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Isolation and Characterization of Symbiotes from the Rocky Mountain Wood Tick, *Dermacentor andersoni*[1]

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Wolbachia-like symbiotes in the Rocky Mountain wood tick, *Dermacentor andersoni*, were isolated repeatedly by injection of ovarial tissues into 5-day-old chick embryos. In Giemsa-stained smears of infected embryo tissues, the organisms appeared as blueish or pink-stained coccal bodies indistinguishable from those seen in the ovaries of ticks, where they are located in the luminal epithelium and funicle cells, as well as in oocytes.

Electron microscopy revealed that these symbiotes are highly pleomorphic and vary in size from 0.6 to 3.4 μm in diameter. Their fine structure in tissue cells is differentiated into a granular, cortical region, which contains densely stained ribosomes, and a medullary region consisting of a diffuse reticulum partially or completely devoid of granular material or ribosomes. Multiplication is by binary fission. Each organism is delimited by a distinct plasmalemma; a cell wall as in bacterial and rickettsial agents was not observed in organisms from ovarial tissues.

Symbiotes cultivated in chick embryos and then injected intracoelomically into adult *D. andersoni*, developed rapidly and produced massive infestations in hemocytes, hypodermal tissues, salivary glands, and in connective tissues surrounding midgut, Malpighian tubules, and ovary. In hypodermal tissue, organisms with a distinct bilayered cell envelope were occasionally detected. The massive invasion of tissues by injected symbiotes invariably proved fatal for ticks.

Results of complement-fixation tests and of fluorescent antibody staining indicated that symbiotes in *D. andersoni* are closely related to *Wolbachia persica*, previously isolated from *Argas arboreus*.

INTRODUCTION

Ever since tick tissues have been subjected to microscopical examination, scientists have been puzzled by the presence of highly pleomorphic bacillus-like microorganisms in certain tissues, particularly Malpighian tubules and ovary. Some investigators considered them as subcellular structures, such as mitochondria, others described them as symbiotes, and still others included them as developmental stages in the life cycle of pathogens they were studying.

Cowdry (1925) published on a group of microorganisms hereditarily transmitted in ticks and apparently unassociated with disease. Based on observations with 16 species of ticks, he described the organisms as follows: "In Giemsa-stained smears the microorganisms stained light red or pink, much more faintly than most bacteria, and they did not possess the sharp contours often seen in the case of bacteria. They were very pleomorphic varying from spherical to straight and curved rods and filaments. They were found to be larger than most rickettsiae particularly in diameter."

Since Cowdry's paper, numerous reports (reviewed by Roshdy, 1968) have been published on distribution and microscopical appearance of symbiotes for various species of ticks. Brinton (1969) briefly described their distribution and fine structure in ovarian tissues of *Dermacentor andersoni*.

The first isolation of such a tick symbiote was reported by Suitor and Weiss (1961), who successfully recovered, in chick embryos, the microorganisms from Malpighian tubules and ovaries of the argasid tick, *Argas persicus* (later determined to be *A. arboreus*) collected from heron rookeries near Cairo, Egypt. They identified their isolate as a new species within the tribe Wolbachiae of the family Rickettsiaceae, and proposed the name *Wolbachia persica*.

At the Rocky Mountain Laboratory (RML), we have been engaged in studies concerning the relationship of symbiotes to pathogenic and nonpathogenic rickettsiae. During these investigations, symbiotes have been isolated repeatedly from various species of ticks. It is the purpose of this paper to describe recovery and preliminary characterization of such symbiotes from the Rocky Mountain wood tick, *Dermacentor andersoni*.

MATERIALS AND METHODS

Specimens of *D. andersoni* used in this study came from RML's normal colony which is free of detectable pathogens. Distribution of symbiotes in this tick was first established by conventional microscopy of Giemsa-stained tissues. For this purpose, unfed females whose exoskeleton had been decontaminated by immersing ticks for 1 hr into a 0.1% aqueous solution of Merthiolate and subsequent rinsing in phosphate-buffered-saline, pH 7.35, were dissected for removal, smear preparation and staining of hypodermal tissues, salivary glands, midgut, Malpighian tubules, and genital organs.

