Distribution of Sterol-specific Complexes in a Continually Shearing Region of a Plasma Membrane and at Procaryotic-Eucaryotic Cell Junctions

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ABSTRACT A narrow zone of plasma membrane between the head and body of a protozoan from termites undergoes continual in-plane shear because the head rotates continuously in the same direction relative to the cell body (Tamm, S. L., and S. Tamm, 1974, *Proc. Natl. Acad. Sci. USA* 71:4589–4593). Using filipin and digitonin as cytochemical probes for cholesterol and related 3-β-hydroxysterols, we found a high level of sterol-specific complexes, visible as membrane lesions in thin sections, in both shearing and nonshearing regions of the membrane, indicating no difference in sterol content. This confirmed previous observations that any region of the fluid membrane can undergo shear, but that this occurs only at certain locations due to cell geometry and proximity to rotating cytoskeletal structures. Filipin and digitonin did not disrupt the plasma membrane at the junctions with ectosymbiotic rod and fusiform bacteria (i.e., membrane pockets and ridges). However, pepsin degradation of dense material coating the junctional membranes resulted in a positive response of these regions to filipin. Fluorescence microscopy revealed a bright halo around each rod bacterium, due to filipin-sterol binding in the sides of the membrane pockets, but no fluorescence at the bottom of the pockets; the same fluorescence pattern was found in pepsin-treated cells despite the presence of sterols throughout the pocket membrane, as shown by electron microscopy. These findings indicate that (*a*) regional constraints may restrict the ability of filipin to interact with sterols or form visible membrane lesions, and (*b*) a negative response to filipin, assayed by either electron or fluorescence microscopy, is not sufficient to demonstrate low membrane sterol concentration, particularly in membrane domains characterized by closely associated proteins.

We recently discovered a dramatic demonstration of the fluid behavior of cell membranes in a devescovinid flagellate from termites (42, 45, 46). The anterior part or head of this protozoan continually rotates in the same direction relative to the rest of the cell. Ectosymbiotic bacteria attached to the surface provided visible markers for showing that in-plane shear occurs continually between neighboring regions of the plasma membrane, and that the shear zone is localized to a narrow belt of bacteria-free membrane between the head and cell body. Freeze-fracture replicas revealed that the lipid bilayer is continuous across the shear zone (42).

In view of current evidence for the existence of planar domains of distinct structure, composition, and function within the membranes of many cells (16, 21), the question arises whether a membrane region that allows continuous shear must be biochemically and/or structurally different from the remainder of the plasma membrane. Our freeze-fracture images showed no differences in intramembrane particle distribution between the shear zone and other membrane regions (42), but this method does not show possible heterogeneities in lipid content within the bilayer. Measurements of kinetic or thermal motions of molecules in membranes, coupled with calculations of viscosity coefficients, have shown that the lipid bilayer behaves as a two-dimensional fluid when the hydrocarbon chains are disordered (above the phase transition temperatures), and therefore the lipid components should not contribute to the surface shear rigidity of the membrane (14). Thus, the ability of the lipid bilayer to undergo continuous shear deformation is to be expected of a fluid system, and can indeed be demonstrated

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directly by our observation of head rotation in devescovinids.

One might predict, then, that the lipid composition of the shearing region would be no different from that of the rest of the protozoan's plasma membrane. However, membrane "fluidity", as calculated from diffusive motions of labeled molecules within membranes, is a qualitative and oversimplified term that must be used with caution (14, 39). It is possible, for example, that the shearing region of the devescovinid membrane has an unusually high degree of fluidity due to local differences in lipid content and microviscosity, and that this increases the efficiency of the system.

In this report we investigate whether the lipid composition of the shearing region of the devescovinid membrane is indeed similar to that of the surrounding plasma membrane. Because sterols exert a key influence on membrane fluidity (10, 19), we used a recently developed cytochemical technique (1, 12, 13, 17, 23, 34, 48, 49) employing the sterol-binding agents filipin and digitonin (8, 22, 28) to examine the planar distribution of sterols in the plasma membrane of the devescovinid.

We also investigated whether the plasma membrane at the attachment sites of the ectosymbiotic bacteria contains a distinct sterol distribution. Previous ultrastructural work showed specializations in the devescovinid membrane at the bacterial junctions, including dense material underlying the membrane and a high density of intramembrane particles in freeze-fracture replicas (43).

