ELECTRON MICROSCOPY OF SYMBIOTIC BACTERIA IN DEVELOPING OOCYTES OF THE AMERICAN COCKROACH, *PERIPLANETA AMERICANA*

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The symbiotic microorganisms found in some groups of insects present many stimulating problems from both the evolutionary and the physiological standpoint. Of particular interest are the symbiotes, usually referred to as bacteroids, of the Blattaria, which were first reported by Blochmann in 1887. These microorganisms have been found in every species of cockroach thus far investigated and also in the primitive termite, *Mastotermes darwinensis* Frogg, which apparently evolved from a Blattaria-like ancestor (Holmgren, 1909–13, cited by Brooks, 1954).

It is now generally agreed that these symbiotic organisms are bacteria, although their systematic classification is in doubt. Gier (1936), after a detailed study, concluded that they were closely related to both the diptheroids and the Rickettsiae. Richards and Brooks (1958), and Roth and Willis (1960) have presented a rather complete summary of our present knowledge of these microorganisms. Thus, the bacteroids have been reported to be about 0.9 μ in diameter and are from 1.5 to 9 μ in length. They are gram-positive or gram-negative, depending on their physiological state. They give a positive, although diffuse, Feulgen reaction and stain after Giemsa and hematoxylin. They are not stained after Sudan dyes, the Baker acid hematin test for phospholipides, nor the periodic acid-Schiff reaction for polysaccharides, and they are not detectably fluorescent or birefringent. They give an intense reduction of neotetrazolium. Using shadowed material, de Haller (1955) concluded after electron microscopic examination that they were neither ciliated nor did they possess a capsule.

Brooks (1954, 1956, 1957) and Brooks and Richards (1955, 1956) have studied the physiology of the bacteroids and their response to antibiotics. These workers successfully produced aposymbiotic progeny from adults by regulating certain mineral dietary constituents as well as by feeding antibiotics directly with the laboratory diet. Such investigations have given further support to the understanding of their bacterial nature.

To the knowledge of the authors, only two papers have appeared describing the fine structure of these bacteria after ultrathin sectioning and electron microscopy. Meyer and Frank (1957, 1958) have made a study of the bacteroids as found in the mycetocytes of the fat bodies in the oriental roach, *Blatta orientalis* L. They were unable to find evidence that would suggest any reaction between the host and symbiote and so concluded that the two existed in a completely balanced state. The state of preservation of their material, as revealed by their figures, is, however, somewhat disappointing. Little of the more intricate detail of structures in either the host cells or the bacteria has been preserved.

The object of the present study has been to investigate the nature of the host-symbiote relationship within the ovary and to reveal more clearly the fine structure of the bacteroids.

MATERIALS AND METHODS

The species used as a source of the bacteroids throughout the course of this investigation was the American cockroach, *Periplaneta americana* L. The roaches were fed Purina lab chow (Ralston Purina Company) and given access to water at all times. They were maintained at a temperature of 24 C and a relative humidity of 60 per cent. Ovaries of last instar nymphs were removed as rapidly as possible under Pringle's (1938)

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isotonic cockroach Ringer solution. Each ovary of this species consists of 8 panoistic ovarioles. These ovarioles were carefully separated before fixation in order to preserve the delicate ovariole sheath intact. Dissected ovarioles were then transferred directly to a modified Palade's (1952) fixative as used by Willey and Chapman (1960) for neurosecretory tissue in the same species of cockroach. This consisted of 2.5 ml acetate Veronal buffer (9.714 g sodium acetate · 3 H_2O, and 14.714 g sodium Veronal, made up to 500 ml of solution), 1.0 ml of 8.5 per cent NaCl, 1.0 ml of 0.11 M $CaCl_2$, 1.0 ml of 0.1 N HCl, 1.75 ml distilled water, and 6.25 ml of 2 per cent OsO_4 in distilled water. The buffered fixative was chilled in an ice water bath before use. The final pH of the solution was about 8.3. Ovarioles were fixed for 4 hr at 2 to 4 C, then washed for 30 min in each of two changes of cold buffered fixing solution in which the 2 per cent OsO_4 solution had been replaced with an equal volume of distilled water. The tissue was then dehydrated through a graded cold ethanol series, beginning at 50 per cent and allowing 30 min in each concentration. After two changes of absolute ethanol, the ovarioles were infiltrated for 2 hr in two changes of a catalyzed methacrylate mixture (2 parts ethyl methacrylate, 3 parts *n*-butyl methacrylate, and 1.5 per cent Luperco CDB). The tissue was transferred to no. 1 gelatin capsules with a fresh methacrylate mixture and polymerization was carried out at 70 C for 24 hr. Ultrathin sections were cut with glass knives, using a Servall Porter-Blum ultramicrotome. Sections were floated onto the surface of a 40 per cent solution of acetone and distilled water in a collecting trough. Compression artifacts were minimized by expanding sections with xylol vapors following the method of Satir and Peachey (1958). Sections were then mounted on collodion-coated 200 mesh copper screen and examined in an RCA EMU-2D electron microscope fitted with a 0.015-in. externally centerable (Canalco) condenser aperture and a 50 μ aperture in the standard objective pole piece. Some sections were stained with uranyl acetate for 1 hr immediately after sectioning, using the methods outlined by Watson (1958).