For isolation of symbiotes, ovarian tissues of 5 to 10 ticks were triturated in sucrose-phosphate-glutamate solution (Bovarnick et al., 1950) or Brain-Heart-Infusion broth containing 100 units of penicillin/ml, and were injected via yolk sac into 8 5-day-old hens' eggs, each receiving 0.25 to 0.5 ml of the suspension. Inoculated eggs were then incubated at 35°C and examined daily for death of embryos. Eggs that died during the first 4 days after inoculation were discarded. From those that died later, and from surviving eggs, smears were prepared from yolk sac tissues, stained by Giemsa's method, and examined microscopically.

For electron microscopy, ticks and their tissues were treated as outlined by Brinton and Burgdorfer (1971).

Antigenic relationship of *D. andersoni* symbiotes to *W. persica* and to rickettsiae of the spotted fever and typhus groups was explored by complement-fixation tests according to standard methods employed at RML. Antigens were prepared by ether extraction of saline solution suspensions of infected yolk sacs, and sera were obtained from convalescent guinea pigs and hamsters. Also used was direct fluorescent antibody (FA) staining with conjugates prepared according to Peacock et al. (1971) from sera of immunized hamsters.

To study development of symbiotes in *D. andersoni*, 0.001–0.003 ml of a 10% suspension of infectious yolk sac were injected intracoelomically into 75 unfed females. On day 5 after inoculation and at 5-day intervals thereafter, 3 to 5 ticks were dissected for microscopical examination; smears of hemolymph and of various tissues were stained either by conventional or by FA techniques. On day 7 after intracoelomic inoculation, 30 infected females together with normal males were placed for feeding on guinea pigs. Upon repletion, females were held individually in glass vials for oviposition.

[1] A portion of this material was presented at the Second International Congress of Parasitology, Washington, D.C., September 6-12, 1970.

Results

Distribution and Morphology of D. andersoni *Symbiotes*

Although all organs of ticks were thoroughly examined, symbiotes were detected only in the ovary. After staining with Giemsa, they appeared as blueish or pink, and morphologically varied from coccal to occasionally bacillary (Fig. 1). Many of the bacillary forms upon close examination were found to represent organisms undergoing binary fission and consisting of two not yet completely divided coccal organisms. Pleomorphism of these symbiotes was even better discernible in smears stained by FA (Fig. 2).

Electron microscopy revealed that the symbiotes of *D. andersoni* varied in size from 0.6 to 3.4 μm in diameter. Their fine structure is differentiated into a cortical granular region which contains densely stained ribosomes, and a medullary region consisting of a diffuse reticulum partially or completely devoid of granular material or ribosomes. The symbiotes are unique in that they do not possess a cell wall. Each organism, regardless of the degree of pleomorphism, is delimited by a narrow, trilayered unit membrane approximately 75 Å in dinmeter (Fig. 3). In some instances the granular cortical region of the cytoplasm exhibited some shrinkage away from the fragile appearing plasmalemma and probably represents a fixation artifact.

Distribution of symbiotes in ovarian tissue of *D. andersoni* varied with the stage of development and the nutritional state of the tick (Brinton, 1969). In unfed and engorged nymphs, the symbiotes have thus far been observed only in interstitial cells (Fig. 4) of the ovary. Occasionally, they were also found in oocytes of newly molted females. Symbiotes were observed with considerably greater frequency in oocytes of fasting adult ticks, yet were relatively sparse compared with their numbers in interstitial cells and their cellular processes. During early feeding of female ticks, i.e., 24–48 hr after attachment to host, when interstitial cells complete their transition to form the lumenal epithelium and funicle cells (Brinton and Oliver, 1971), symbiotes, either in foci or individually dispersed, multiply by binary fission (Fig. 5) and increase considerably in number in the lumenal epithelium. They are most frequently observed in oocytes 3–6 days after attachment of ticks to their hosts—the period when oocyte enlargement is primarily due to intrinsic development (Brinton and Oliver, 1971). In oocytes the symbiotes varied in numbers up to 8 per section, and were dispersed individually in the peripheral ooplasm rather than as small colonies. During this period rounded forms were common (Fig. 6). When oocytes are enlarging rapidly as a result of micropinocytosis during the latter half of the extrinsic phase of development (Brinton and Oliver, 1971), symbiotes become obscure, partially due to the enlargement of vitelline spheres and the increasing volume of these cells. Symbiotes were never observed in an intranuclear association with the host cell.