Finally, we have been forced by our findings to address some of the problems associated with the use of filipin and digitonin as cytochemical probes for localizing membrane sterols. In particular, whether the absence of labeling in a given region of membrane is due to lack of sterols, or to local constraints on the formation of sterol-specific complexes, is investigated by a new combination of methods utilizing fluorescence and electron microscopy.

MATERIALS AND METHODS

Organism: The protozoan is the same (as yet unnamed) devescovinid flagellate from the Florida termite *Cryptotermes cavifrons* used in previous studies (40–46, 50).

Filipin Treatment for Electron Microscopy: Protozoa were released from termite hindguts and fixed in 1.25% glutaraldehyde, 2% paraformaldehyde, 0.1 M $NaPO_4$ buffer (pH 7.0) for 30 min at room temperature. Cells were washed briefly in 0.1 M $NaPO_4$ buffer (pH 7.0), and split into two groups: experimentals were incubated in fresh buffer containing 200 μg/ml filipin (kindly provided by J.E. Grady, Upjohn Co., Kalamazoo, MI) and 1% dimethyl sulfoxide for 3–4 h at room temperature; controls were placed in buffer containing 1% dimethyl sulfoxide without filipin for the same period. Both groups were then washed in buffer, and postfixed in 2% OsO_4, 0.1 M $NaPO_4$ (pH 7.0) for 1 h at room temperature. Cells were rinsed in water and block-stained with 0.5% uranyl acetate in veronal-acetate buffer at 4°C overnight. Following dehydration in acetone and flat-embedding in Araldite, individual cells were mounted in known orientations for thin sections. Sections were stained with uranyl and lead salts and examined in a Zeiss 10C A electron microscope at 80 kV.

Digitonin Treatment: Protozoa were fixed in glutaraldehyde-paraformaldehyde for 30 min as described above, and then put into fresh fixative containing 1 mg//ml digitonin (Sigma Chemical Co., St. Louis, MO) for 7 min at room temperature. Controls were left in fixative without digitionin. Cells were washed in buffer, postfixed in osmium tetroxide, and processed further as described above.

Pepsin-Filipin Treatment: Protozoa were prefixed in glutaraldehyde-paraformaldehyde for 15 min, then washed thoroughly in buffer. One group of cells was incubated in 0.5% pepsin (Boehringer Mannheim Biochemicals, Indianapolis, IN) in 0.1 N HCl for 20 min at 37°C (14). Enzyme controls were incubated in 0.1 N HCl for 20 min at 37°C. After washing in buffer, some of the pepsin-treated cells and all the controls were fixed in glutaraldehyde-paraformaldehyde containing 200 μg/ml filipin and 1% dimethyl sulfoxide for 1.5 h at room temperature. The remaining pepsin-treated cells were placed in fixative containing 1% dimethyl sulfoxide without filipin for the same period. All batches were processed for electron microscopy as described above.

Fluorescence Microscopy: The fluorescence properties of filipin (2, 3, 11, 27, 36) were used to monitor its binding to sterol-containing membranes in living and pepsin-treated cells. Cells were examined with Zeiss epifluorescence optics using wide-band ultraviolet excitation (Zeiss UG1 filter set) and a 100×/1.3 Neofluar phase objective. A red-free barrier filter (Zeiss KP 560) was tested but not used routinely since ultraviolet light did not excite autofluorescence of glutaraldehyde-fixed cells. Filipin fluorescence was recorded on Kodak Tri-X Pan 35 mm film pushed to 6400 ASA with Perfection XR-1 developer (Perfection Photographic Products, Inc., Los Angeles, CA).

Living protozoa were placed in insect Ringer's (0.57% NaCl, 0.005 M $NaPO_4$ buffer, pH 6.95) containing 200 μg/ml filipin and 1% dimethyl sulfoxide. Control cells were placed in Ringer's containing 1% dimethyl sulfoxide without filipin. Slide preparations were examined at various times after exposure to filipin by phase-contrast and fluorescence microscopy.

To compare the effect of pepsin treatment on the patterns of filipin labeling observed by electron microscopy vs. fluorescence microscopy, protozoa were prefixed in glutaraldehyde-paraformaldehyde, incubated in pepsin or HCl without pepsin, and placed in fixative containing filipin or 1% dimethyl sulfoxide as described above. After washing in buffer, cells were transferred to slides for fluorescence microscopy. In one experiment, pepsin/filipin-treated cells were split into two groups: one group was processed further for electron microscopy as described above, while the other batch was observed by fluorescence microscopy.