RESULTS AND DISCUSSION

General ovariole structure. Figure 1 shows a low magnification view of a portion of the ovariole. The structures seen here correspond to those described with the light microscope by Bonhag (1959) for the American cockroach. The outermost "membrane" is the one, or sometimes two, layered ovariole sheath (*OS*). No function has yet been attributed to this sheath. In all the material studied it was never found in contact with the tunica propria (*TP*), which has many of the characteristics of insect connective tissue. Beneath the tunica propria lie the follicular epithelial cells (*FE*) which function as intermediaries in the transport of metabolites from the blood to the egg and secrete the chorion before ovulation, or resorb the yolk if the egg has not completed its development in the vitellarium (Bonhag, 1959). These cells generally have a considerable number of mitochondria (*M*). The bacteroids (*B*) are always located between the egg (*E*) and the follicular epithelial cells. Microvilli (*MV*) can be seen extending from the periphery of the egg and in close apposition with the symbiotes. The possible significance of this association will be discussed subsequently.

Bacterial cell wall. A rather thin, single layered cell wall (*CW*) of the bacteria can be seen in figure 2, at higher magnification in figure 3, and in the other figures. The thickness of the wall varies from 50 to 90 A. It should be noted that the cell wall stained intensely with uranyl acetate (figure 5) and was always more electron dense following the uranyl acetate staining than were the adjacent cytoplasmic membranes of the egg and follicular epithelial cells.

The chemical composition of the bacteroid cell wall is unknown. Disclosure of its nature will have to await analysis of isolated cell walls. Although the bacteroid cell wall appears only slightly thinner than the cell wall of *Escherichia coli* described by Kellenberger and Ryter (1958), it is the thinnest bacterial cell wall yet encountered by the authors. It, therefore, seems appropriate to speculate briefly as to the possible significance of this thinness. It is quite clear that, in a relationship such as that of the host and the symbiote, at least part of the function of the cell wall (presumably the protective part) may be assumed by the host. In the course of evolution, then, the cell wall may have become reduced in thickness. It would be most interesting to observe whether or not the cell wall thickness changes when the bacteroids are maintained in pure culture.

Along these same lines, one may wonder whether it is, indeed, the thinness of the wall which contributes to the difficulty met in attempting to culture these microorganisms on artificial media (Richards and Brooks, 1958 and Roth and Willis, 1960).

Observation of bacteroids in the process of cellular division reveals that a centripetal deposi-