Isolation of D. andersoni *Symbiotes in Chick Embryos*

Isolation of symbiotes in yolk sac tissues of chick embryos was accomplished from suspensions of tick ovaries. None of the originally inoculated embryos died but microscopical examination of yolk sac tissues from two live embryos examined on day 7 after inoculation revealed cocci indistinguishable from those in ovarial tissues. Yolk sac suspensions of these two eggs passed into fresh lines of 8 eggs each killed all embryos from 5 to 7 days after inoculation. Yolk sac smears of dead eggs regularly contained masses of organisms which appeared similar if not identical to those in tick tissues although they were predominantly coccal and not as pleomorphic as those in ovarial tissues.

Subsequently, three additional isolates of symbiotes were obtained from suspensions

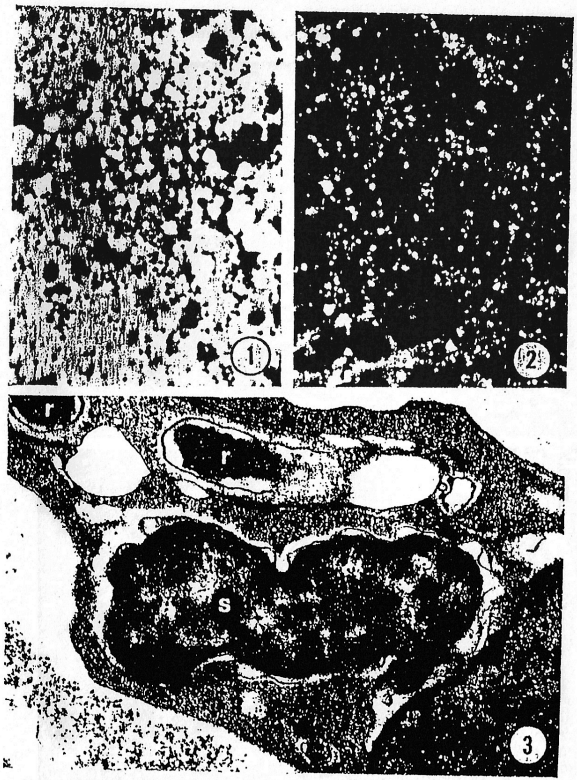
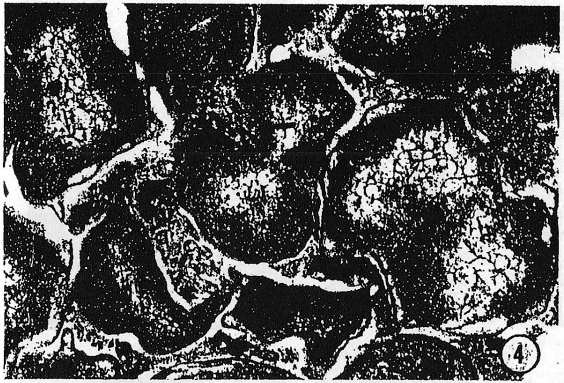


FIG. 1. *Wolbachia*-like symbiote in smear of ovarian tissue of *Dermacentor andersoni*. Giemsa stain. ×1000.

FIG. 2. Smear of ovarian tissue of *Dermacentor andersoni* showing pleomorphic appearance of symbiotes. Fluorescent antibody staining. ×1000.

FIG. 3. Symbiote (s) in ovarian interstitial cell of *Dermacentor andersoni* infected with *Rickettsia rickettsi* (r). ×25,000.



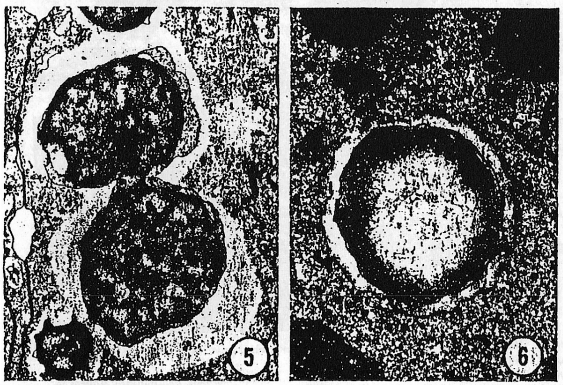


FIG. 4. *Wolbachia*-like symbiotes exhibiting granular cortical cytoplasm and fibrillar (arrows) medullary region, in ovarian interstitial cell of nymphal *Dermacentor andersoni*. ×57,000.

