tissues and no organisms were detected with the light microscope.

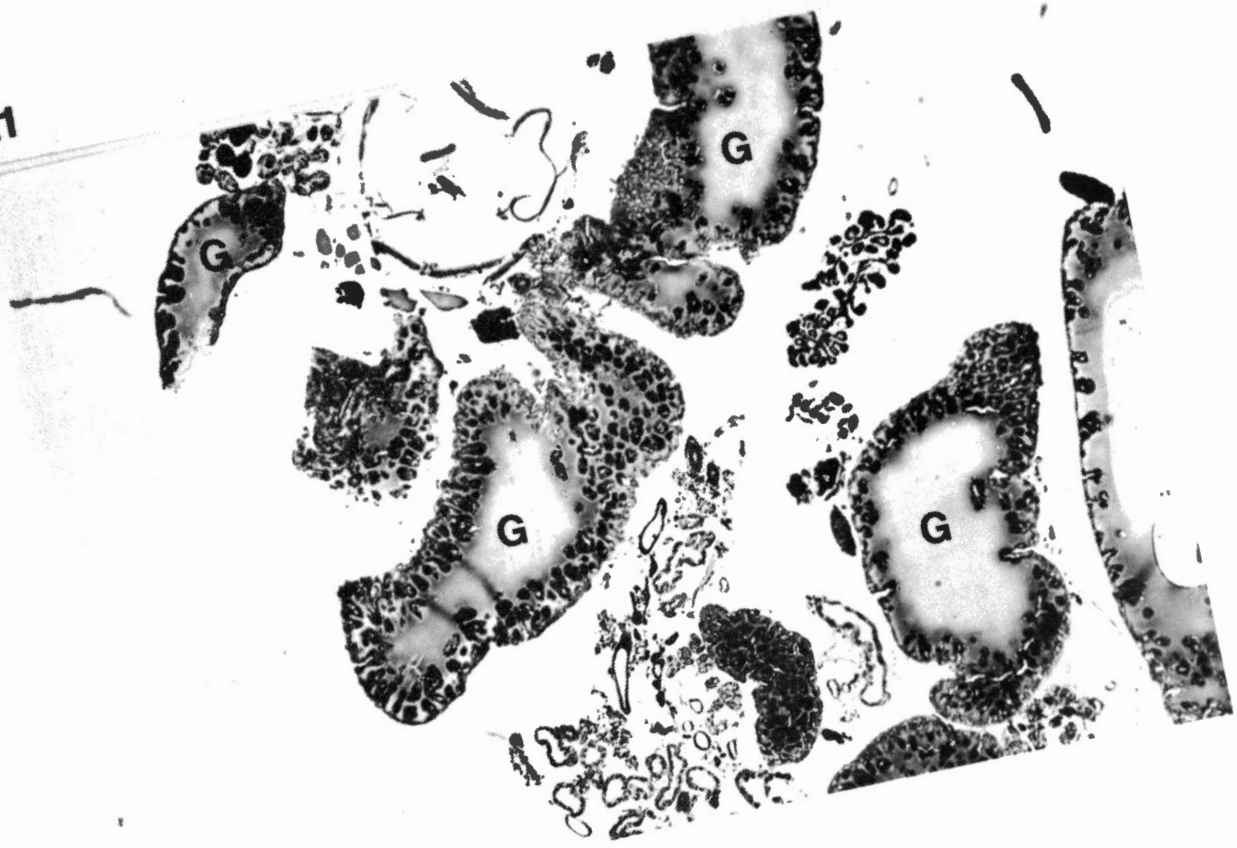
Plastic-embedded sections (1.5 μ) of the gut cells had a more distinct morphology. Figure 23 is a plastic-embedded section of gut collected at day 5 of feeding. Concentrations of granules can be seen clearly along with portions of sloughed cells within the lumen. Material in the lumen stained darkly with Richardson's blue. No differences in morphology were noted in control and infected tissues and rickettsia-like organisms were not seen in either.

Electron Microscopy Studies. Figure 24 is an electron micrograph of the luminal portion of gut epithelial cells. The substance filling the lumen is electron dense and invaginations can be seen in the plasmalemma, suggesting the occurrence of pinocytosis. The cells contained many morphologic types of granules as well as common organelles (mitochondria, endoplasmic reticulum, and nuclei). The basal portion of the typical gut

Figure 21. Light Microscope Photomicrograph of a Cross Section of Midgut From an Adult Tick at Five Days of Feeding. Giemsa stained; paraffin embedment. x 420.

Figure 22. Light Microscope Photomicrograph of Cross Section of Midgut From an Infected Adult Tick at Seven Days of Feeding. Four cross sections of gut (G) are visible. Giemsa stain; paraffin embedment. x 120.

21



22

Figure 23. Light Microscope Photomicrograph of a Cross Section of Midgut From an Infected Adult Tick at Five Days of Feeding. Two cross sections of gut (G) contain sloughed cells (SC). Plastic embedment; Richardson's blue stain. x 320.

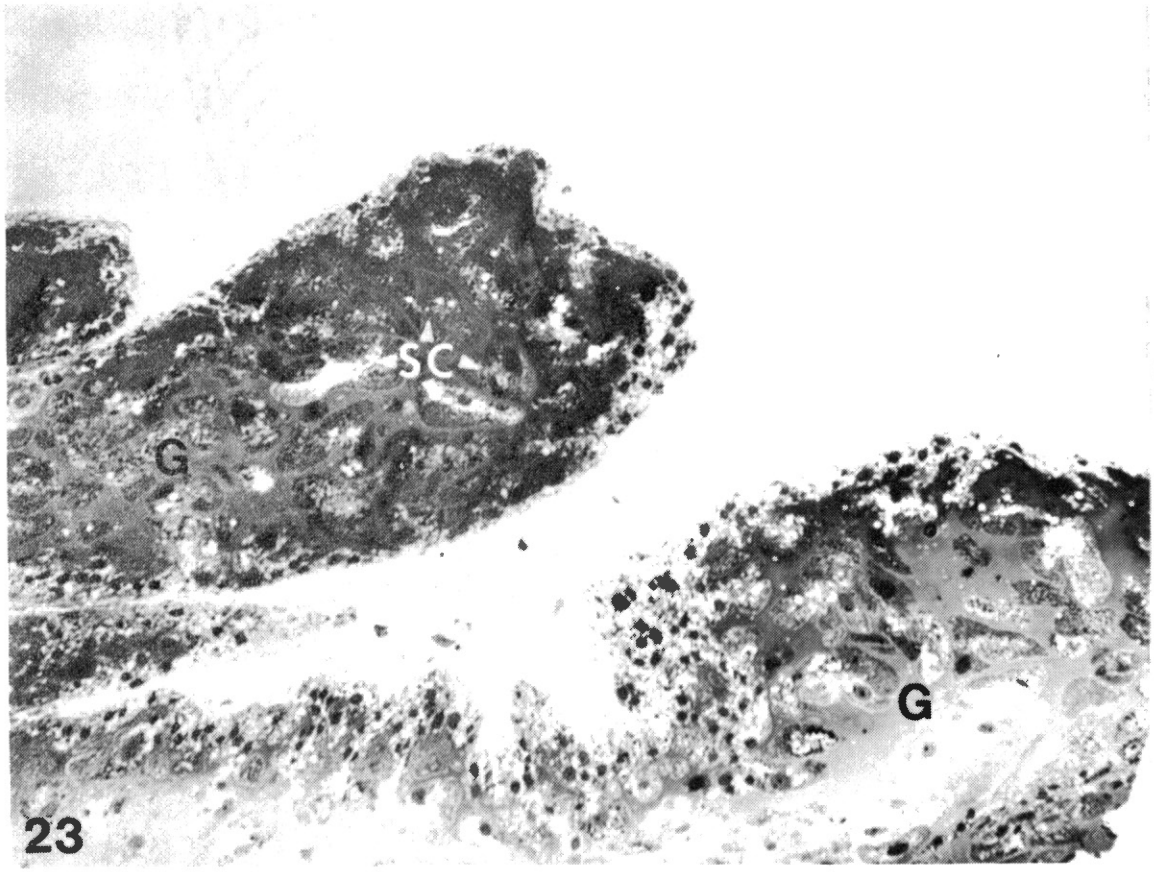
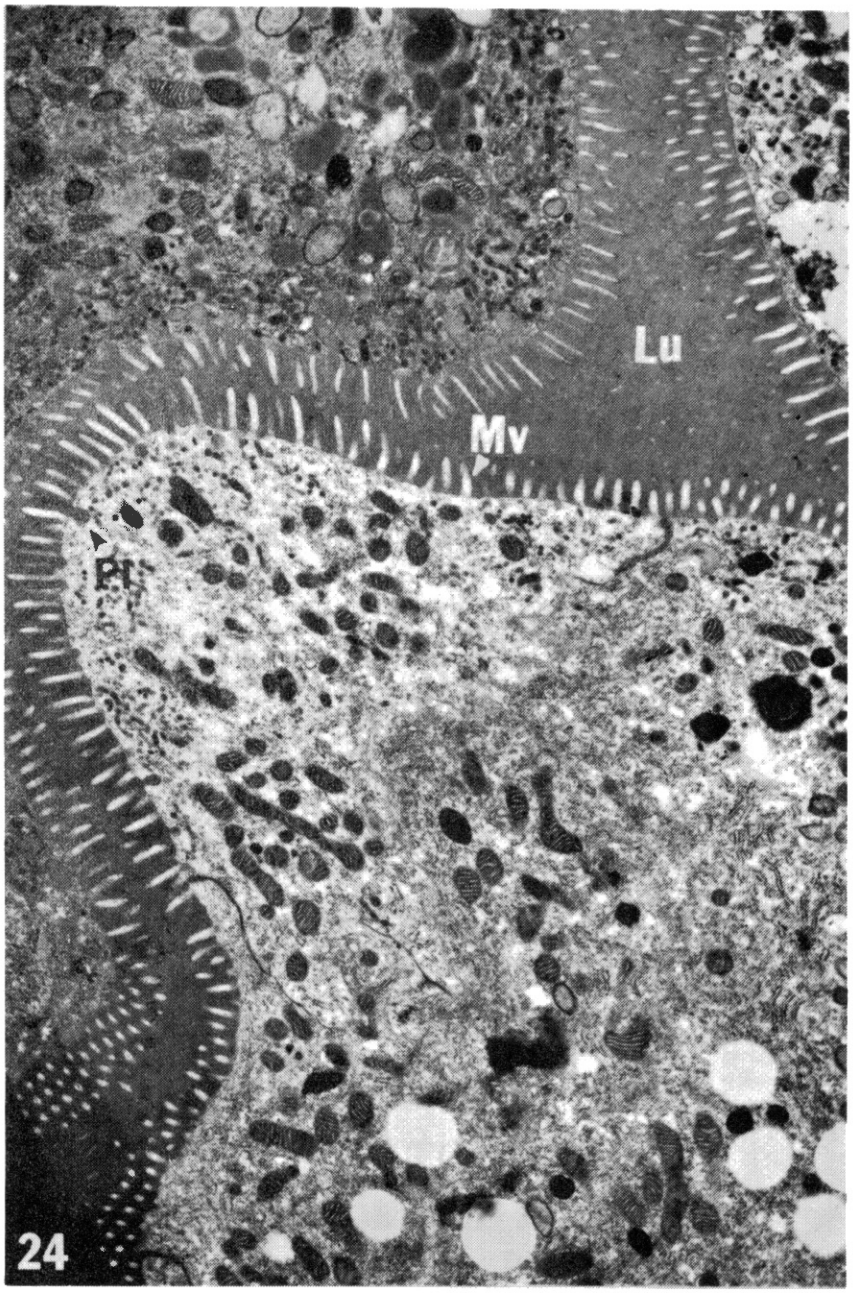

SC
G
G
23

Figure 24. Electron Micrograph of the Luminal Border of Midgut Epithelial Cells From a Feeding, Adult Tick. The lumen (Lu) contains electron-dense material. Numerous microvilli (Mv) are on the luminal cell border. Invaginations of the plasmalemma (PI) suggest the occurrence of endocytosis. x 6,800.


Lu
Mv
Pl
24

epithelial cell is depicted in Figures 25 and 26. On the luminal side, projections of the epithelial cells interdigitate along a very thick basement membrane. Granules can also be observed in the basal portion of the cells (Figure 25). Cells containing muscle fibers were present on the outer surface of the basement membrane.

Anaplasma-like organisms were observed most frequently in the basal portion of the gut epithelium of infected ticks (Figures 27 and 28). They appeared in the cytoplasm and were seen in close apposition to electron-translucent cellular deposits (Figure 27), but were not observed within the cell nuclei. The organisms were similar in size and structure to those observed in nymphal ticks. They were shaped somewhat irregularly, had two cell membranes, and contained dense aggregates and a filamentous protoplasmic network. On occasion, organisms appeared to be in the process of dividing (Figure 28). Anaplasma-like organisms were not seen in control adult ticks.