RESULTS

Membrane Structure and Rotational Movements

The devescovinid is 100–150 μm long and zeppelin-shaped when freshly isolated from termites. The caplike anterior end or head continually rotates in a clockwise direction (as viewed anteriorly) relative to the cell body, which may either turn in the opposite direction or remain stationary (Fig. 1) (42, 45). Head rotation occurs at speeds up to 0.5–0.7 rotation/s when the cell body is not turning. Rotational movements are caused by a rodlike axostyle complex that extends through the cell body and generates torque along its length (41).

Two types of ectosymbiotic bacteria, rod-shaped and fusiform, are attached to the surface of the devescovinid by specialized junctional complexes (Fig. 1) (43). The protozoan's plasma membrane is deeply invaginated into pockets surrounding the rod bacteria, and is elevated into ridges underlying the longer and more slender fusiform bacteria. At both types of procaryotic-eucaryotic junctions, the host membrane is coated by electron-dense material on the cytoplasmic side (43). Freeze-fracture replicas revealed additional specializations, including a higher density of intramembrane particles in the pockets enclosing the rod bacteria, and particle aggregations along the ridges underlying the fusiform bacteria (43).

The rod bacteria provide visible markers for showing that rotation of the anterior end involves continual unidirectional rotation of the entire plasma membrane of the head relative to that of the cell body (42, 45). Membrane shear is localized to a narrow zone of bacterial-free membrane, 1–1.5 μm wide, between the head and body (Figs. 1 and 2) (42–45). Since the circumference of the shear zone is ~30 μm, the maximal rate of membrane shear is ~20 μ/s. Freeze-fracture electron microscopy demonstrated that the lipid bilayer was unbroken across the shear zone, and that no regional differences in density or pattern of intramembrane particles existed in the shear zone membrane (42).

Filipin and Digitonin Labeling: Electron Microscopy

Filipin, a polyene antibiotic, and digitonin, a saponin, react

specifically with cholesterol and other 3-β-hydroxysterols (8, 22, 28) to produce distinctive structural alterations in membranes, "sterol-specific complexes", which are readily detected by freeze-fracture or thin-section electron microscopy (1, 12, 13, 17, 23, 34, 48, 49). Thin sectioning of individually oriented cells rather than freeze-fracture was used here because this method allowed the relatively small amount of shear zone membrane to be more readily examined.

Filipin and digitonin induced characteristic deformations in all regions of the devescovinid plasma membrane except at the junctions with the ectosymbiotic bacteria. Filipin-affected membranes appeared wavy or scalloped in outline, with frequent loss of clear unit membrane structure (Figs. 2, *A* and *C*, and 3 *B*). Digitonin caused more gross corrugations in membrane profiles, often with sharp creases or folds. In addition, digitonin-treated membranes typically displayed only a single leaflet of the bilayer (Fig. 4).

No differences in the degree or extent of filipin or digitonin-induced lesions were observed between the shear zone membrane (Fig. 2 *A*) and other regions of the extra-junctional plasma membrane on the head (Fig. 2 *C*) and cell body (Fig. 3 *B*). The plasma membrane of controls appeared smooth with a distinct trilaminar image (Figs. 2 *B* and 3 *A*). Filipin and digitonin also disrupted the parallel array of closely packed membranous tubules that extend from the helical Golgi apparatus to directly under the shear zone membrane (Figs. 1 and 2). In contrast, the coated junctional regions of the plasma membrane at the attachment sites of the rod and fusiform bacteria were not disrupted by either filipin (Figs. 2 *C* and 3 *B*) or digitonin (Fig. 4).

Pepsin treatment completely removed the dense material coating the membrane pockets and ridges, as well as degrading most of the cell cytoplasm (Fig. 5). Pepsin also detached the fusiform bacteria from the surface ridges. Subsequent exposure to filipin resulted in marked deformation of the now bare junctional membranes, demonstrating the presence of filipin-sterol complexes in these regions (Fig. 5 *A*). The pocket membranes displayed variable degrees of labeling, but usually were not so strongly affected by filipin as were the extra-junctional regions of the membrane (Fig. 5 *A*).