FIG. 5. Division form of symbiote in ovarian interstitial cell. ×18,000.

FIG. 6. Spherical symbiote in ooplasm of enlarging oocyte of *Dermacentor andersoni*. ×33,000.

of ovarial tissues and were maintained by serial egg passages.

Pathogenicity for Laboratory Animals and Serology

Guinea pigs and golden hamsters shown free of antibodies to antigens prepared from yolk-sac grown symbiotes invariably died within 24 hr after i.p. injection of 0.5 ml of 50% suspensions of infected yolk sac. However, when inocula consisted of 0.1 or 0.25 ml, all animals survived. Guinea pigs responded in most instances with elevated temperatures (>40°C) for 2 to 4 days beginning within 24 hr after inoculation. Neither guinea pigs nor hamsters displayed other signs of illness.

Sera obtained 28 days after inoculation and tested by CF yielded titers as high as 1:1024 against homologous antigens but were negative for antibodies to spotted and typhus fever antigens. However, titers of similar magnitude were detected against antigens prepared from *Wolbachia persica* that had been isolated from *Argas arboreus*. Similarly, sera of guinea pigs immunized with *W. persica* reacted against antigens from the *D. andersoni* symbiote. Antigenic similarities between these two organisms were also demonstrated by FA staining; conjugates against *D. andersoni* symbiotes regularly stained *W. persica* and vice versa.

Behavior of Symbiotes in Intracelomically Inoculated D. andersoni

Microscopical examination of hemolymph and tissues on day 5 after injection of infectious yolk sac suspensions revealed symbiotes in practically all hemocytes. Degree of infection was still mild although some cells already were heavily infected. Tissues of hypodermis, Malpighian tubules, and salivary glands contained few organisms. By day 10, all hemocytes had masses of symbiotes often to the extent that cells were greatly enlarged. Development of the organisms appeared to occur intracellularly as well as extracellularly and was so intense that the hemolymph took on a "milky" appearance. All tick organs, but particularly hypodermis and Malpighian tubules, showed massive invasion which appeared limited to connective tissues surrounding each organ. By day 15 and regularly thereafter, infection was generalized and massive throughout the tick.

The cellular fine structure of these highly invasive organisms appeared in general similar to that of the naturally occurring forms confined to the ovary. However, two exceptions were occasionally noted (Fig. 7). One was the presence of dense-staining bodies considerably larger than ribosomes in the granular or cortical cytoplasm of some organisms, and the other, less consistently observed, was the presence of a cell wall in addition to the plasmalemma of the protoplast.

Invasion of Malpighian tubules by symbiotes was limited to the basement membrane, which, due to growth and multiplication of organisms, was found to undergo progressive distention (Fig. 8). The symbiotes occurred characteristically in foci which probably resulted from clonal development; scattered or isolated organisms could not be detected in the basement membrane of this organ.

Interaction between symbiotes and hemocytes occurred primarily with plasmatocytes (nongranular hemocytes) in which invasion and phagocytosis was pronounced, and secondarily in granular hemocytes, with symbiotes exhibitng two forms of intracellular association. They occurred either in large numbers in membrane-limited vacuoles or as individual forms in the cytoplasm (Fig. 9) in the absence of vacuolar or restricting membranes. However, in one instance, a single organism in the cytoplasm of a granular hemocyte was found encased in a thickened, membrane-limited capsule. Enlargement of the vacuoles as result of intensive multiplication of vacuolized symbiotes gradually led to complete disruption of the cytoplasm (Fig. 10) and to eventual disintegration of infected hemocytes.