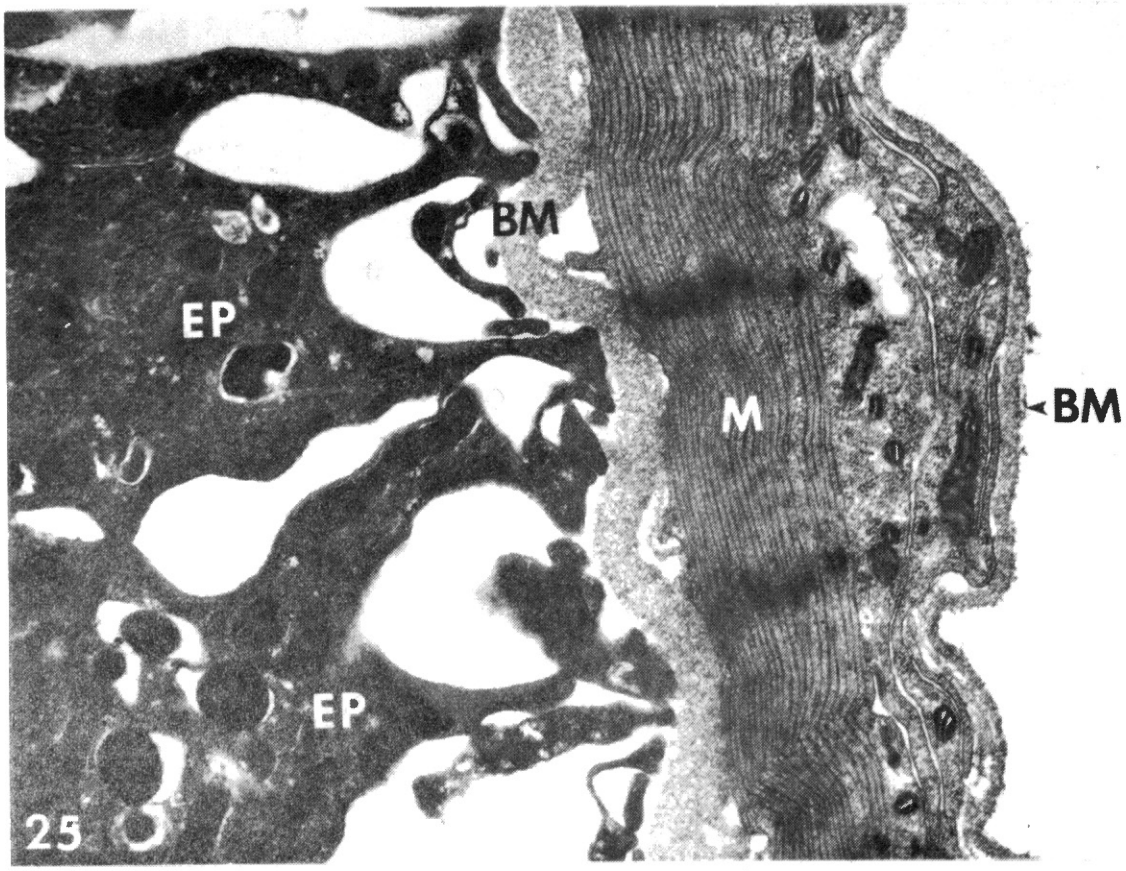
Morphologic Studies of Gut From Flat Adult Ticks

Light Microscope Studies. Cross sections of gut tissues from infected ticks that had been incubated are presented in Figures 29 through 32. Small colonies of organisms were seen in infected ticks which were not present in ticks that were not incubated. These colonies were usually located near the basement membrane and were often surrounded by clusters of dark-staining granules (Figures 31 and 32). The colonies had specific staining characteristics and were easily observed in the light microscope.

Electron Microscope Studies. Anaplasma-like organisms which were very electron dense were seen in gut tissues of unincubated, infected,

Figure 25. Electron Micrograph of the Basal Portion of Midgut Epithelial Cells From a Feeding, Adult Tick. Basal projections (EP) of gut epithelial cells interdigitate along a thick basement membrane (BM). Cells on the outer surface of the gut contain muscle fibers (M) and are surrounded by basement membrane. x 21,000.

Figure 26. Electron Micrograph of the Basal Portion of a Midgut Epithelial Cell From a Feeding, Adult Tick. Basal projections (EP) of epithelial cells interdigitate along a thick basement membrane (BM) which surrounds a muscle cell (M). x 42,500.


BM
EP
M
BM
EP
25

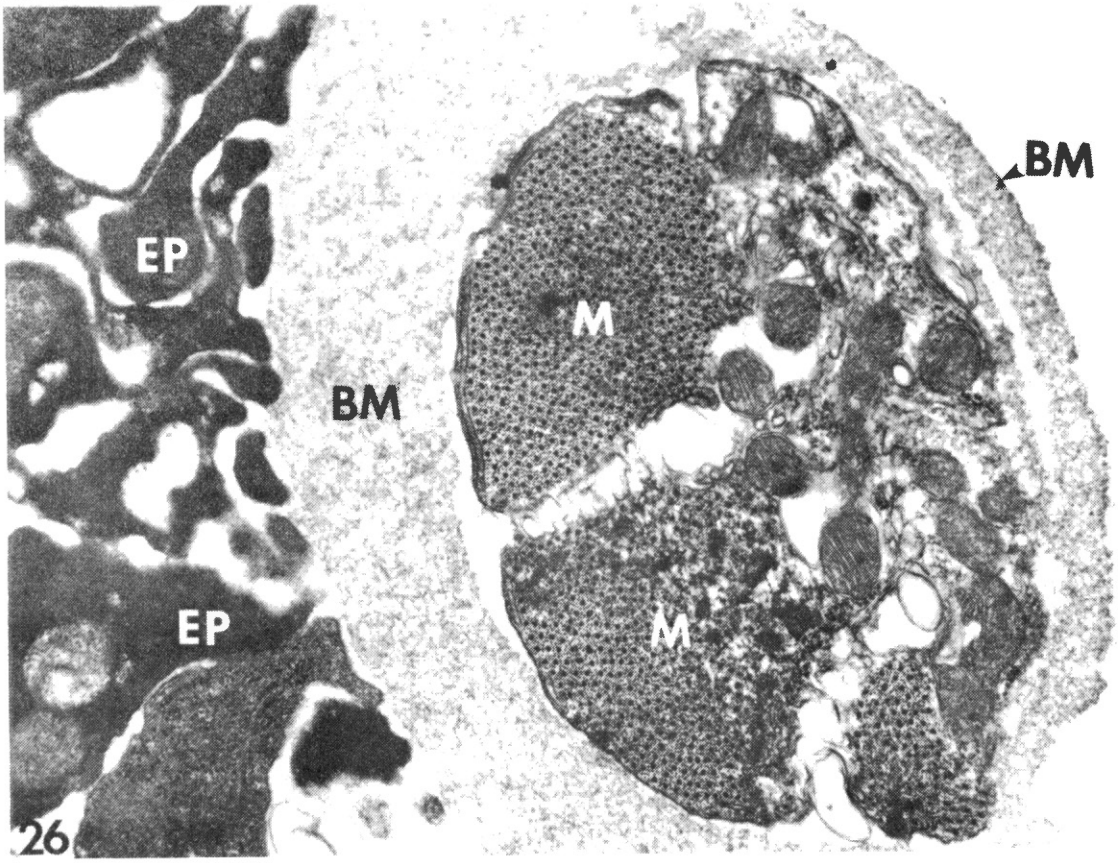
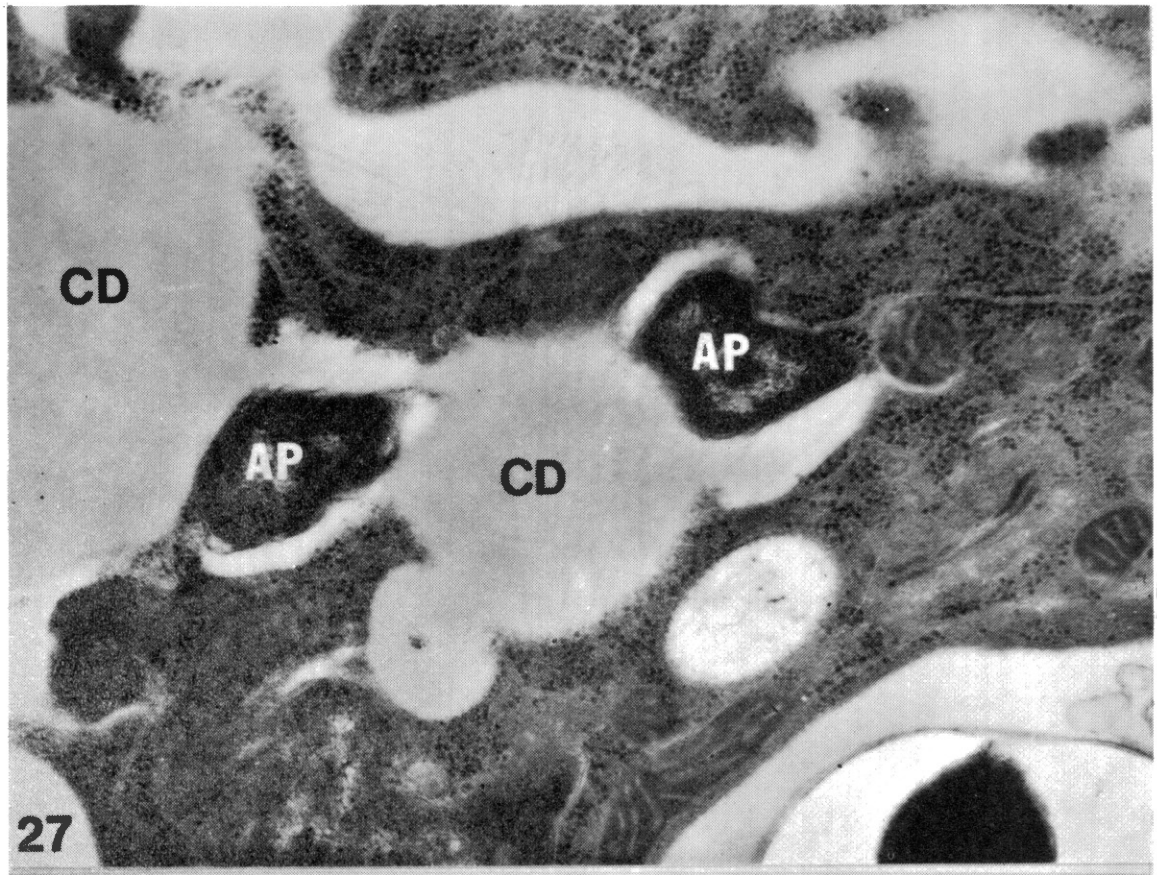
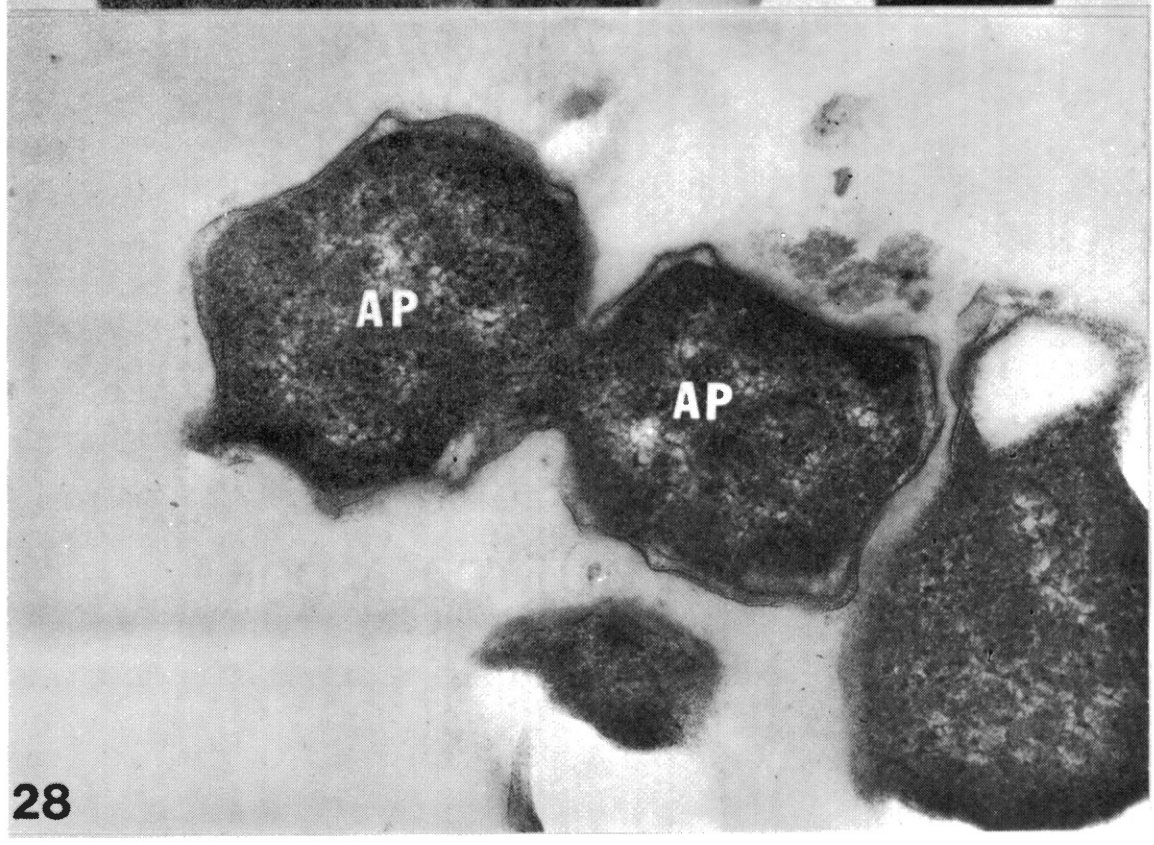

BM
EP
M
BM
EP
M
26

Figure 27. Electron Micrograph of an Anaplasma-Like Organism in the Cytoplasm of a Midgut Epithelial Cell of a Feeding, Adult Tick That was Infected in the Nymphal Stage. Anaplasma-like organisms (AP) adjacent to electron-translucent cellular deposits (CD). x 43,000.

Figure 28. Electron Micrograph of an Anaplasma-Like Organism in the Cytoplasm of a Midgut Epithelial Cell of a Feeding, Adult Tick that was Infected in the Nymphal Stage. The Anaplasma-like organism (AP) appears to be dividing by binary fission. x 86,000.