Controls treated with pepsin but not filipin showed clear trilaminar membranes (Fig. 5 *B*), confirming that the alterations in the filipin-treated membranes represent sterol-specific complexes. Controls incubated without pepsin displayed a pattern of filipin labeling similar to that of standard filipin treatment (i.e., Fig. 3 *B*), indicating that the positive responses of the junctional membranes are due to action of the enzyme alone.

Preliminary analysis of the sterol content of the protozoan fauna from *Cryptotermes cavifrons* (60–70% devescovinids by cell counts) indicated that cholesterol and sitosterol are the major sterols present in nearly equal amounts (R. L. Conner, personal communication).

Fluorescence Microscopy

Living flagellates placed in filipin-containing Ringer's soon ceased all rotational and flagellar movements. When compressed by the coverslip these cells began to glide, propelled by the flagellar activity of their adherent rod bacteria as reported previously (44).

Cells in filipin/Ringer's displayed a characteristic honeycomb pattern of fluorescence that faded slowly (Fig. 6). The entire extra-junctional plasma membrane, including the shear

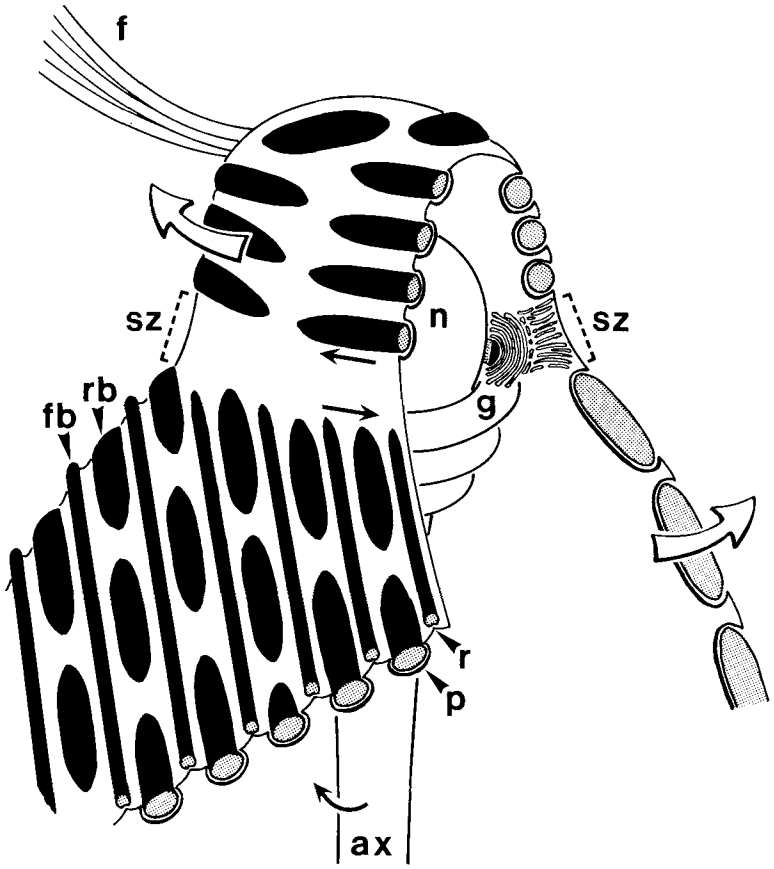

FIGURE 1 Diagram of the anterior end of the devescovinid. Ectosymbiotic rod (*rb*) and fusiform (*fb*) bacteria are attached to the surface by specialized junctional complexes consisting of coated pockets (*p*) and ridges (*r*), respectively, of the host membrane. The rotary axostyle (*ax*, arrow) turns the caplike head in a clockwise direction relative to the cell body which rotates counterclockwise (large arrows). As a result, a narrow belt of bacteria-free membrane between the head and body undergoes continual unidirectional shear (shear zone, *sz*; arrows). One gyre of the helical Golgi apparatus (*g*) is drawn in cross-section to show the closely-packed membranous tubules that extend from the Golgi cisternae to the shear zone membrane. *f*, flagella; *n*, nucleus.