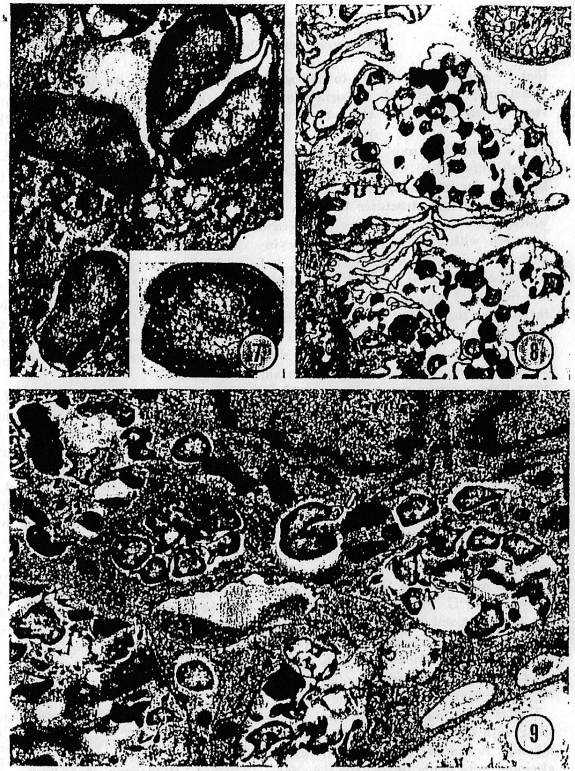


FIG. 7. Invasive forms of *Wolbachia*-like symbiotes in hypodermis of *Dermacentor andersoni*. ×46,000. Inset shows large, dense bodies in the cytoplasm of a symbiote. ×60,000.

FIG. 8. Distention of Malpighian tubule basement membrane caused by invasive forms of *Dermacentor andersoni* symbiotes. ×8000.

FIG. 9. Free and intravacuolar invasive forms of symbiotes in plasmatocyte. Cross and longitudinal views show fingerlike extensions of the plasma membrane of intravacuolar symbiotes (arrows). ×17,500.

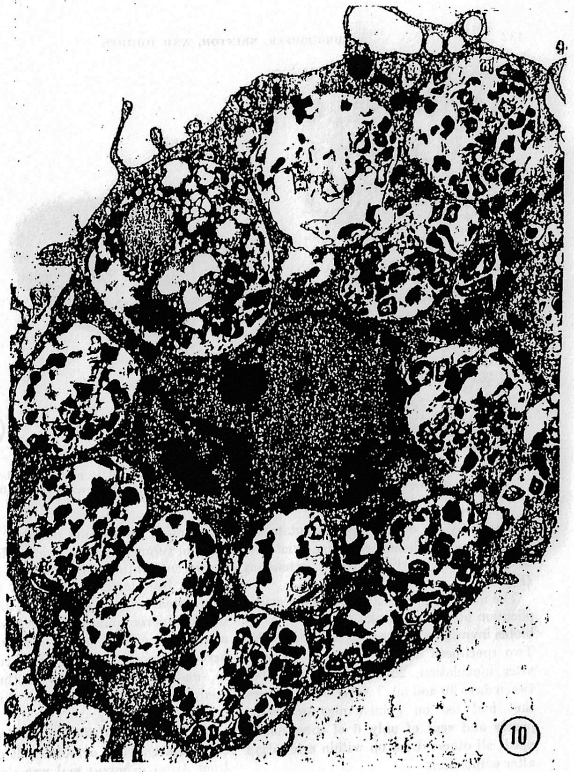


FIG. 10. Extensive intravacuolar growth of invasive *Wolbachia*-like symbiotes in plasmatocyte. ×9500.

Cytoplasmic organization of intravacuolar symbiotes in hemocytes was as described for naturally occurring forms in the ovary, with the exception that the cell membrane of these organisms often appeared folded in one area to form short to elongate extensions. In cross sections, these extensions exhibited a tubular morphology.

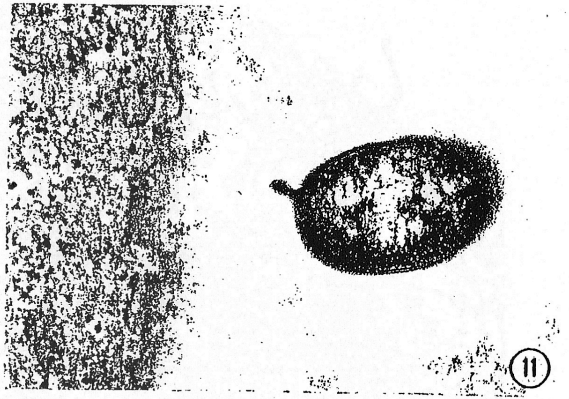


FIG. 11. Free invasive form of *Wolbachia*-like symbiote adjacent to plasma membrane of granular hemocyte. Note fingerlike extension of symbiote's plasma membrane. ×72,000.