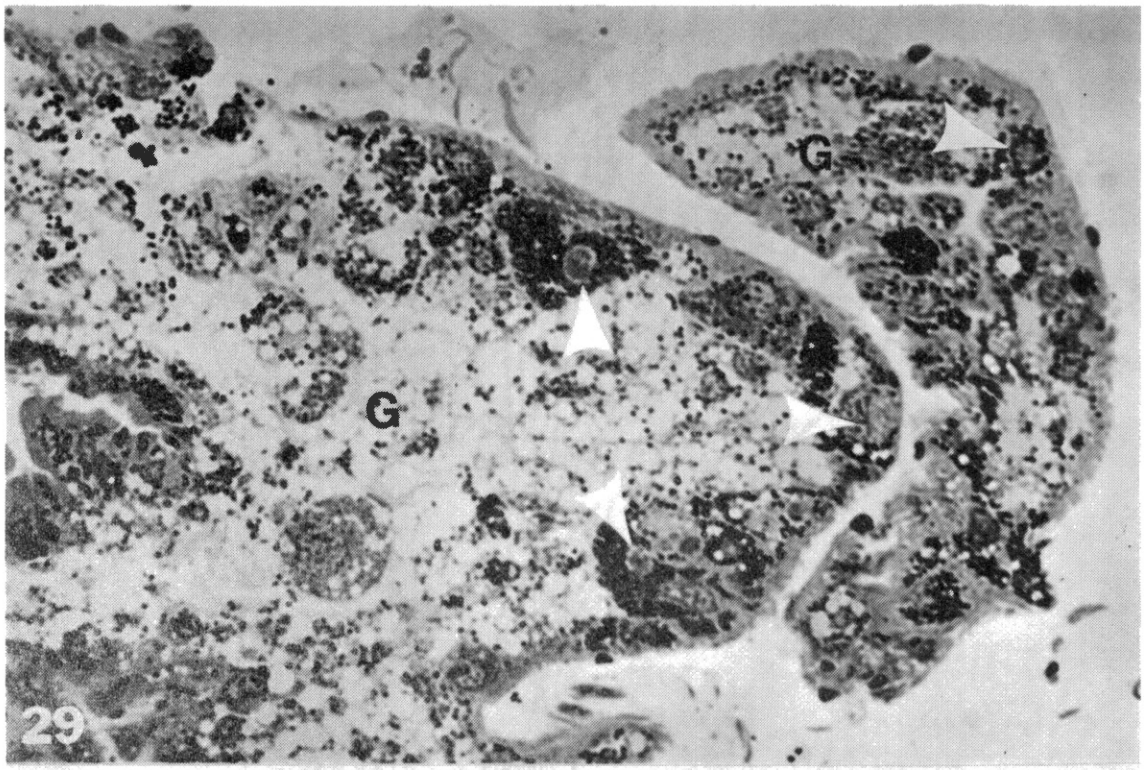
27



28

Figure 29. Light Photomicrograph of a Cross Section of Midgut From an Infected, Flat, Adult Tick That was Incubated. Arrows point to colonies of Anaplasma-like organisms at the periphery of a cross section of midgut (G). Plastic embedment, Richardson's blue stain. x 150.

Figure 30. Light Photomicrograph of a Colony of Anaplasma-Like Organisms in Midgut of an Infected, Flat Adult Tick That was Incubated. Three colonies (C) are near the basement membrane of the gut cross section (G). A limiting membrane is not visible with the light microscope. Plastic embedment; Richardson's blue stain. x 400.


G
G
29

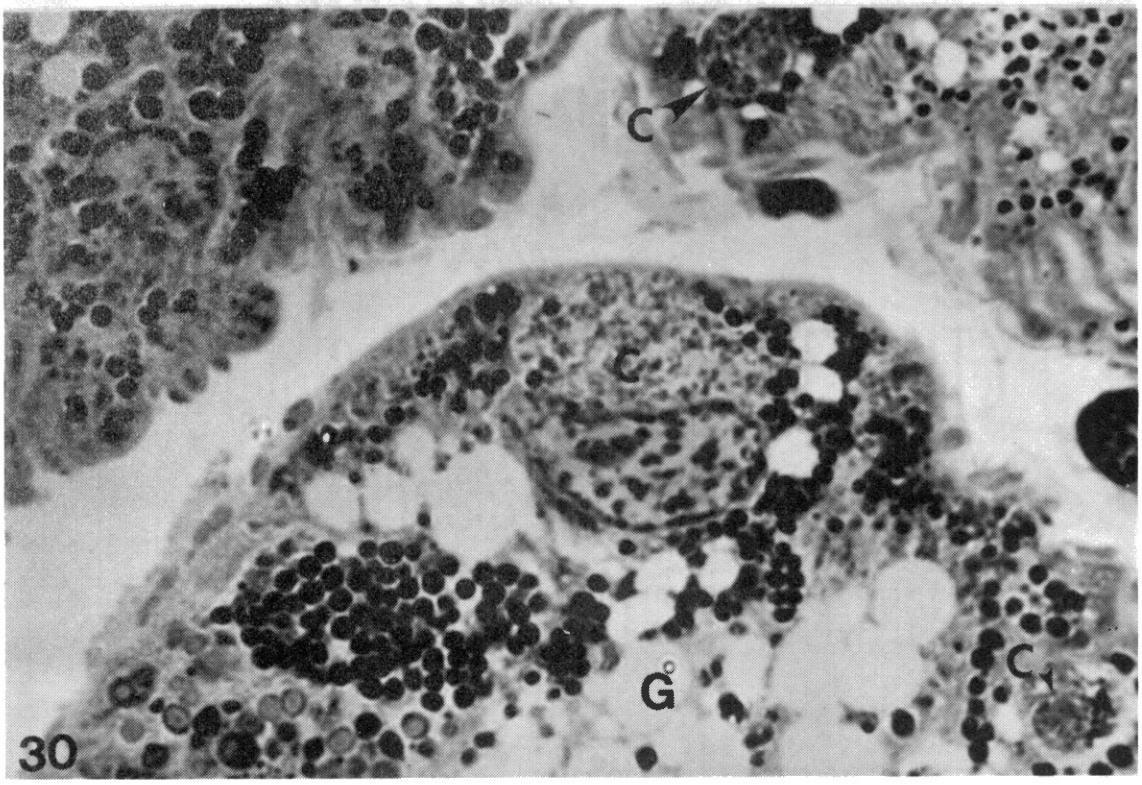
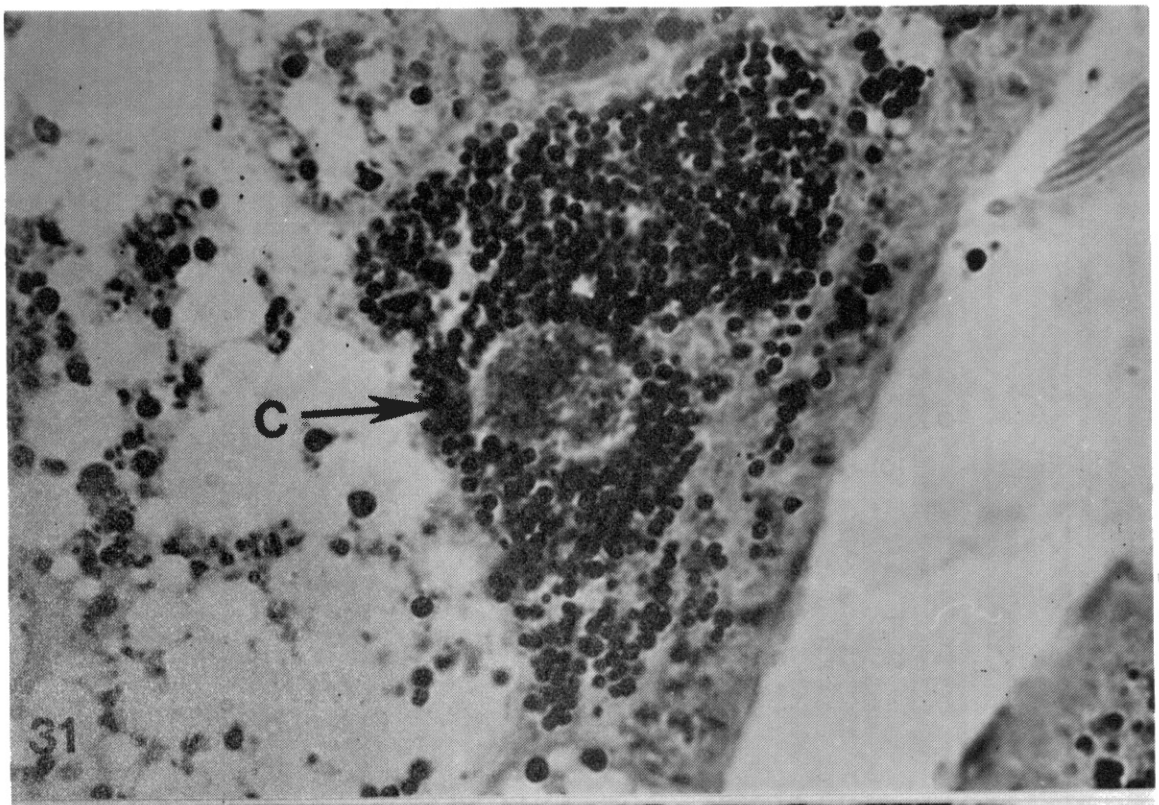
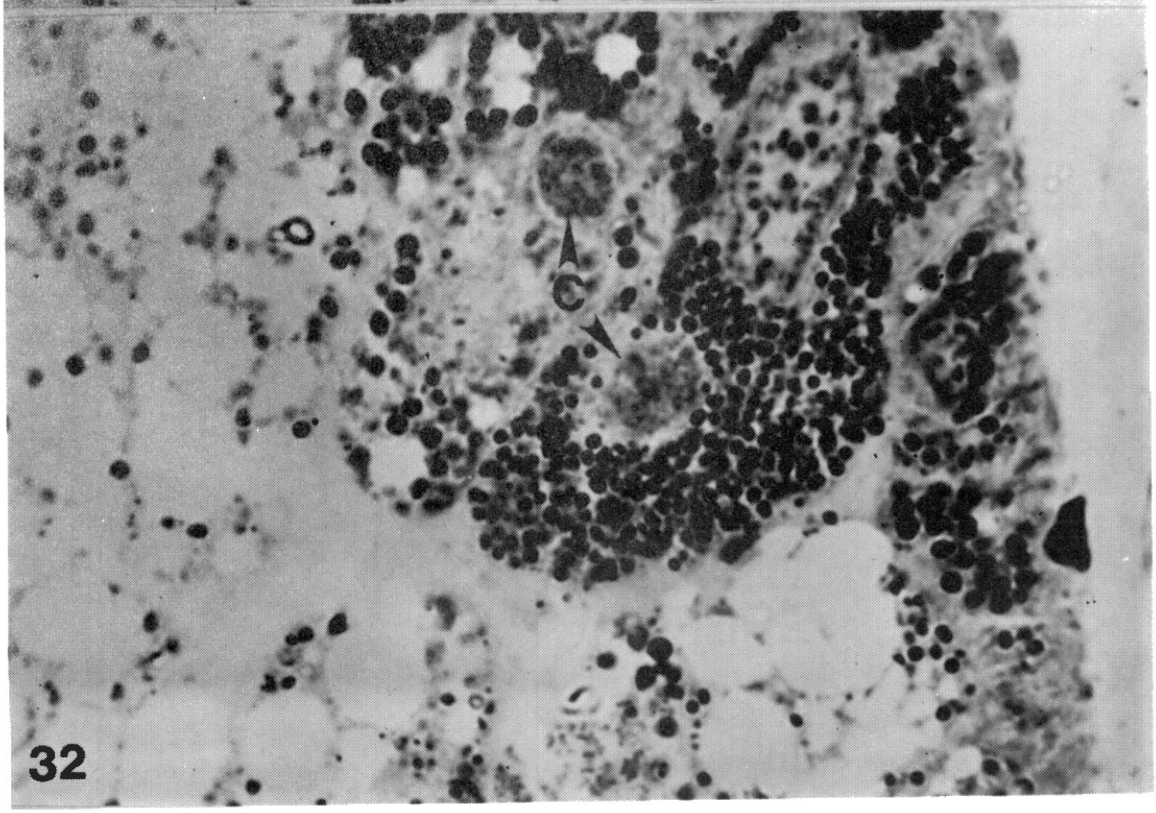

C
C
G
C
30

Figure 31. Light Photomicrograph of a Colony of Anaplasma-Like Organisms in Midgut of an Infected, Flat Adult Tick That was Incubated. The colony (C) is in a cell with many dark granules. Plastic embedment, Richardson's blue stain. x 380.

Figure 32. Light Photomicrograph of two Colonies of Anaplasma-Like Organisms in Midgut Epithelium of an Infected, Flat, Adult Tick That was Incubated. The two colonies (C) are in cells with many dark granules. Plastic embedment, Richardson's blue stain. x 380.


C
31


C
32

flat adult ticks (Figures 33A and B). The organisms had a light halo around them. Small, electron-dense particles often occurred near the organisms (Figure 33A and B). Many small tubular forms were also observed (Figure 33B). Organisms were not found in incubated and unincubated control flat adult ticks.