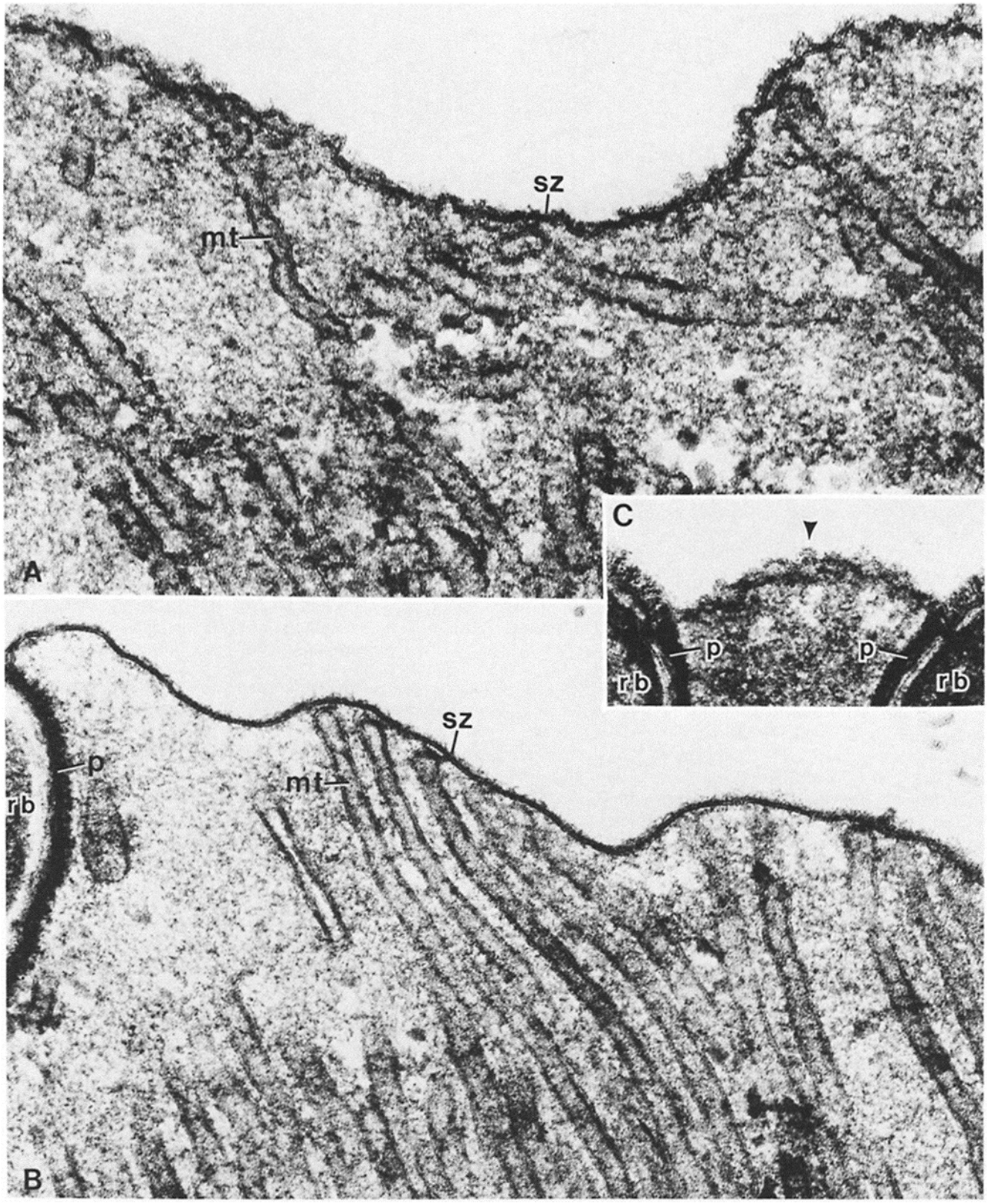

FIGURE 2 (*A* and *B*) Thin sections cut transversely through the shear zone membrane (*sz*), with underlying array of parallel membranous tubules (*mt*) as depicted in Fig. 1. Anterior is to the reader's left. (*A*) Filipin-treated cell. The shear zone membrane and membranous tubules are wavy and scalloped showing the presence of numerous filipin-sterol complexes. The Golgi cisternae themselves were not disrupted by filipin (not shown). (*B*) Control cell. All membranes appear smooth with a typical trilaminar image. The plasma membrane invaginates to form a coated pocket (*p*) enclosing a rod bacterium (*rb*) on the head. (*C*) Thin section through the plasma membrane on the head of a filipin-treated cell. The extra-junctional membrane is corrugated by filipin-sterol complexes (arrowhead) similar to the labeling pattern of the shear zone (*A*). The coated pockets (*p*) surrounding the rod bacteria (*rb*) are not affected by filipin. × 121,000 (*A* and *B*); × 88,200 (*C*).