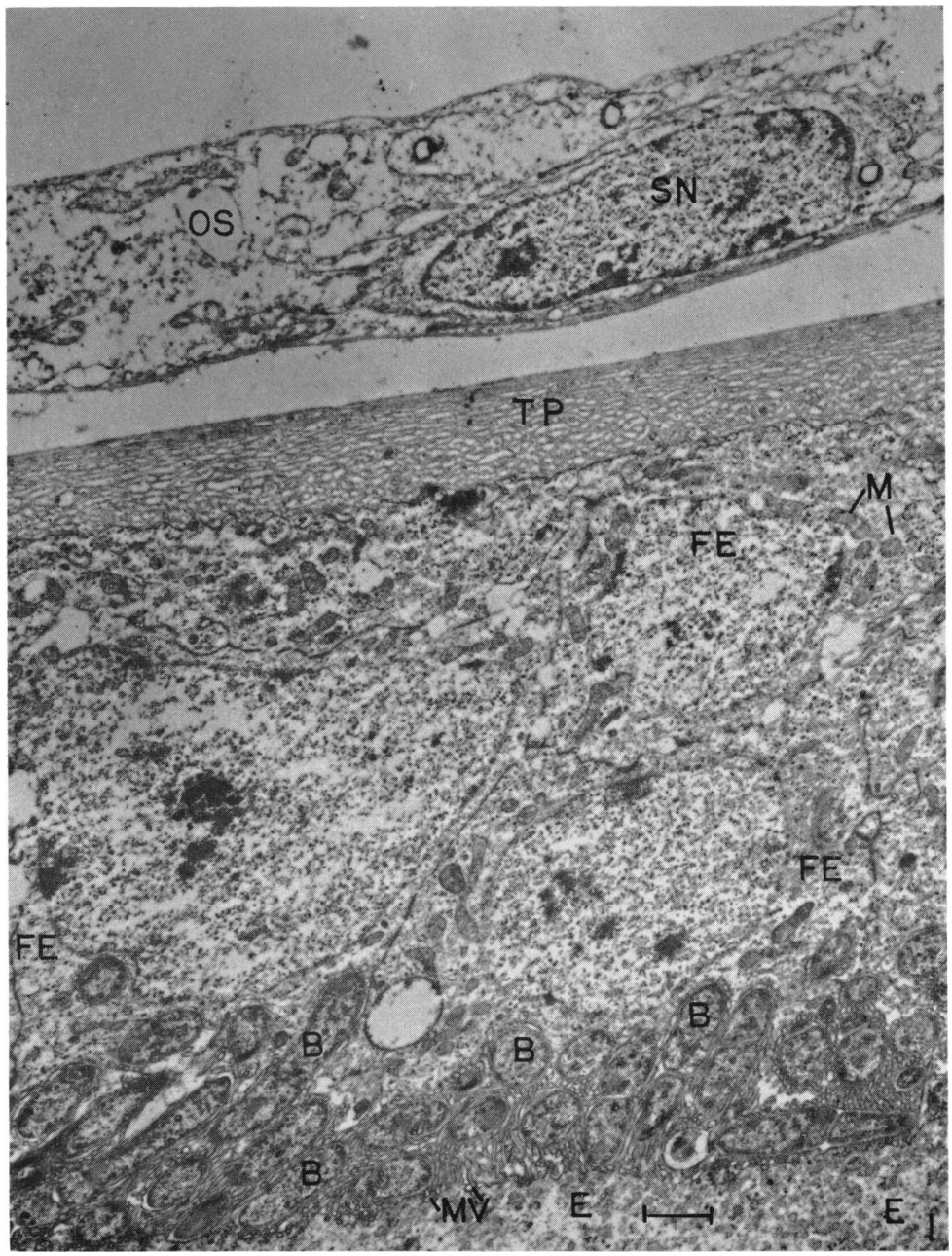

Figure 1. Low magnification electron micrograph of a portion of an ovariole showing the disposition of the bacteroids. *B* = bacteroid; *OS* = ovariole sheath; *SN* = sheath nucleus; *TP* = tunica propria; *FE* = follicular epithelial cells; *M* = mitochondria; *E* = egg; *MV* = microvilli. In each figure the magnification mark equals 1 μ.