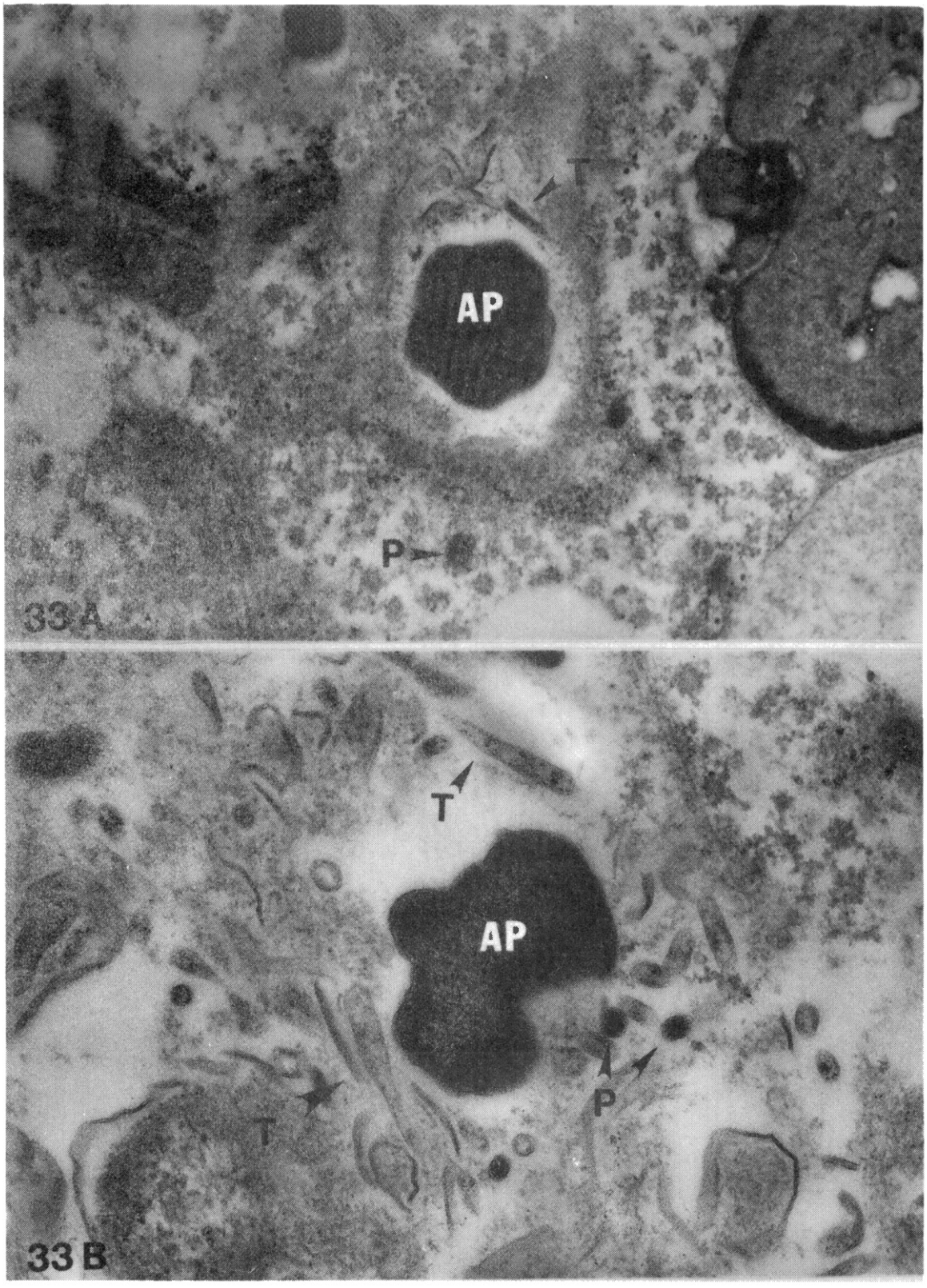
Rickettsia-like organisms, presumably A. marginale, were seen in ultrathin sections of gut from infected flat adult ticks that were incubated (Figures 34 through 37). Although clusters of organisms were seen in the cytoplasm of gut epithelial cells near the basement membrane (Figure 34), most Anaplasma-like organisms were seen in colonies in the basal portion of the epithelial cells (Figures 35 through 37). As observed in the light microscopy studies, many colonies were surrounded by granules which were very electron dense (Figure 35A, B, and D). A surrounding membrane was not evident in most colonies (Figure 35B and C), but a very thin limiting membrane was seen in a few instances (Figure 35D).

The organisms are rickettsia-like in structure, having two distinct cell membranes and containing both electron-dense aggregates and a filamentous protoplasmic network (Figure 36A-D). However, these organisms differ from typical rickettsiae in that they are very pleomorphic. Long protoplasmic extensions protrude from some organisms (Figure 36A) and sometimes completely surround other organisms (Figure 36B). Shapes of organisms range from round or concave to very irregular (Figure 36A-D). Size varies greatly (Figure 37A-D). In some instances colonies contain many very small particles as well as larger pleomorphic organisms (Figure 37A). At higher magnification (Figure 37B-D), it appears that many of the smaller particles possess features common to the larger organisms.

Figure 33. Electron Micrographs of Anaplasma-Like Organisms in Midgut Epithelial Cells of Infected, Adult Ticks That Were not Incubated.

(A) An Anaplasma-like organism (AP) is very electron-dense and is surrounded by a light area and tubular structures (T). An electron-dense particle (P) is near the organism. x 43,000.

(B) An Anaplasma-like organism (AP) surrounded by many tubular structures (T) and electron-dense particles (P). x 64,000.


T
AP
P
33A
T
AP
P
T
33B

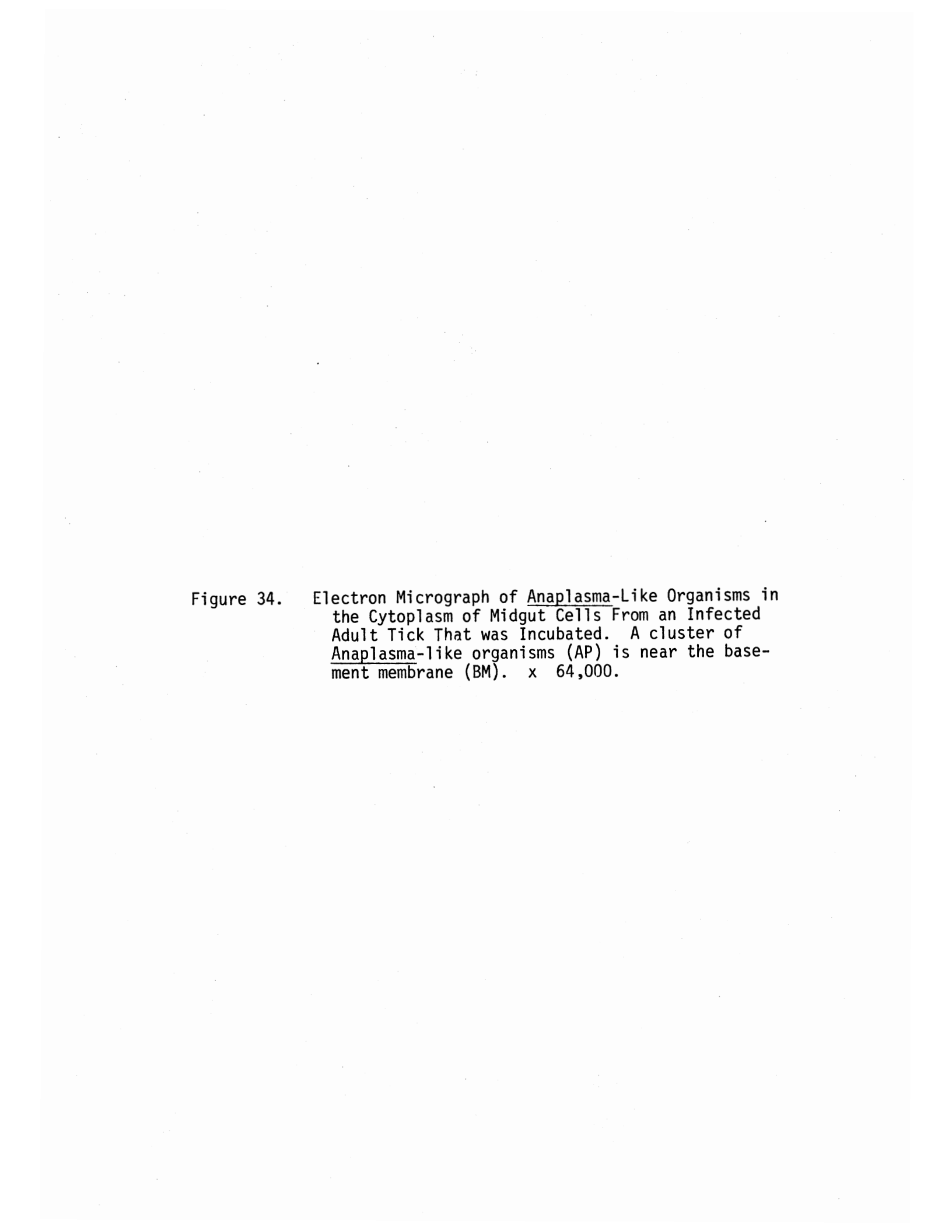


Figure 34. Electron Micrograph of Anaplasma-Like Organisms in the Cytoplasm of Midgut Cells From an Infected Adult Tick That was Incubated. A cluster of Anaplasma-like organisms (AP) is near the basement membrane (BM). x 64,000.

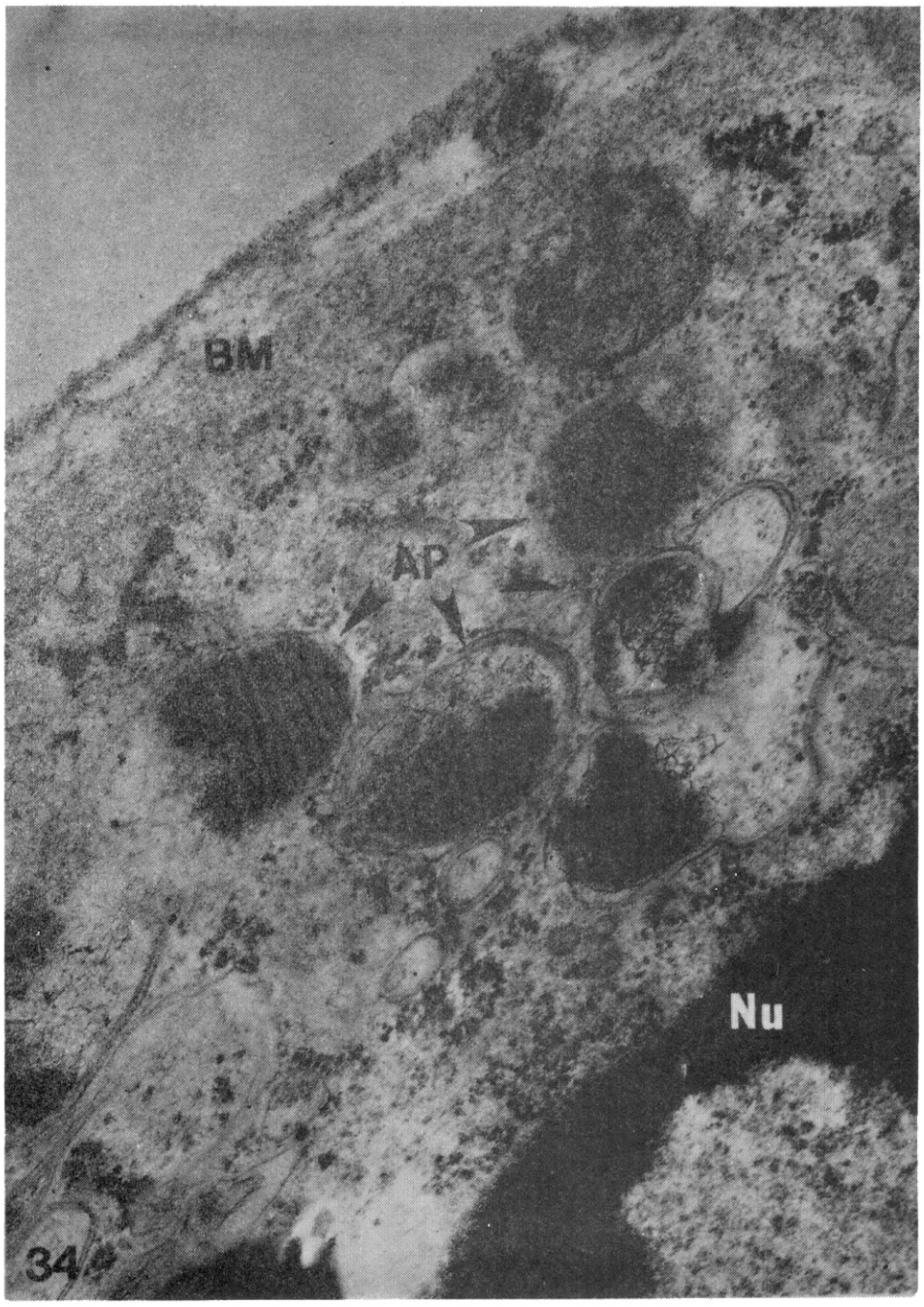

BM
AP
Nu
34

Figure 35. Electron Micrographs of Colonies of Anaplasma-Like Organisms in the Midgut Cells of Infected, Adult Ticks That Were Incubated. Colonies were in cells with many dark granules (DG). A delicate, limiting membrane (LM) was sometimes visible as seen in 35D. (A) x 5,400; (B) x 5,400; (C) x 12,300; (D) x 19,500.