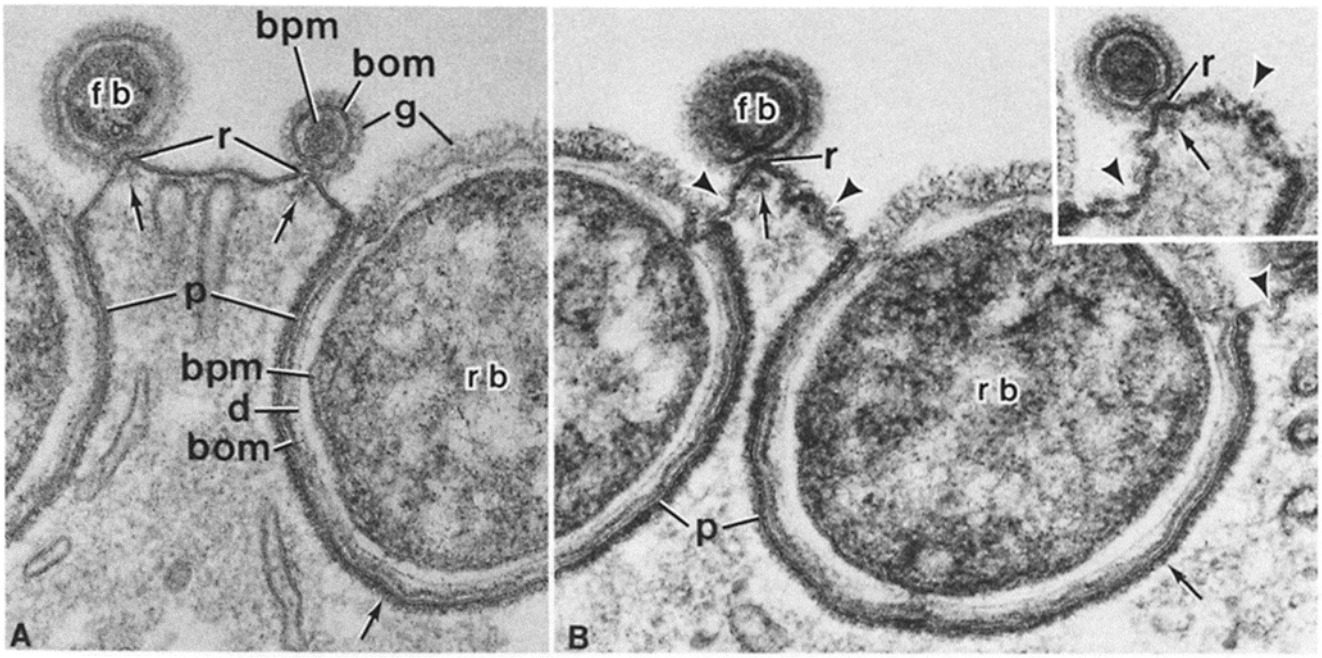

FIGURE 3 Transverse thin sections through junctional complexes with ectosymbiotic rod (*rb*) and fusiform (*fb*) bacteria on the body of devescovinids. (*A*) Control cell. The envelopes of the gram-negative bacteria consist of a plasma membrane (*bpm*), peptidoglycan layer (*d*), outer membrane (*bom*), and glycocalyx (*g*). The devescovinid plasma membrane forms pockets (*p*) around the rod bacteria, and ridges (*r*) under the fusiform bacteria. At both types of junctions the host membrane is underlain by fuzzy electron-dense material (arrows). (*B*) Filipin-treated cells. The extra-junctional plasma membrane is scalloped by filipin-sterol complexes (arrowheads), similar to the labeling pattern of the shear zone membrane (Fig. 2*A*) and extra-junctional membrane of the head (Fig. 2*C*). The coated junctional membranes (arrows) of the pockets (*p*) and ridges (*r*) are devoid of visible lesions, and display a clear trilaminar structure (*inset*). Note that the bacterial membranes are unaffected by filipin. × 88,200.