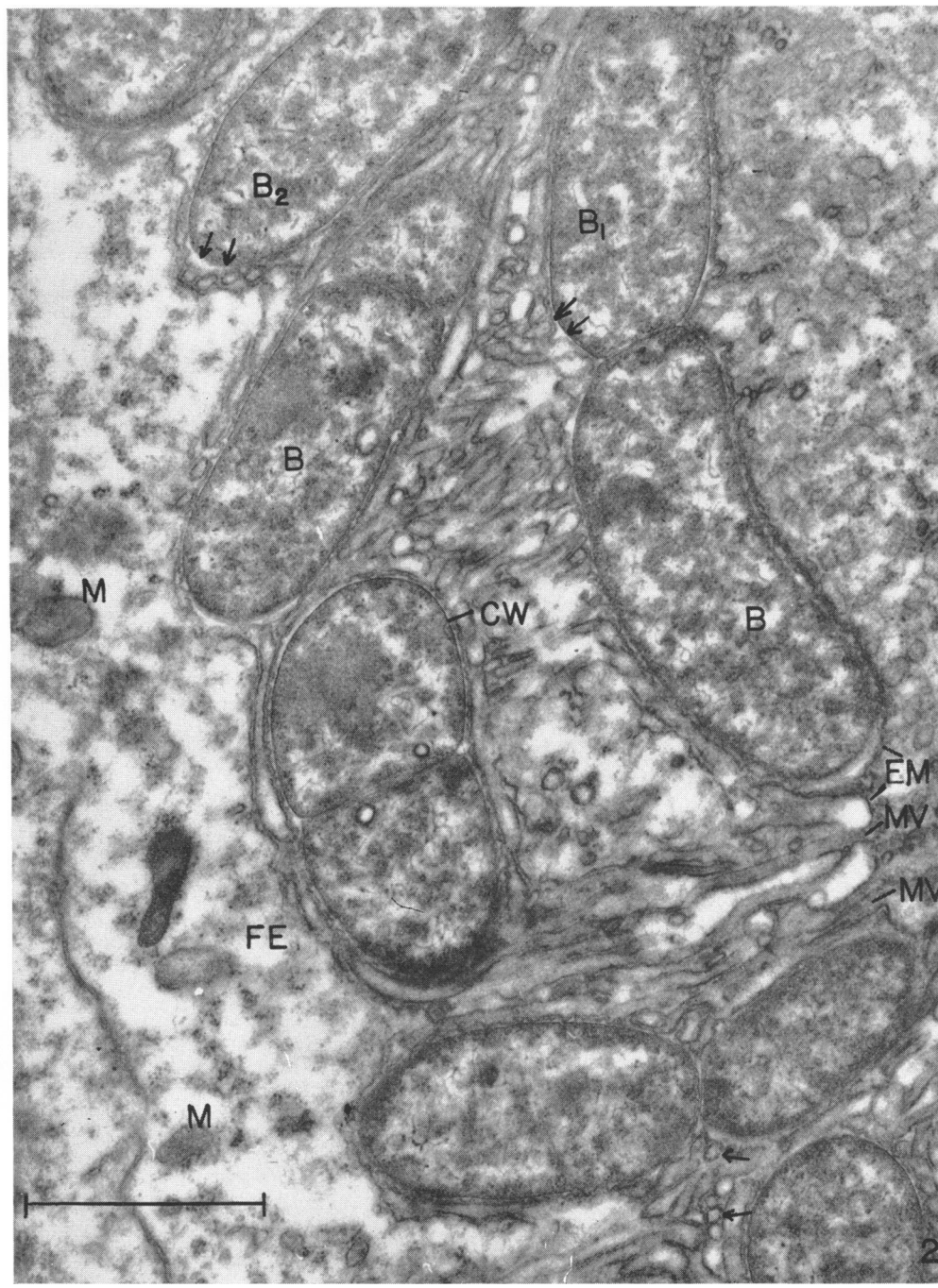

Figure 2. Electron micrograph showing the nature of the relationship among the bacteroids (*B*), egg membrane (*EM*) and follicular epithelial cells (*FE*). *MV* designates particularly prominent microvilli which extend from the egg surface to entwine about the bacteroids. *Arrows* designate several microvilli cut transversely. At B_1, *double arrow*, the microvilli are separated from the bacteroid by the egg membrane; at B_2, *double arrow*, there is no such separation. *M* = mitochondrion; *CW* = cell wall.