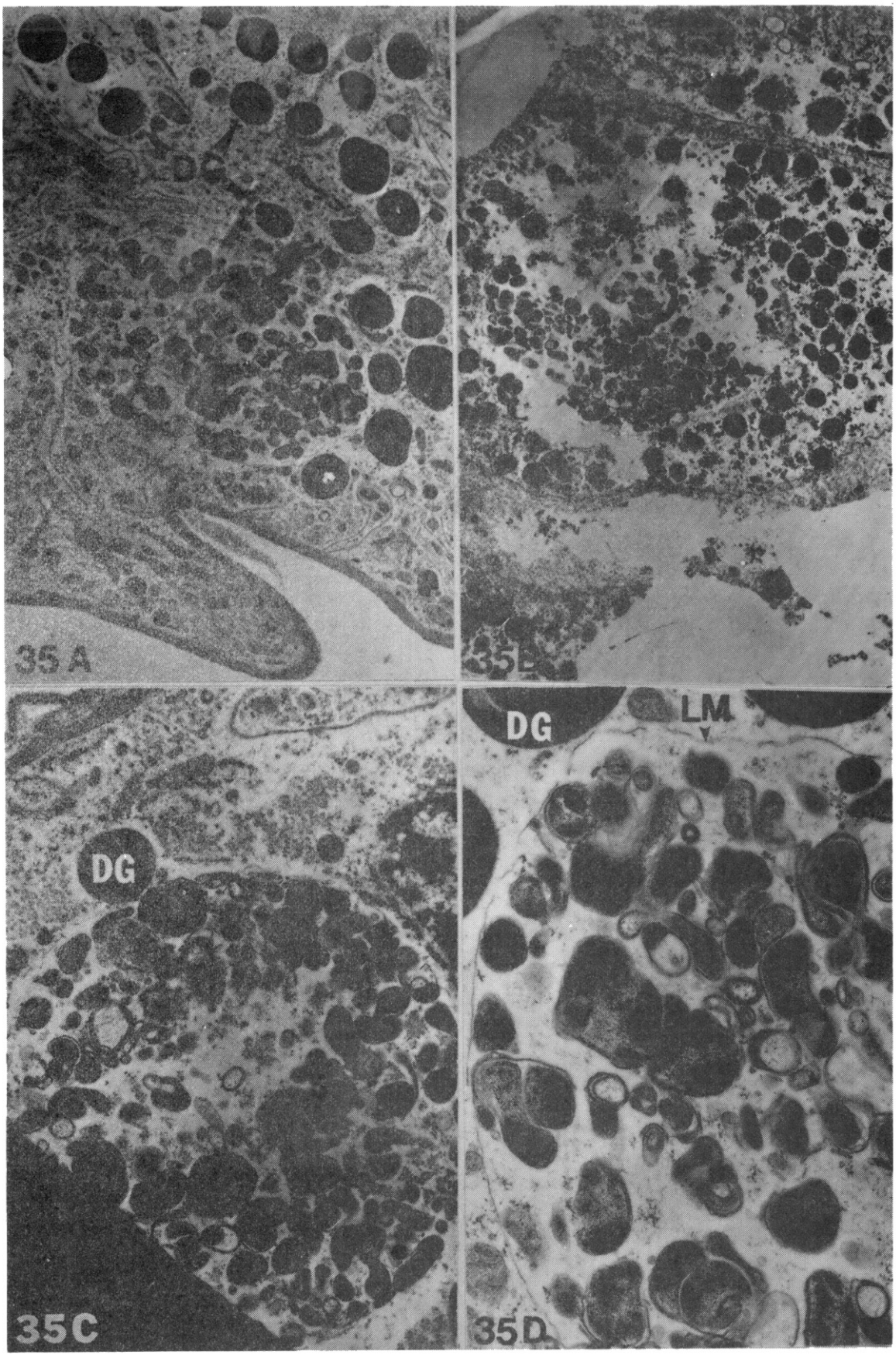

DG
35 A
35B
DG
35C
DG
LM
35D

Figure 36. High Magnification Electron Micrographs of Anaplasma-Like Organisms From Colonies in the Midgut of Cells of Infected, Adult Ticks That Were Incubated. The colonies contained organisms of many shapes (Figure A-D). Long protoplasmic extension (arrows) protrudes from an organism in 36A and completely surrounds an organism (arrows) in 36B. A crystalline body (CB) is in an organism in 36B. (A) x 22,600; (B) x 22,600; (C) x 20,500; (D) x 20,500.

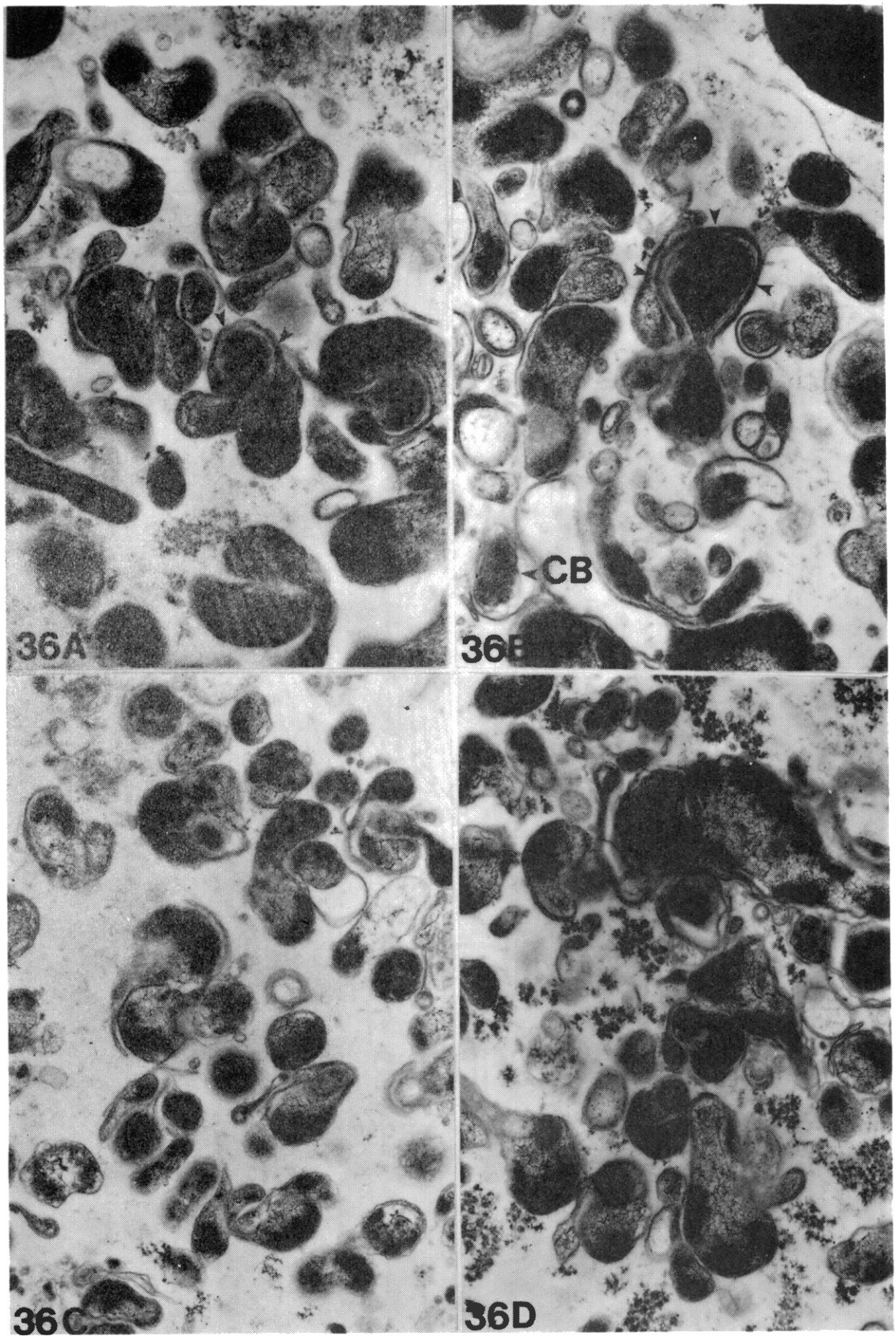
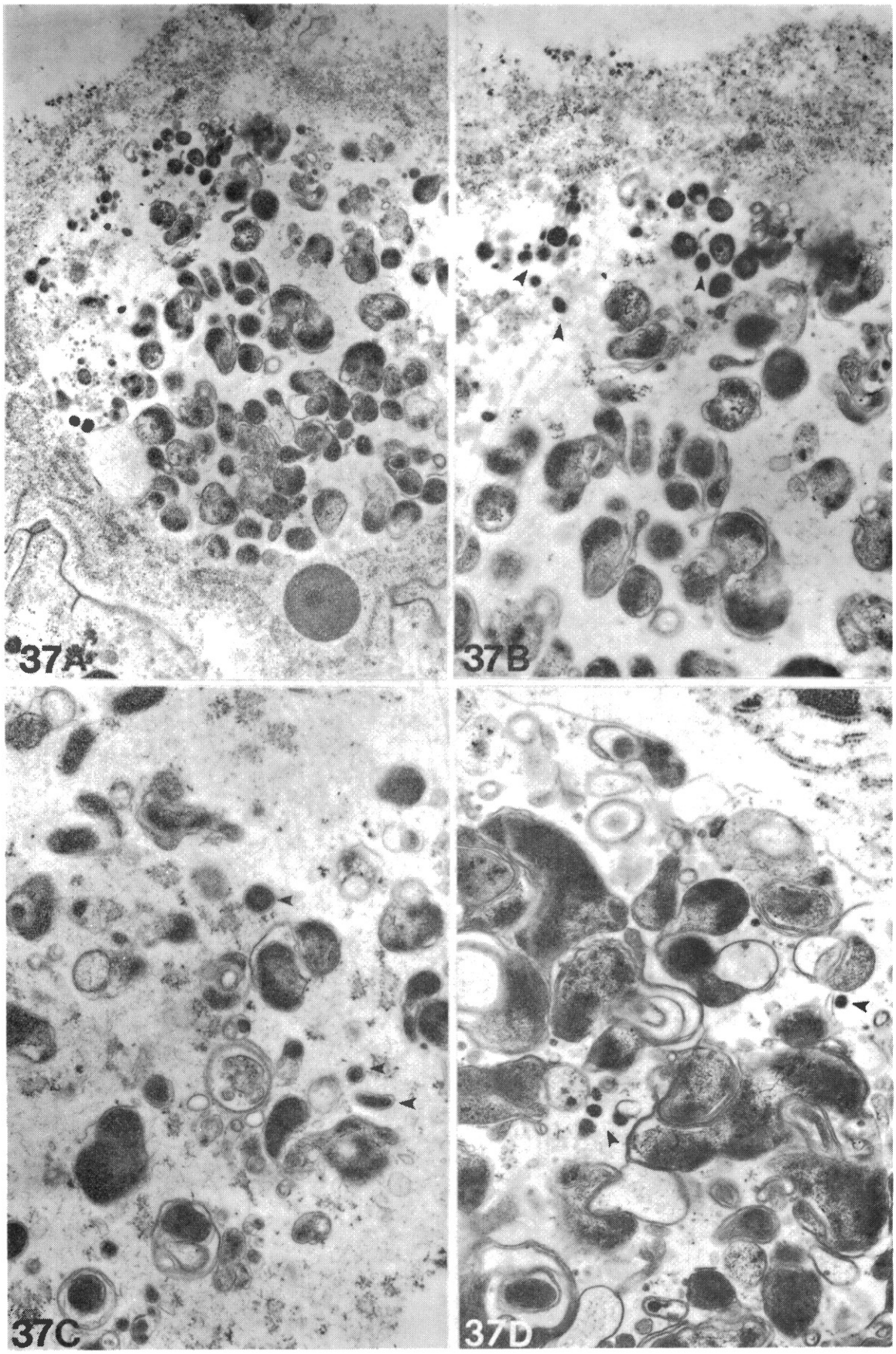

36A
CB
36B
36C
36D

Figure 37. Electron Micrographs of Colonies of Anaplasma-Like Organisms With a Wide Range in Size of Organisms. Very small organism-like bodies are seen in A-D with arrows pointing to specific bodies in B-D. (A) x 7,800; (B) x 14,000; (C) x 14,000; (D) x 20,000.


37A
37B
37C
37D

The internal components are very electron dense and the particles are bound by two membranes. The large electron-dense granules were not similar enough to be confused with these particles; furthermore, they were found in both infected and control tissues, were much larger and were not observed within colonies (Figure 35A and B).

Other rickettsia-like organisms were observed in both the infected and control gut tissue (Figure 38A and B) but their morphology differed from that of those organisms presumed to be A. marginale. These organisms were much larger than those seen in the colonies, were not very pleomorphic or electron dense and the two limiting membranes were in close apposition to one another.

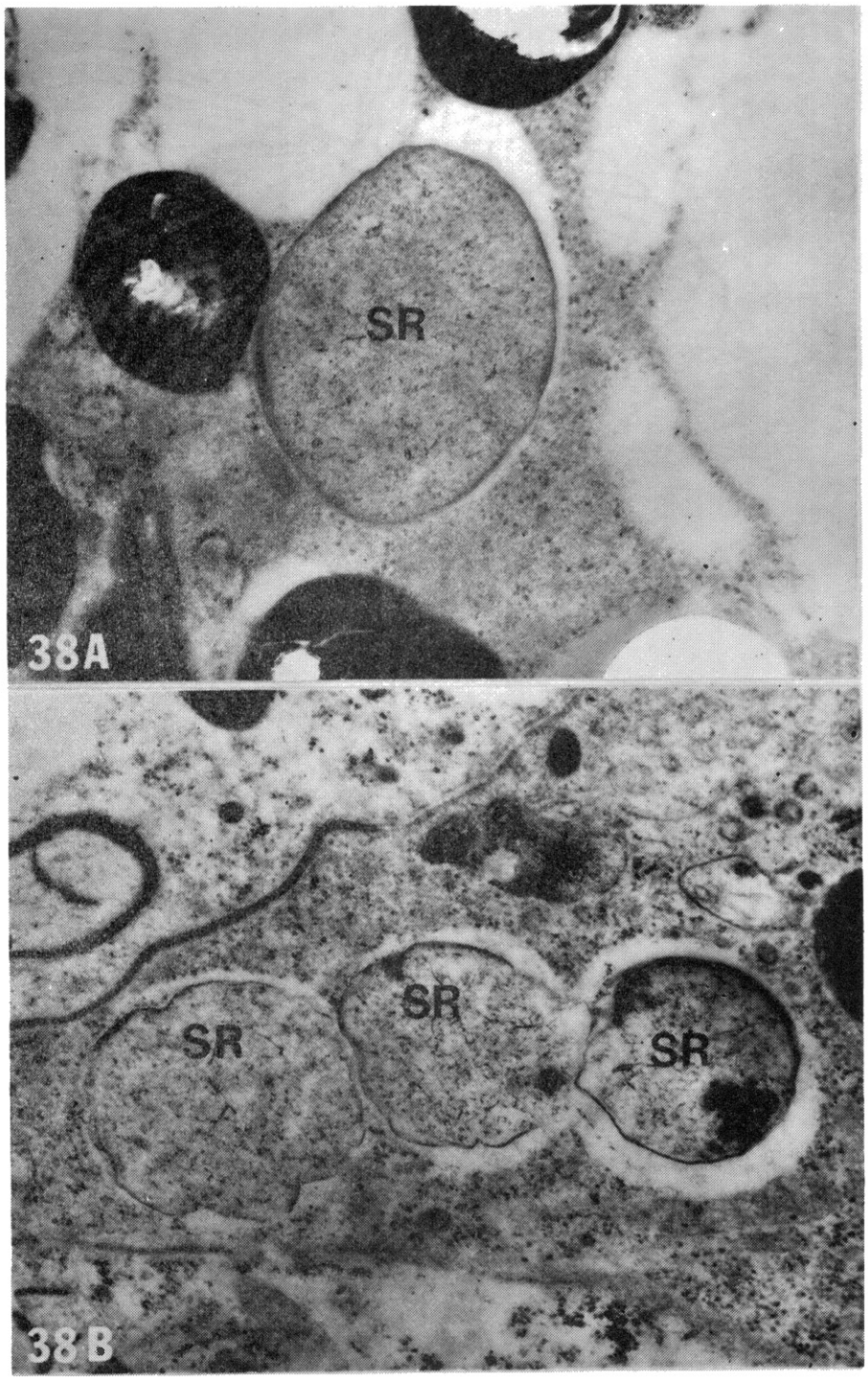
Morphologic Studies of Tick Salivary Glands

Rickettsia-like organisms were not seen in either paraffin or plastic-embedded sections with the light microscope nor in electron micrographs of salivary glands. This included sections of salivary glands from replete nymphs, flat adults (incubated and unincubated) and feeding adult ticks.