zone, exhibited a uniformly weak fluorescence. However, each rod bacterium was outlined by an intensely fluorescent envelope that coincided with the edges of the membrane pocket enclosing the bacterium (Fig. 6*A*). The part of the pocket membrane that lay directly under the rod bacteria did not fluoresce but appeared dark like the background. By focusing through partially disrupted cells, the same fluorescence pattern was observed on both upper and lower surfaces, regardless of whether the pocket membrane lay below or above the bacterial cell with respect to the direction of the incident light. Therefore, the nonfluorescence of the bottom of the pocket membrane was not a trivial result due to masking by the bacterial cell body. Insertion of a red-free barrier filter resulted in a more bluish emission, but did not alter the fluorescence pattern.

In addition to showing the surface pattern of fluorescence, cells in filipin/Ringer's exhibited a brightly fluorescent cytoplasmic collar encircling the shear zone region (Fig. 6*B*). Phase contrast microscopy showed that this fluorescent ring lay under the cell surface, and corresponded to the location of the parallel array of membranous tubules which extends from the helical Golgi apparatus to just under the shear zone membrane (Figs. 1 and 2).

Control cells treated with 1% dimethyl sulfoxide/Ringer's without filipin showed very brief fluorescence emission throughout the cytoplasm, which faded rapidly (<1 s) after the onset of ultraviolet irradiation. No honeycomb pattern of surface fluorescence resembling that observed in the presence of filipin could be detected.

Fixed cells treated with pepsin and then filipin exhibited a honeycomb fluorescence pattern indistinguishable from that of controls without enzyme. The edges of the membrane pockets were intensely fluorescent, but the invaginated membrane lying directly under the rod bacteria did not fluoresce. Because fixed cells could not be easily flattened on slides, extra-junctional regions of the plasma membrane were difficult to discern. In addition, cytoplasmic fluorescence arising from filipin binding to internal membranes was better preserved in fixed cells, obscuring somewhat the surface pattern of fluorescence. Nevertheless, the overall pattern of fluorescence was similar to that of nonfixed cells described above.

Fixed controls treated with pepsin without filipin showed only a brief faint fluorescence of the whole cell which rapidly faded to extinction in <1 s. This brief emission occurred with or without a red-free barrier filter, indicating that ultraviolet irradiation did not excite autofluorescence in glutaraldehyde-fixed cells.

DISCUSSION

Shear Zone Membrane

Filipin and digitonin bind specifically to cholesterol and related 3-β-hydroxysterols (8, 22, 28) to produce recognizable deformations at membrane sites containing free sterols (1, 23, 48, 49). By applying this cytochemical method (12, 13, 17, 34) to termite flagellates, we found that a region of plasma membrane which undergoes continual unidirectional shear exhibits a high density of sterol-specific complexes similar to the labeling pattern shown by the nonshearing plasma membrane.

A clear correlation between the amount of cholesterol experimentally inserted into membranes and the numerical density of filipin-induced lesions has been shown in cultured cells (17). Also, the response of certain membranes to filipin

agrees well with biochemical data on cholesterol content (12, 13, 25, 31). As yet, no cases of false positive responses to filipin or digitonin (i.e., induction of lesions in sterol-poor membranes) have been reported (37).

Therefore, the pronounced reaction of both shearing and nonshearing regions of the devescovinid membrane to these agents indicates that the entire extra-junctional plasma membrane has a high content of cholesterol and/or sitosterol, the major sterols present in the gut protozoa of *C. cavifrons*.

Our use of thin sections rather than freeze-fracture replicas, although allowing more convenient access to the narrow shear zone region, precluded a quantitative comparison of sterol concentration in shearing vs. nonshearing regions of the cell membrane. Nevertheless, the similarity in labeling patterns between the shearing and nonshearing areas suggests that no regional differences in sterol distribution exists within the extra-junctional plasma membrane. The absence of any detectable microheterogeneity in sterol distribution in a region of continually shearing membrane emphasizes that exhibition of fluid properties in certain regions does not require specializations in the lipid content of the bilayer (14, 39). The present results confirm our previous interpretation of membrane organization based on observations of living cells (42, 45). We noted that an additional membrane shear zone, not present in freshly-isolated cells, appeared at the base of the rotating axostylar projection when cells change shape in vitro. Moreover, the primary shear zone may be displaced posteriorly under certain in vitro conditions (45, 50). Because almost any region of the protozoan's plasma membrane is thus capable of undergoing such shear, we proposed that the entire cell membrane is potentially as fluid as the membrane between head and body, but that shearing is only manifested in certain regions due to cell shape and proximity to rotating cytoskeletal structures (42).