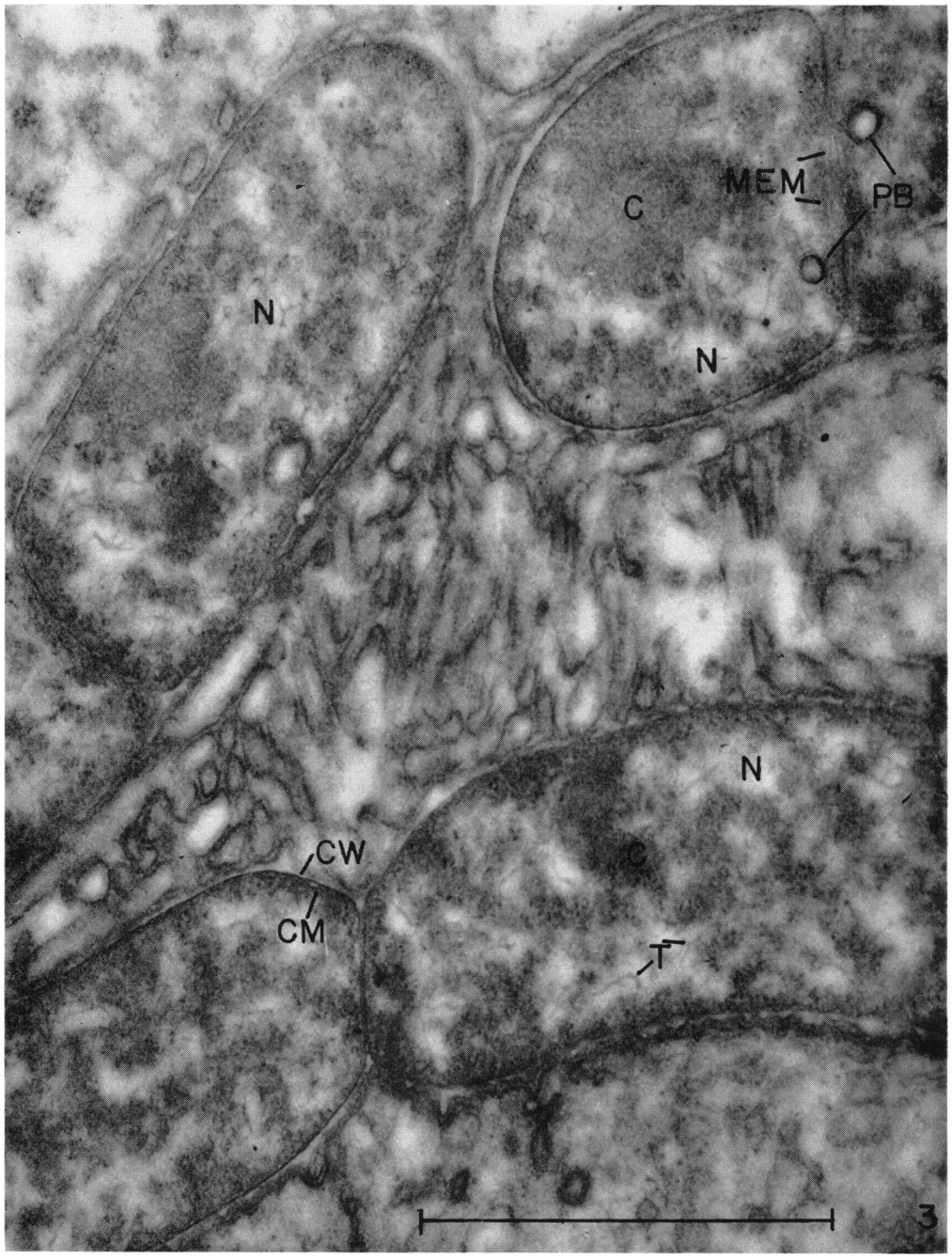

Figure 3. High magnification electron micrograph of a portion of figure 2 to show details of bacterial fine structure. *CW* = cell wall; *CM* = cell membrane; *N* = nuclear zones; *T* = threadlike structure; *C* = granular ground cytoplasm; *PB* = peripheral bodies in a dividing bacteroid; *MEM* = membrane structures at site of transverse wall and membrane septum formation.