Discussion

Of the many types of organisms found in ticks (viruses, bacteria, sporozoans, and rickettsiae), the organisms observed in these studies are most similar to the rickettsiae and so-called rickettsia-like organisms. Organisms with similar morphologic features to the organisms described herein and the diseases they cause include Rickettsia sp. (Rocky Mountain spotted fever) (14) (90), Ehrlichia canis (canine ehrlichiosis) (79), and Cowdria ruminantium (heartwater disease) (91).

Figure 38. Electron Micrographs of Symbiotic Rickettsia Found in the Midgut Epithelial Cells of Infected and Uninfected Adult Ticks That Were Incubated. Figure 38A is a symbiotic rickettsia (SR) in an infected midgut epithelial cell while 38B contains three symbiotic rickettsiae (SR). (A) x 32,000; (B) x 31,500.


SR
38A
SR
SR
SR
38B

The studies reported herein have involved examination of gut and salivary gland tissues from various stages of ticks. Although other tissues were occasionally examined, they were not consistently studied in both infected and control groups. The organisms described herein as Anaplasma-like in gut from all tick stages were not found in corresponding control tissues. Anaplasma-like organisms were seen only in gut tissues which is in contrast to reports regarding Rickettsia sp. and Ehrlichia sp. infections in ticks. Rickettsia sp. organisms have been reported in all tissues (14) (92) (the infection referred to as "generalized") and Ehrlichia canis demonstrated in the gut, salivary gland and hemocytes (79).

The data from the present study do not firmly implicate salivary glands as being important in transmission of anaplasmosis. Although homogenates of salivary gland were shown to be infective for susceptible cattle (Chapter II, Tables II and III), oral secretion from the same lot of ticks was not infective (Chapter II, Table II). Furthermore, rickettsial organisms were not observed in salivary glands of any tick stage studied (Chapter III, page 81). The possibility exists that infectivity of salivary gland homogenates could have resulted from contamination. Tick gut is very fragile and it is difficult to dissect ticks without rupturing this organ with resultant diffusion of gut contents and cells.

Tick transmission of rickettsial organisms via routes other than the salivary gland has been demonstrated. Řehàček reviewed transmission of rickettsial agents via tick feces (13). Ticks eliminate large quantities of fecal material which contains crystallized hematin and viable rickettsiae. In studies regarding transmission of Coxiella burnetii (93), rickettsiae have been shown to persist in tick feces stored for 65 days at

room temperature. Daiter and Amosenkova have infected guinea pigs with C. burnettii recovered from tick feces that had been stored at room temperature in 70 percent relative humidity for 744 days (94). Regurgitation of gut contents during feeding may be another mode of transmission of infectious agents. Studies on the feeding mechanisms of ticks have confirmed that regurgitation does occur during the feeding process (24). This type of transmission is thought to occur in Cowdria ruminantium infection (95). In the present study, rickettsia-like organisms were not observed free in the lumen of the gut. However, transmission via gut regurgitation could have occurred when organisms were present with apical portions of midgut epithelial cells that had sloughed.

Anaplasma-like organisms demonstrated in nymphal, flat adult, and adult feeding ticks were scattered loosely within the cytoplasm of midgut epithelial cells; most organisms observed in incubated flat adult ticks were in membrane-bound colonies. Similar colony formations of rickettsia-like organisms have been described in Cowdria sp. infections in cattle (91) and for Ehrlichia sp. infections in canine leukocytes (96) (97). Colonies observed in Ehrlichia sp. infections are referred to as "morulae" and have been described within tick gut and salivary gland tissues as well as in the vertebrate host. It is possible that, in the present study, colonies of organisms were overlooked in adult ticks that had fed because there was a much larger quantity of tissue to sample. The samples from ticks that had not fed but had been incubated were small and colonies of organisms were easily demonstrated.

All rickettsial and rickettsia-like organisms share common features: (1) two or more limiting membranes, and (2) internal components consisting of dense aggregations and a filamentous protoplasmic network. These

features were noted in the organisms studied, but there was much greater pleomorphism than has been reported for rickettsiae. The organisms observed in gut tissues of infected nymphs were morphologically very similar to those described as A. marginale in infected bovine erythrocytes (73). Organisms observed in gut tissues of flat, unincubated, adult ticks were extremely compact and electron dense; organisms demonstrated in colonies of gut epithelial cells from incubated flat adult ticks were extremely pleomorphic in terms of size, shape and electron density of internal components. Many morphologic descriptions of Rickettsia sp. (92) (98) (99) have included some pleomorphic forms; however, the organisms described herein were predominantly pleomorphic in both size and shape. Pleomorphism was the rule, not the exception.

Different morphologic types of rickettsial organisms have been reported. Brinton and Burgdorfer described three rickettsial forms in hypodermal cells of ticks, viz., growth forms, atypical forms, and forms containing crystalline bodies (92). In the present study, crystalline bodies were seen in some Anaplasma-like organisms in colonies in gut cells from flat adult ticks that had been incubated (Figure 36B). Avakyan et al. also described three stages of rickettsia-like symbiotes in ticks; these included: (1) reproductive forms, (2) conservative forms, and (3) activation forms (100). Although none of these descriptions seems to apply to the organisms studied here, the morphology of the organisms varied somewhat in each tick stage. These observations presume that the organisms seen in each stage were A. marginale. The morphologic form observed in nymphal ticks is most similar to that found in bovine erythrocytes; and these were observed in samples collected from ticks 10 days after they fed to repletion on an infected cow. The morphologic form observed in

the gut of flat adult ticks was very compact and electron-dense and was similar to a resting or conservative form described by Avakyan et al. The organisms observed in flat adult ticks that were incubated were very pleomorphic and in membrane-bound colonies and may represent a reproductive form of the organism.

The occurrence of small, electron-dense particles in electron micrographs of all tick stages was of interest. The author described similar structures in infected bovine erythrocytes (73). The occurrence of these bodies in nymphal ticks (Figure 20), unincubated flat adult ticks (Figure 33A and B) and incubated flat adult ticks (Figure 37A-D), all of which were shown to be capable of transmitting anaplasmosis, suggests that these structures are a part of the life cycle of Anaplasma marginale. More detailed studies of the development of colonies in incubated ticks may help to clarify the origin and function of these dense particles.

Many authors have described binary fission as the mode of reproduction for rickettsial organisms (98) (100) (101). Weiss stated "there is overwhelming evidence from observations (too numerous to be cited) . . . that members of the genus Rickettsia multiply by transverse binary fission" (102). Some morphologic evidence in the present study supports the occurrence of binary fission (Figure 28); however, the small electron-dense particles may represent an alternate mode of reproduction. The extreme pleomorphism observed in colonies of A. marginale-like organisms does not appear to be consistent with reproduction by binary fission.

Many nonpathogenic, intracellular, rickettsia-like organisms have been described in ticks (17 through 23). One problem encountered in describing the anaplasmal organism in the tick is differentiation from these symbiotic forms. Evidence pointing to positive identification of

the anaplasmal organism includes positive fluorescence and ferritin-conjugated antibody studies (Chapter IV). In addition, no organisms that were sufficiently similar to be confusing were observed in tissues from control ticks. Adult ticks that had been incubated soon after molting from the nymphal stage, but that had not fed, provided the most substantial evidence that the Anaplasma-like organisms were, in fact, A. marginale. In these tissues, the organisms were in colonies which could be detected with the light microscope, thus enabling study of a larger sample of tissue. Chances of postive identification of the organism would be improved by testing Koch's postulates for the identification of an infectious agent. This process includes isolation and growth of the organism in in vitro and, upon inoculation into a susceptible cow, production of clinical anaplasmosis. Rickettsia-like organisms seen in both infected and control ticks probably are symbiotic rickettsiae (Figure 38).

If the colonies of rickettsia-like organisms observed in the incubated, flat adult ticks do represent colonies of A. marginale, it may be possible also that these structures exist in extra-erythrocytic sites in infected vertebrate hosts. Since these structures are detectable with the light microscope, much larger quantities of tissue than can now be studied could be examined, thus providing a more thorough sampling. Colonies detected with the light microscope could then be studied with the electron microscope in order to confirm identification.

CHAPTER IV

LABELED ANTIBODY STUDIES OF *ANAPLASMA MARGINALE* IN SELECTED IXODID TICKS

Introduction

Labeled antibody techniques have been developed and widely used for the identification of microorganisms (103) (104). Fluorescein-labeled antibody was developed by Coons (103) for identification of specific microorganisms with the light microscope. Ferritin particles attached to antibody can be visualized in the electron microscope. Ferritin-labeled antibody techniques have been developed more recently and used to identify a wide variety of antigens (104). This technique permits identification of specific antigenic structures of cells or organisms.

The intraerythrocytic inclusion body has been recognized as an antigenic component of *Anaplasma marginale* since the development of immunologic tests for detection of the disease. The antigenic nature of the inclusion body in the bovine erythrocyte also has been confirmed by fluorescein-labeled antibody studies of serum collected from cattle with acute or carrier infections (75) (105-108).

Fluorescent antibody studies of *A. marginale* in the tick, *Dermacentor andersoni*, have been undertaken by several investigators (33) (109) (110). *A. marginale* has been demonstrated in the gut contents, excreta, and malpighian tubules of nymphal ticks for a limited time after feeding on an infected cow, but not after host erythrocytes were digested (1).

Studies on tissues of engorging adult ticks removed from infected cattle after attachment have not been reported.

Direct and indirect ferritin-labeled antibody techniques have been used to study bovine erythrocytes infected with A. marginale. Highly specific labeling of the anaplasmal organism has been demonstrated including the initial-body pellicle and the inclusion appendage (75) (Figure 39). Ferritin-labeled antibody has also been used to identify erythrocytic membrane antigens in bovine erythrocytes infected with A. marginale (76). These techniques have not been utilized previously to identify A. marginale in ticks.