Occasionally we observed, adjacent to the plasmalemma of hemocytes, free symbiotes with similar fingerlike tubular extensions (Fig. 11).

The invasive propensity and rapid multiplication of intracoelomically injected symbiotes invariably proved fatal for the ticks. Two specimens died as early as 20 days after inoculation, 26 additional ones between days 30 and 50. Twelve of 30 females that had fed on guinea pigs oviposited poorly and eggs of only 6 of these developed; all others dried up within a few days after oviposition.

DISCUSSION

Observations by various investigators (reviewed by Roshdy, 1961, and Balashov, 1968) with numerous species of ticks led to the concept that all ticks, argasid and ixodid regardless of geographic origin, contain symbiotes. These microorganisms according to most investigators are limited in localization to various parts of the Malpighian tubules and the ovaries. Exceptions are recent findings by Hecker et al. (1968) and by Burgdorfer and Brinton (unpubl.), who noted a more generalized distribution of such microorganisms in the tissues of the argasid ticks *Ornithodoros moubata* and *O. hermsi*, respectively. On the other hand, in *D. andersoni*, as shown in the present study, symbiotes appeared limited to ovarian tissues only.

Until recently *Wolbachia persica* was the only isolate of tick symbiotes. It originated from *Argas arboreus* and was classified as a member of Rickettsiaceae because of basic similarities (size, intracellular growth, and susceptibility to broad range antibiotics) to rickettsial agents. Inclusion in the tribe Wolbachiae was suggested because this tribe contains organisms adapted to existence in arthropods as symbiotes but not in vertebrates as highly pathogenic parasites (Philip, 1957). Metabolic activity studies by Weiss et al. (1967) have shown

that *W. persica* is unrelated to pathogenic rickettsiae; in contrast to rickettsiae it respires vigorously in the presence of glucose.

The symbiote from *D. andersoni*, although different in its distribution within the tick, shows similarities in gross morphology and fine structures to *W. persica* which has been described as a spherical organism with granular, vacuolated cytoplasm delimited by a cell membrane, but without a cell wall as is known for bacterial and rickettsial organisms. In addition, cross reactions in the CF test and by FA staining indicate antigenic relationship between the two symbiotes. However, absence of such cross reactions with symbiotes from certain other ticks suggests the existence of different *Wolbachia* species and warrants further investigations into the classification of these organisms.

Also, there is evidence of differences among tick symbiotes in regard to their physiologic characteristics. Thus, Suitor (1964) in his study on the relationship of *W. persica* to its host, failed to infect by intracoelomic injection of infectious yolk sac suspensions, *A. persicus* that had been rendered aposymbiotic by prolonged incubation at 40°C. Feeding of such ticks on the air-sac membrane of infectious hens' eggs resulted in the development of *Wolbachia* in the intestinal tract only, and in no instance did such experimental infection have any adverse effects on the ticks.

Contrary findings were obtained in our study with the symbiotes of *D. andersoni*, although it should be emphasized that no attempts were made to free the ticks from their symbiotes before they were inoculated intracoelomically with infectious yolk sac. Within as early as 10 days after inoculation, all ticks showed massive multiplication of organisms particularly within plasmatocytes and in the connective tissues of the various organs. The infection invariably proved fatal for the ticks. Whether the symbiotes after ingestion would have developed in gut tissues only or in a fashion similar to that described above, was not investigated. Our findings, however, are in agreement with those obtained by Weyer (1973) with *W. persica* in experimentally infected body lice (*Pediculus humanus humanus*), ticks (*Ornithodoros moubata*) and mealworm larvae (*Tenebrio molitor*). After intracoelomic injection of infectious yolk sac, all these arthropods died as result of intensive multiplication of this symbiote. On the other hand, Weyer noted no adverse effect when he infected lice intrarectally; the symbiotes penetrated the gut wall and multiplied in the hemocytes, but never to such an extent as recorded after intracoelomic injection.