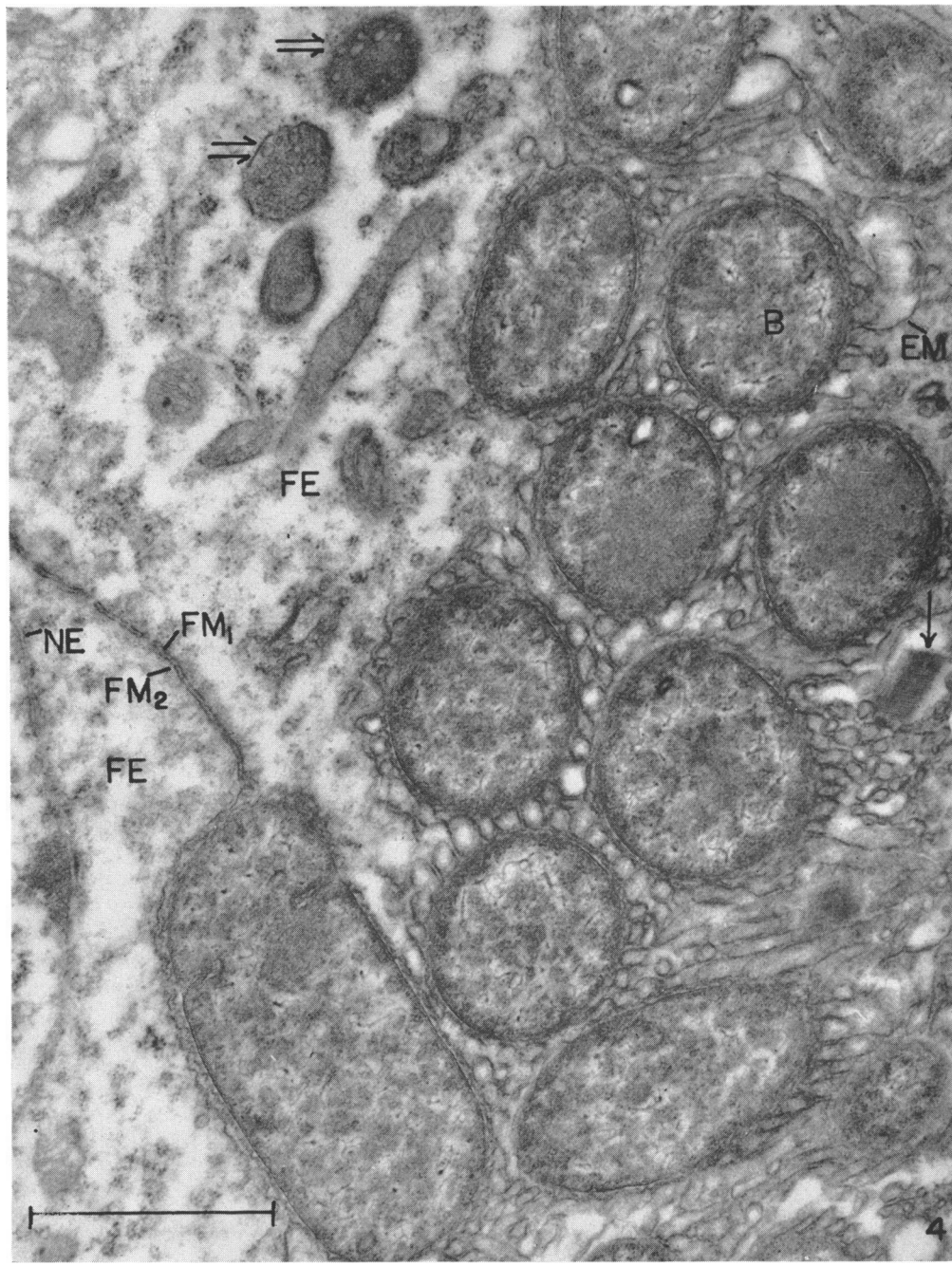

Figure 4. Electron micrograph showing relationship among bacteroids, egg membrane, and follicular epithelial cells. NE = nuclear envelope of follicular epithelial cell; FM_1, FM_2 = apposing plasma membranes of two follicular epithelial cells. The *arrow* indicates what is thought to be a section tangential to the surface of the cell wall. The *double arrows* indicate inclusions which are apparently identical to multivesicular bodies.