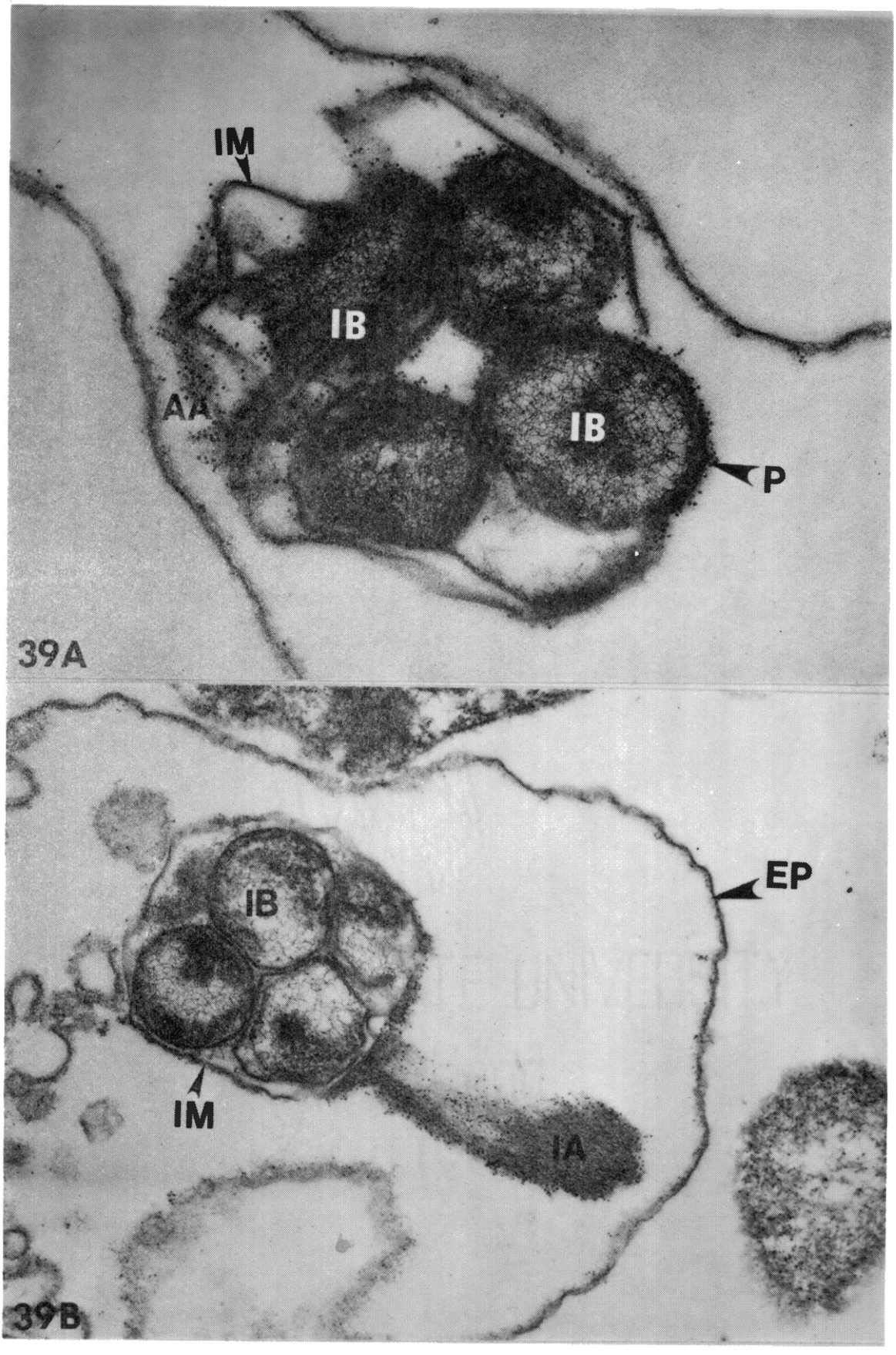
Materials and Methods

Fluorescent-Antibody Studies

Collection and Preparation of Tick Tissues. Tissues were collected from adult ticks on the 5th and 7th day following attachment to a complement fixation (CF) negative cow. Salivary gland and gut tissues were removed from approximately 30 ticks, rinsed in tick culture medium followed by phosphate buffered saline (PBS) and placed onto preformed frozen Sephadex 2.5 percent gelatin blocks. Sections were cut, frozen, and stored according to the procedures of Kimber et al. (111). Oral secretions were collected as described in Chapter II (p. 22) and smears were made on coverglasses, air dried for 30 minutes, fixed in acetone for 10 minutes at room temperature, and stored at -50°C until they were studied.

Indirect Fluorescent Antibody Test Procedures. Bovine serum with a CF titer of 1:70 to A. marginale was collected from a carrier cow to be used as antiserum for these studies. Bovine serum with no CF titer was

Figure 39. Electron Micrographs of Ferritin-Labeled Anitbody on the Pellicle and Inclusion Appendage of Anaplasma marginale in Hemolyzed Erythrocytes. In Figure 39A, the ferritin labeling is on the initial body (IB), pellicle (P) and on the inclusion membrane (IM) where the appendage is attached (AA). When the inclusion membrane (IM) remained intact (39B), the large ferritin complex could not enter the inclusion to label the initial bodies (IB) and only labeled the inclusion appendage (IA). The erythrocyte plasmalemma (EP) did not label with ferritin. (A) x 80,000; (B) x 50,000.


IM
IB
AA
IB
P
39A
IB
EP
IM
IA
39B

used as a negative control. Gut tissue sections were flooded with the anti-A. marginale serum and incubated in a moist chamber at 37°C for 25 minutes. After incubation, sections were washed with three changes of PBS (pH 7.2) for 20 minutes each, rinsed with distilled water and air dried. The sections were then reacted with fluorescein-conjugated anti-bovine IgG[1] in the same manner, washed, dried and mounted with buffered glycerine before being examined. The same procedures were used for the salivary gland sections. In order to adsorb nonspecific staining components, the bovine anti-A. marginale serum was mixed 1:1 with a 10 percent suspension of salivary gland tissues from control ticks in PBS, pH 7.2. To reduce autofluorescence, aqueous amido black diluted to 0.02 V/V was incorporated into the conjugate before the second incubation (112). Tissue sections from infected and control ticks were stained with either positive or negative sera for comparison and evaluation of the stained tissue fluorescence. The slides were examined using a Zeiss microscope with epi-illumination.

Ferritin-Antibody Studies

Collection and Preparation of Tick Tissues. Tick gut homogenates were prepared in order to disrupt the midgut cells and cause release of the organisms. This step was necessary because the ferritin-antibody complex was too large to pass through an intact plasmalemma. Gut tissues were collected from 50 adult D. variabilis on the 6th day following attachment to a CF-negative cow, placed in tick culture medium and homogenized in a tissue grinder. The homogenate was centrifuged at 500 rpm

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[1]Miles Laboratories, Inc., Research Products, Elkart, Indiana.

for 10 minutes for removal of intact cells and gut debris. The supernatant was collected and sedimented with a microcentrifuge in 400 µℓ tubes. Homogenates from infected and control ticks were collected and prepared in this manner. Similar homogenates of gut tissue from infected and uninfected ticks were prepared and each inoculated into a susceptible cow for determination of infectivity.

Ferritin-Conjugated Test Procedure. Bovine serum with a CF titer of 1:70 to A. marginale was collected from a carrier cow to be used as antiserum for these studies. Bovine serum with no CF titer was used as a control. The globulin portion of the serum (both anti-A. marginale and control) was removed by precipitation with anhydrous sodium sulfate and conjugated with ferritin according to the procedure of Andres et al. (104).[2] Prepared homogenates were sedimented in 400 µℓ centrifuge tubes with a microcentrifuge, providing samples for each experimental group. Samples were washed three times with 0.01 M PBS and then reacted with the ferritin conjugate for 30 minutes at room temperature. After several washes with PBS, the samples were prepared, sectioned and examined with the electron microscope, as described in Chapter III. The ferritin conjugate test consisted of the following experimental groups for control and infected samples: (1) plain sample, (2) sample reacted with unlabeled antiserum followed by reaction with the anti-A. marginale ferritin conjugate, (3) sample reacted with control ferritin conjugate, and (4) sample reacted with anti-A. marginale ferritin conjugate. The sample groups were compared, using electron microscopy for distribution of

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[2]Serum conjugated by Dr. Konrad Hsu, Department of Microbiology, College of Physicians and Surgeons, Columbia University, New York, New York.

ferritin particles to determine specific sites of antigen-antibody complex.

Results

Indirect Fluorescent Antibody Studies

Results of the fluorescent antibody studies are summarized in Table VII. Positive fluorescence was demonstrated by indirect FA techniques. Organisms appeared as bright, particulate fluorescence; this type of fluorescence was not observed in control tissue sections or infected tissue sections stained with negative serum. Smears of oral secretion did not fluoresce.

Ferritin Conjugate Antibody Studies

The results obtained by injecting homogenates of gut from infected and control ticks into susceptible cows are presented in Table VIII. Homogenates from infected ticks produced anaplasmosis (28-day prepatent period and peak parasitemia of 24.3%) while the homogenate prepared from control ticks did not. The 28-day prepatent period was not remarkably different from that induced by feeding of adult ticks (25 days) which were infected as nymphs.

The gut homogenates from infected ticks contained Anaplasma-like organisms that were labeled with ferritin conjugate (Figure 40A-D). The organisms did not label with control ferritin conjugate (Figure 41A and B) and reaction with unlabeled anti-A. marginale serum inhibited subsequent ferritin labeling almost completely (Figure 42A and B). Anaplasma-like organisms were not seen in gut homogenate preparations from control ticks.

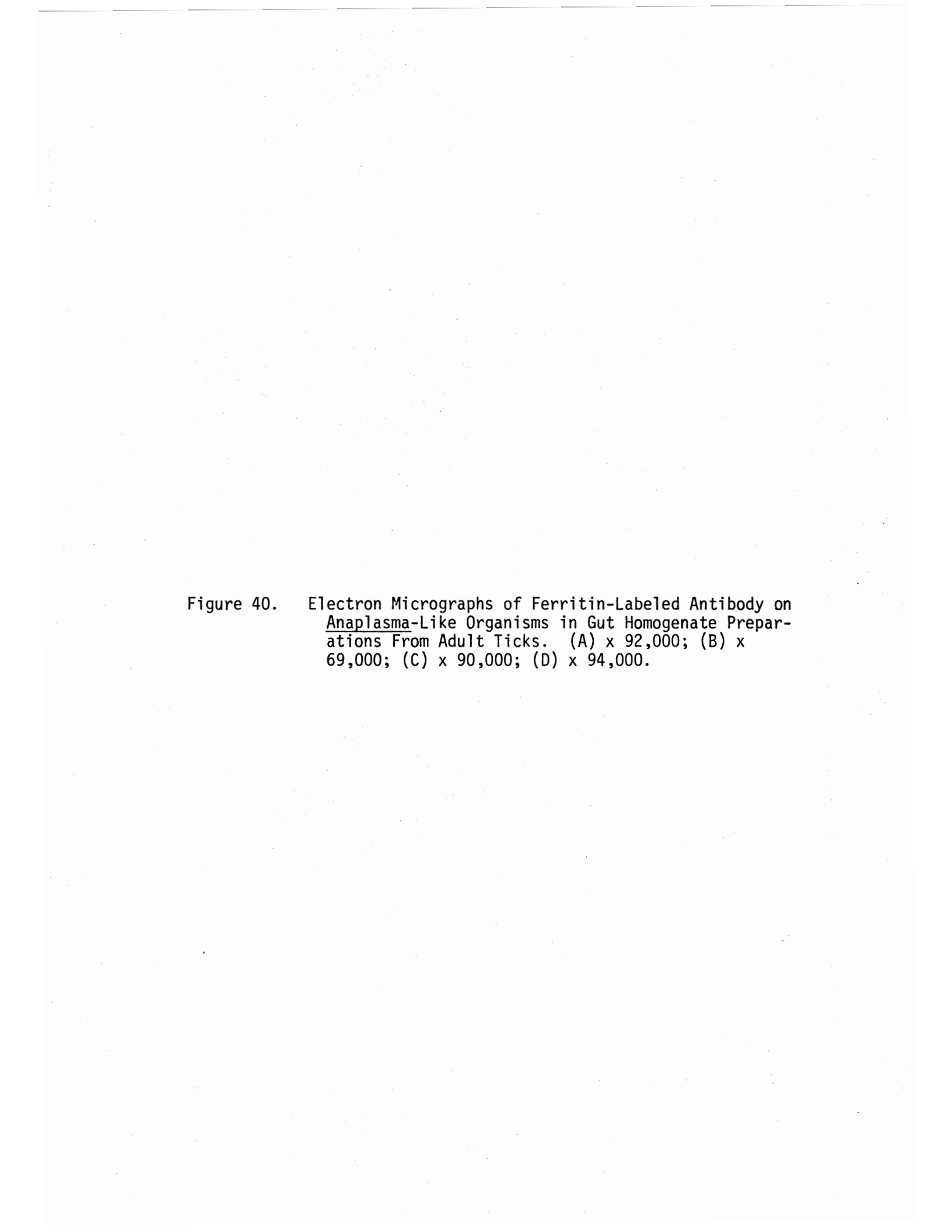

Figure 40. Electron Micrographs of Ferritin-Labeled Antibody on *Anaplasma*-Like Organisms in Gut Homogenate Preparations From Adult Ticks. (A) x 92,000; (B) x 69,000; (C) x 90,000; (D) x 94,000.

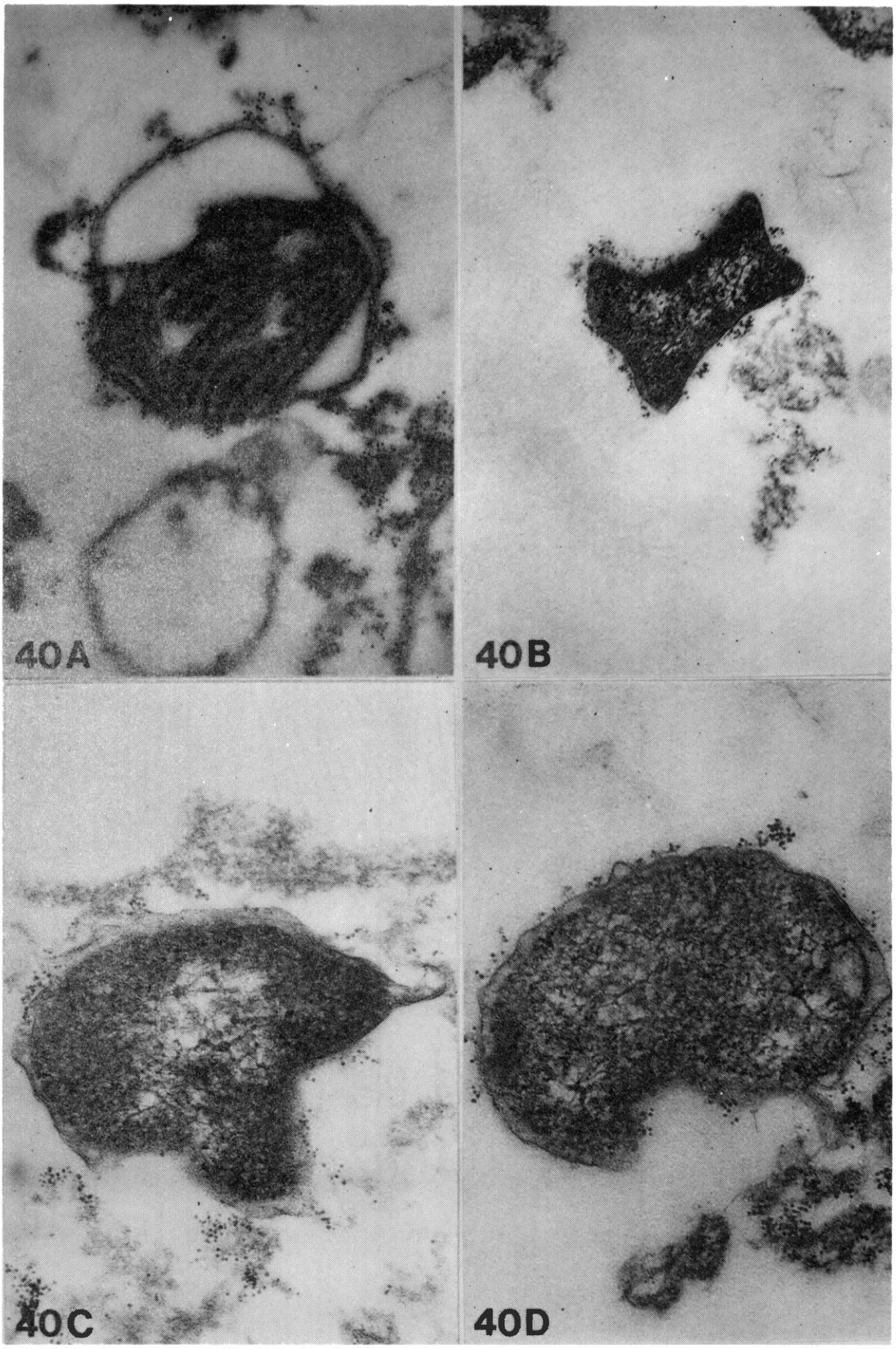
40A
40B
40C
40D

Figure 41. Electron Micrographs of Control Preparations for Ferritin-Labeled Antibody Studies in Gut Homogenates. Organisms reacted with control ferritin-conjugate were not labeled with ferritin. (A) x 93,500; (B) x 93,500.