Practically nothing is known of the role *Wolbachia* play in the ticks' biology, and it is only assumed that they are involved in certain aspects of the ticks' metabolic activities. Experimental studies with other hematophagous arthropods (Brooks, 1964) have shown that there exists a delicate balance in the relationship between symbiotes and their hosts. Disturbance of this balance either by removal of symbiotes from their natural cellular environment or by transferring them to other tissues, as shown in the present study, may result in severe effects on development and biologic processes of the arthropod hosts.

REFERENCES

BALASHOV, YU. S. 1968. "Bloodsucking Ticks (Ixodoidea)—Vectors of Diseases of Man and Animals." Nauka Publishers, Leningrad Department, Leningrad, 1967, pp. 319 [In Russian]. (NAMRU-3, T. 500) *Entomol. Soc. Amer., Misc. Publ.* **8**, 159-376.

BOWMNICK, M. R., MILLER, J. C., and SNYDER, J. C. 1950. The influence of certain salts, amino acids, sugars and proteins on the stability of rickettsiae. *J. Bacteriol.*, **59**, 509-522.

BRINTON, E. P. 1969. Developmental anatomy, histology, and cytological fine structure of the nymphal and adult ovary in *Dermacentor andersoni* Stiles, before and after engorgement and mating, with related observations on the oviducts. Ph.D. Thesis, Univ. of California, Berkeley, California. pp. 213.

BRINTON, L. P., AND BURGDORFER, W. 1971. Fine structure of *Rickettsia canada* in tissues of *Dermacentor andersoni* Stiles. *J. Bacteriol.*, 105, 1149–1159.

BRINTON, L. P., AND OLIVER, H., JR. 1971. Fine structure of oogonial and oocyte development in *Dermacentor andersoni* Stiles (Acari: Ixodidae). *J. Parasitol.*, 57, 720–747.

BROOKS, M. A. 1964. Symbiotes and the nutrition of medically important insects. *Bull. WHO.*, 31, 555–559.

COWDRY, E. V. 1925. A group of micro-organisms transmitted hereditarily in ticks and apparently unassociated with disease. *J. Exp. Med.*, 41, 817–830.

HECKER, H., AESCHLIMANN, A., AND BURCKHARDT, M. J. 1968. Contribution à la connaissance des symbiotes chez *Ornithodorus moubata* (Ixodoidea). Etude au microscope électronique. *Acta Tropica*, 25, 256–262.

PEACOCK, M., BURGDORFER, W., AND ORMSBEE, R. A. 1971. Rapid fluorescent-antibody conjugation procedure. *Infect. Immunol.*, 3, 355–357.

PHILIP, C. B. 1957. In "Bergey's Manual of Determinative Bacteriology" (R. S. Breed, E. G. D. Murray, and N. R. Smith, eds.) 7th ed., pp. 931–934. Williams & Wilkins, Baltimore, Maryland.

ROSHDY, M. A. 1961. Observations by electron microscopy and other methods on the intracellular rickettsia-like microorganisms in *Argas persicus* Oken (Ixodoidea, Argasidae). *J. Insect. Pathol.*, 3, 148–166.

ROSHDY, M. A. 1963. A rickettsialike microorganism in the tick, *Ornithodoros sarignyi*; observations on its structure and distribution in the tissues of the tick. *J. Invertebr. Pathol.*, 11, 155–169.

SUITOR, E. C., JR. 1961. The relationship of *Wolbachia persica* Suitor and Weiss to its host. *J. Insect. Pathol.*, 6, 111–124.

SUITOR, E. C., AND WEISS, E. 1961. Isolation of a rickettsialike microorganism (*Wolbachia persica*, n.sp.) from *Argas persicus* (Oken). *J. Infect. Dis.*, 108, 95–106.

WEISS, E., REES, H. B., JR., AND HAYES, J. R. 1967. Metabolic activity of purified suspensions of *Rickettsia ricketsi*. *Nature* (*London*), 213, 1020–1022.

WEYER, F. 1973. Versuche zur Übertragung von *Wolbachia persica* auf Kleiderläuse. *Z. Angew. Zool.*, 60, 77–93.