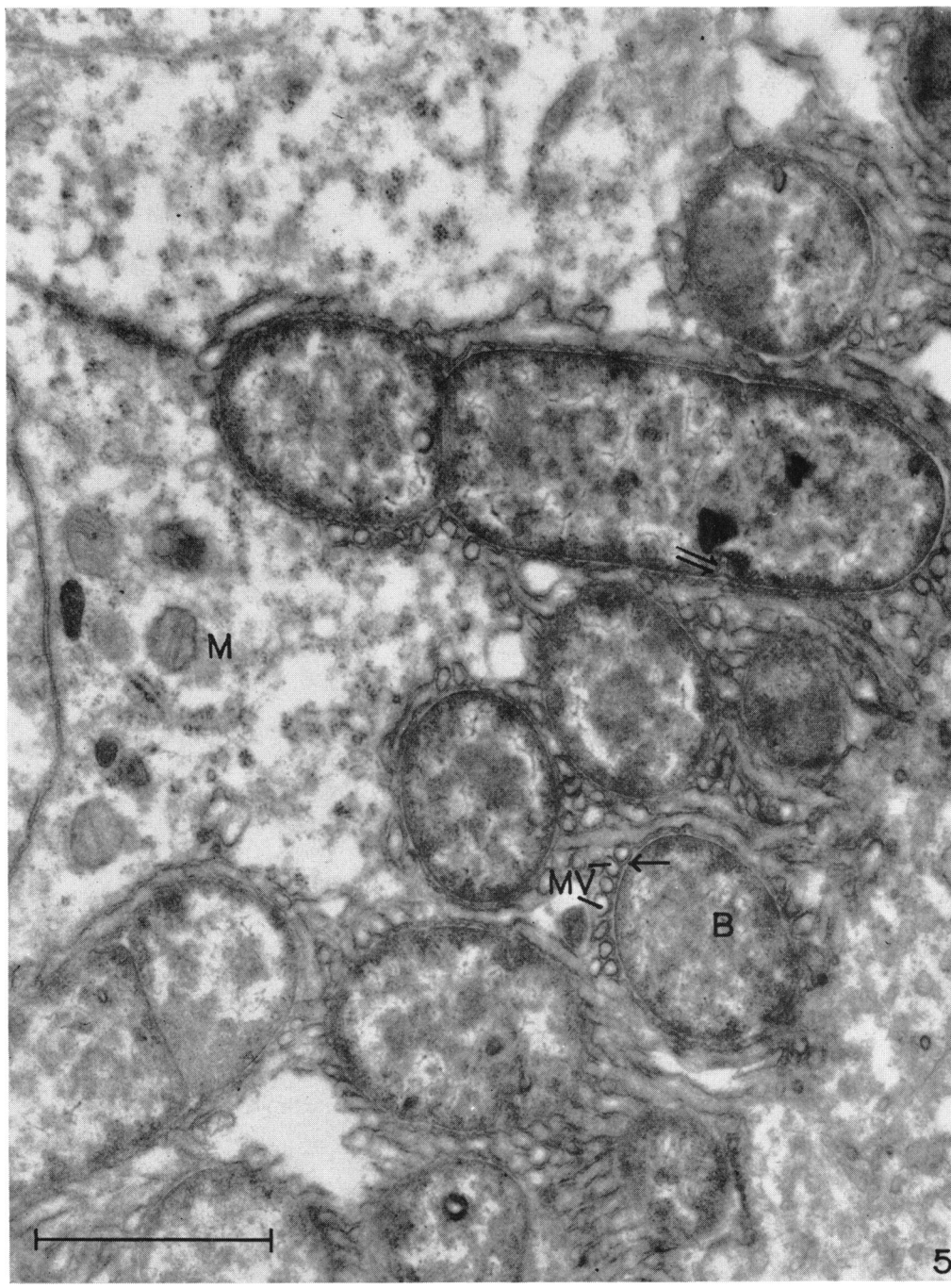

Figure 5. Electron micrograph showing clearly a bacteroid (*B*) which is enclosed within the egg plasma membrane (*arrow*). Egg microvilli (*MV*) remain closely apposed to the bacteroid surface, but the additional egg membrane now encloses the bacteroids. The centripetal nature of the transverse cell wall deposition may be seen at the *double arrows*. *M* = mitochondrion.

tion of cell wall material, reminiscent of that described for *Bacillus cereus* by Chapman and Hillier (1953) and for an unidentified bacterium by Chapman (1959*b*), occurs. This observation of the nature of the cellular division process in the bacteroids strongly supports the case for their bacterial nature.

Cytoplasmic membrane. A very thin cytoplasmic membrane (*CM*) is visible just below the cell wall in figure 3. This single membrane measures from 17 to 35 A in thickness. It is considerably thinner than the bacterial cell membranes described by other authors (Tomlin and May, 1955; Murray, 1957; Chapman and Kroll, 1957; Chapman, 1959*a*; Kellenberger and Ryter, 1958; and Kellenberger, Ryter, and Séchaud, 1958). Its thickness is also considerably less than that usually reported for animal cell plasma membranes. It was visible only after staining and could be distinguished from the cell wall only in regions where the two had separated. No obvious explanation arises to account for the thinness of the plasma membrane.

Cytoplasm. The internum of the bacteroid can be divided into five well-defined structures or regions under the given conditions of fixation:

1) A low density area, probably representing the nuclear zone (*N*, figure 3), is dispersed in rather diffuse, irregular masses throughout the cytoplasm. This is unlike the centrally oriented nuclear zones reported by most authors (Chapman, 1959*a*; Chapman and Hillier, 1953; Caro, van Tubergen, and Forro, 1958; and Kellenberger et al., 1958). The impression of the dispersed nature of the nuclear material in these bacteria is supported by light microscope studies where a diffuse staining reaction of the bacteria was noted after the Feulgen test for deoxyribonucleic acid (Gier, 1936; Rizki, 1954; and Brooks, 1954). No limiting membrane has been found around the nuclear area of the bacteroids. In this respect, they correspond to both the bacteria and the blue-green algae. The nuclear zones reveal no characteristic division figures such as the dumbbell configurations reported by Chapman and Hillier (1953), Bradfield (1956), Chapman (1959*a*, *b*) and others. In this respect, also, the bacteroids resemble the blue-green algae particularly closely.