Figure 42. Electron Micrographs of Control Preparations for Ferritin-Labeled Antibody Studies in Gut Homogenates. Organisms were reacted first with unlabeled anti-A. marginale serum that inhibited ferritin labeling almost completely. (A) x 89,500; (B) x 89,000.

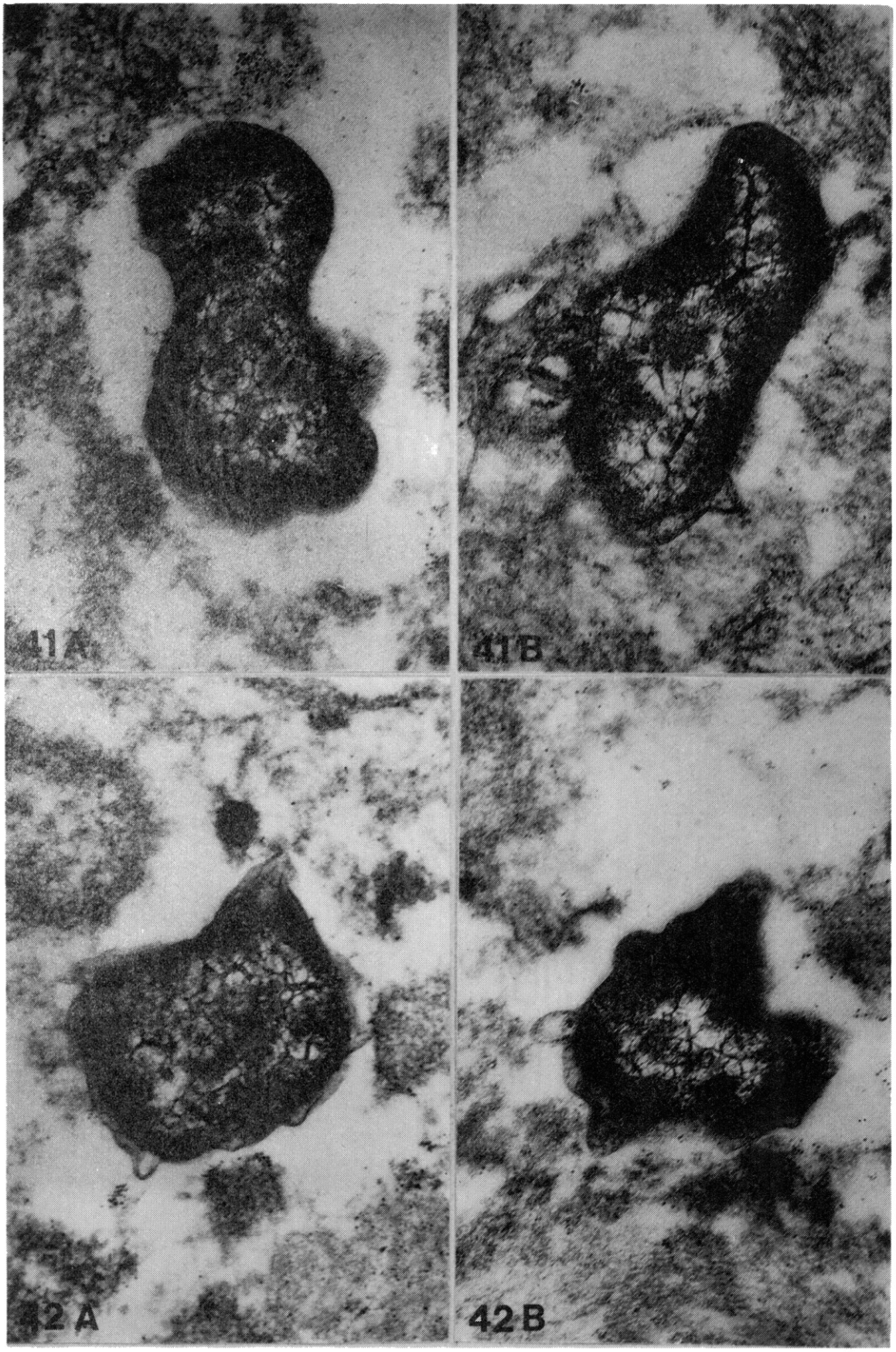
41A
41B
42A
42B

TABLE VII

INDIRECT FLUORESCENT ANTIBODY STUDIES OF *ANAPLASMA MARGINALE* IN GUT AND SALIVARY GLAND TISSUE AND IN SMEARS OF ORAL SECRETION FROM ADULT *DERMACENTOR ANDERSONI* AND *D. VARIABILIS*

Tissue	Days of Feeding	*D. andersoni*	*D. variabilis*
Gut*	5 and 7	+	+
Salivary Gland*	5 and 7	+	+
Oral Secretion	6	-	-

*Data combined from Trials 1 and 2 of tick transmission studies; tissues were pooled from 30 ticks per experiment.

TABLE VIII

ATTEMPTED TRANSMISSION OF *ANAPLASMA MARGINALE* BY INJECTION OF HOMOGENATES OF *DERMACENTOR VARIABILIS* GUT COLLECTED AT DAY 6 OF FEEDING

Treatment	Cow No.	Age (yrs)	Prepatent[a] Period (days)	Peak Parasitemia (%)
Adult Feeding	729[b]	5	25	18.0[c]
Homogenate of Infected Ticks	669	4	28	24.3[c]
Homogenate of Control Ticks	352[d]	9	None	None

[a]Prepatent period determined from day 5 of feeding to the appearance of marginal bodies in the peripheral blood smear.

[b]Nymphal ticks for these studies were infected on Cow No. 401 with a peak parasitemia of 10.0 percent.

[c]Cows found to undergo seroconversion and became CF positive.

[d]Cow was subsequently challenged and found to be susceptible.

Discussion

Previous fluorescent antibody studies of A. marginale in ticks have involved examination of nymphal or adult D. andersoni after engorgement on a cow experimentally infected with anaplasmosis (33) (109-110). Organisms were identifiable for approximately 5 days after repletion and were not detectable after digestion of erythrocytes in the blood meal. Two problems were encountered in these earlier studies: (1) the need to show that the ticks were actually becoming infected, and (2) the ability to separate anaplasmal organisms ingested in the blood meal from those residing in tick tissues. The present study circumvents both of these problems because (1) the ticks were proved to be infected by subsequent transmission of anaplasmosis to a susceptible cow, and (2) the possibility of fluorescence of infected erythrocytes which had been ingested recently was eliminated by studying adults which were infected as nymphs. In addition, the present studies were done at two time periods (5 and 7 days after attachment) during active feeding. Recent studies on other tick-transmitted organisms have suggested the probable coordination of complex feeding physiology and disease transmission (44).

The method of collection of oral secretion used in this study has been used successfully to detect the infective stage of Theileria parva in Rhipicephalus appendiculatus (43). Failure to detect the anaplasmal organism in the salivary gland may indicate that the infective organisms are not passed via the salivary gland. However, oral secretion was collected only on one day of feeding (day 6 post attachment) which may have not been adequate for detection of the organism, even if it was present. Also, the drug, dopamine, used to induce salivation may not cause

release of organisms from potentially infected salivary gland cells even if they were infected.

The animal inoculation study and the associated ferritin conjugate study suggest that the organisms observed in tick tissues by electron microscopy are A. marginale. Organisms similar in structure and size to anaplasmal initial bodies previously described in bovine erythrocytes were detected by the ferritin antibody techniques (73). It should be mentioned, however, that labeling of these organisms in tick tissues is less dense than that described in infected bovine erythrocytes (73).

CHAPTER V

SUMMARY

Anaplasma marginale is one of many types of disease-producing organisms that are transmitted to vertebrates by ticks. The phylogenetic range of organisms transmitted by ticks is great and includes viruses, rickettsiae, sporozoans, and bacteria. Ticks are very suitable vectors of disease, in part, because of the long attachment time for feeding on the vertebrate host and because they feed on blood. Ticks are durable during all stages of their life cycle and individual organisms potentially can persist in the environment for a long time. The transmission of many disease agents has been shown to occur during the feeding process and the virulence and/or development of infective stages may be affected by this process. Tissues from unfed ticks have often been found not to be very infective. Some studies have shown that increased body temperature, which occurs when ticks feed, is the factor that initiates changes in virulence or development of the parasite being transmitted.

Insofar as is known, the route of infection of ticks with an infectious agent is via a blood meal. On the other hand, several routes of transmission of disease agents from ticks include: (1) orally via salivary glands or gut regurgitation, (2) via fecal contamination or by (3) ingestion of ticks by a susceptible host. Some infections may be passed from generation to generation through the egg (transovarial transmission)

and perpetuation of such organisms does not require involvement of a vertebrate host. Other organisms may be passed only from stage to stage (transstadial transmission) and persistence of disease agents is, therefore, dependent on interaction of the tick, disease agent, and vertebrate host.

The purpose of the experiments described in this thesis was to study Anaplasma marginale in two species of ticks belonging to the genus Dermacentor. Although D. andersoni and D. variabilis have been shown previously to transmit anaplasmosis, the location and morphology of A. marginale in the invertebrate had not been demonstrated. Likewise, the mode of transmission to the vertebrate host has not been investigated. Strong evidence is presented that A. marginale exists in these ticks and several parameters examined experimentally support the conclusions. Transmission studies have clearly shown that both species of ticks consistently transmitted anaplasmosis to susceptible cows when they fed as adults after having been infected as nymphs. Furthermore, homogenates prepared from these ticks have shown that gut and salivary gland tissue were infective; both induced anaplasmosis when injected into susceptible cows. Gut tissues studied from all infected stages contained anaplasma-like organisms which were similar to A. marginale described in infected bovine erythrocytes; and they were rickettsia-like in morphology. Anaplasma-like organisms were most easily detected in flat adult ticks that were incubated. Colonies of organisms found in these tissues were detectable by light microscopy, thus providing a larger sampling of tissue than that done with the electron microscope. Similar organisms were not observed in the uninfected control tissues.

Labeled antibody studies provided evidence that the organisms studied were Anaplasma marginale. Infected gut and salivary gland tissues had a positive fluorescence while the respective controls did not. Ferritin-labeled antibody technique was used to demonstrate specific sites of antigen-antibody complex on the organisms; these studies, likewise, suggested that the structures are the tick stage of Anaplasma marginale.

Positive identification of A. marginale in the tick is more difficult than in bovine red blood cells because of the common presence of symbiotic rickettsiae in ticks. Although rickettsiae, presumably nonpathogenic symbiotes, were also observed in both infected and control ticks, the size and structure of these organisms was different from the Anaplasma-like organisms. Unequivocal identification of the organisms as Anaplasma marginale can be accomplished only by fulfillment of Koch's postulates for the identification of a disease-producing agent. This would involve isolation of the organisms from ticks, maintenance in a culture system, and, upon introduction into a susceptible cow, causation of the specific disease syndrome characteristic of anaplasmosis.

The role of the salivary gland in transmission of anaplasmosis has not been determined previously nor has it been clearly ascertained in the present study. Although homogenates of salivary gland were infective and sections of these tissues had positive fluorescence, collections of oral secretion were not infective. Furthermore, morphologic studies on salivary glands of all tick stages failed to reveal any organisms. The data from these studies suggest that the midgut epithelial cells are the primary location of the anaplasmal organisms in the tick. It is possible that the infectivity of the salivary gland homogenate was due to contamination from ruptured gut.

Several stages have been described for rickettsial organisms, viz., reproductive forms, conservative forms, and activation forms (100). The Anaplasma-like organisms studied in the present experiments appeared to be in different morphologic forms and varied with the specific developmental stage of the tick tissue. The organisms found in ticks in this study which most resembled A. marginale in bovine erythrocytes were found in nymphal ticks 10 days after feeding on a cow with clinical anaplasmosis. The Anaplasma-like organisms in flat adult ticks were very compact and electron dense and were similar to a resting or conservative state described by other workers. Colonies demonstrated in incubated adult ticks seemed to represent reproductive forms. Small electron-dense bodies were noted near Anaplasma-like organisms and were similar to those described by the author in infected bovine erythrocytes. Although it is not known what relationship the small electron dense bodies have to the life cycle of A. marginale in the tick, it is possible that they may represent an alternate mode of reproduction of the organism.

The data from these experiments have suggested several interesting relationships in the transmission of anaplasmosis by ixodid ticks. Continued investigations are needed to substantiate these findings and to determine their influence on enzootic transmission of the disease.

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VITA[2]

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