2) A fine, dense, threadlike structure (*T*, figure 3), from 85 to 135 A in diameter, was frequently observed within the nuclear areas. These structures have been observed in other bacteria (Birch-Andersen Maaløe, and Sjöstrand, 1953; Piekarski and Giesbrecht, 1956; Chapman and Kroll, 1957). It has been suggested by Chapman (1959*a*), that these filaments might represent an alternate form of the chromatin material, but their true nature is not known.

3) The ground cytoplasm of the bacteroids frequently appears quite granular. The granules, of varying size, medium density, and ill-defined borders are believed to be ribonucleoprotein particles which are abundant in bacterial cytoplasm (Chapman, 1959*b*).

4) Peripheral bodies (*PB*, figure 3), 600 to 1600 A in diameter and similar to those described by Chapman and Hillier (1953) in *Bacillus cereus*, were found in most dividing cells. They were always associated with the areas of formation of new transverse cell walls and plasma membranes. These bodies usually consisted in the bacteroids of one or several concentric spherical profiles with membranes 20 to 55 A thick, and had low density centers.

5) Associated with the peripheral bodies in some dividing cells are several widely separated membranes 20 to 30 A thick. They are located between the centripetally forming cell wall and membrane (*MEM*, figure 3) and may possibly represent stages in the formation of the new cytoplasmic membrane or cell wall, or they may otherwise be involved in the production of the wall and plasma membrane.

Host and symbiote relationships. The intimate relationship between the egg membrane (*EM*, figure 4) and the bacteria raises several interesting possibilities. The position of the microvilli (*MV*, figure 2) with respect to the bacteria suggests that active synthesis of one or more substances necessary for egg development is possibly being carried out by the symbiotes.

Richards and Brooks (1958) suggest that the bacteroids are involved in the breakdown of urates for protein synthesis and also may supply B vitamins, amino acids, and possibly a tripeptide. They appear to have no direct effect on the endocrine system. The relationships between the egg membrane and symbiote furnish further evidence that one, or possibly all, of these factors and others not yet understood are directly influencing egg development. This factor, or complex of factors, may not be utilized directly

by follicular epithelial cells as these produce no microvilli.

The bacteroids have always been considered to be intracellular microorganisms usually located within the cytoplasm of the egg during embryogenesis or in the mycetocytes of the fat body. The included micrographs reveal instances where bacteroids appear to be included within the egg membrane (B, figure 5; B_1, figure 2) and instances where bacteroids appear to be, at least partially, intercellular (B_2, figure 2). It seems quite likely that these bacteroids reveal two stages in a process by which the bacteroid is drawn into the egg cytoplasm.

Whatever the nature of the association between host membranes and symbiotes may be throughout the life cycles of the host and symbiote, it is obvious that the level of symbiosis is one that involves a very highly integrated system. The fact that the bacteroids have most of the structural features characteristic of bacteria, and divide in a fashion similar to that of the bacteria leaves little or no doubt that the bacteroids are true bacteria and should be regarded as such in the future. A detailed study of the relationships between host and symbiotes through their complete cycle from egg to adult using recent techniques of electron microscopy and physiology would be highly profitable. Such an investigation would provide many avenues of fruitful research.

SUMMARY

Electron microscopy of the symbiotic bacteria of the cockroach has revealed a close association between the egg membrane and the bacteria. Microvilli from the egg surround the symbiotes suggesting active exchange of metabolites.

The bacteroids have a cell wall between 50 and 90 A thick, as well as a very thin cell membrane 17 to 35 A thick. The cytoplasm has a diffuse low density nuclear area which has dense, axially arranged filaments 85 to 135 A wide. A granular ground cytoplasm is interspersed among the nuclear portions of the protoplast.

Multiple membraned peripheral bodies were present in many dividing cells. These bodies were associated with several membranes which were possibly involved in laying down the cell wall and membrane, or represented these structures in an early stage of development.

The association between host and symbiote is one that has evolved into a highly integrated system.

There can be little doubt that these symbiotes are true bacteria as is evident from their characteristic cellular structure and mode of division which are similar to those previously described for bacteria.

ADDENDUM

While this paper was in press an article appeared by R. A. R. Gresson and L. T. Threadgold entitled "An electron microscope study of bacteria in the oocytes and follicle cells of *Blatta orientalis*" (Quart. J. Microscop. Sci., **101**, 295–297, 1960). Although these authors note that the relationship between the bacteroids and dense membrane-like structures forming a sort of outer covering of the bacteroids is not always obvious, they suggest that the membranous structures originate from the bacteroids. Our micrographs indicate that this suggestion is inappropriate for it is clear that these structures arise from the egg membrane and are in reality microvilli.

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