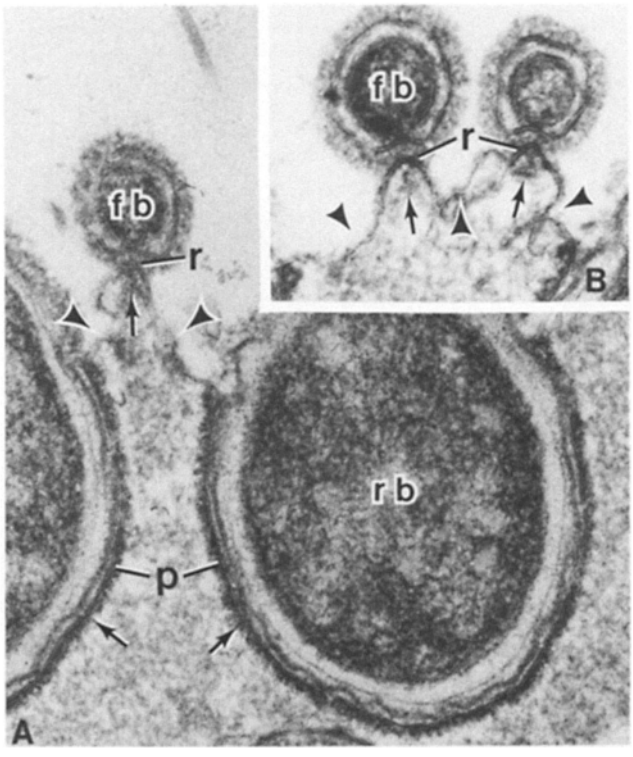

FIGURE 4 Transverse thin sections through junctional complexes with rod (*rb*) and fusiform (*fb*) bacteria on the body of digitonin-treated cells. The extra-junctional plasma membrane is grossly distorted by digitonin, with only a single leaflet of the bilayer visible (arrowheads). The pocket (*p*) and ridge (*r*) membranes are coated by dense material (arrows) and do not respond to digitonin; this demarcation is particularly evident in *B*. The bacterial membranes are unaffected by digitonin. (*A*) × 104,500; (*B*) × 111,000.

With regard to the question of membrane-motility coupling, it is not necessary to postulate specific linkages between the plasma membrane and the cytoskeleton to explain the rotational movements of the membrane observed here (42). Most of the anterior cytoplasm and organelles are firmly attached to the rotating axostyle, and are turned passively by it. Therefore, the plasma membrane of the head may simply follow the movements of the underlying cytoplasm by viscous coupling, rather than being driven by specific connections between cytoskeletal elements and membrane proteins, for example. Better evidence for this view comes from rotation of the plasma membrane surrounding the caudal projection of the axostyle that forms as cells change shape in vitro. Here, membrane that in vivo had been turning counterclockwise with the body cytoplasm (i.e., opposite to the rotation direction of the head and axostyle) rotates clockwise once it becomes closely applied to the axostylar projection. Therefore, close proximity to rotating cytoskeletal structures is all that is required to account for membrane movements in this system.

Although there appears to be nothing unique or interesting about the lipid bilayer in the shearing region of this remarkable unicellular wheel, fascinating problems arise on the cytoplasmic side of the shear zone. The ability of the red cell membrane to sustain in-plane shear and to exhibit elastic deformations, for example, is attributed not to the lipid components, as already discussed, but to the associated cytoskeletal matrix underlying the bilayer (14). Indeed, most cell membranes are believed to have such associated material that contributes to the surface shear resistance or solid character of the membrane by providing structural rigidity and support (14). The intriguing area for future research lies in how this potential barrier to shear deformation is overcome in the case of the devescovinid.

Finally, what is the functional significance of the high sterol content of both shearing and nonshearing regions of the extra-junctional plasma membrane? Sterols are known to modify membrane fluidity by condensing fluid lipids while fluidizing solid ones, leading to a state of intermediate fluidity with suppression of temperature-induced lipid phase transitions (10, 19). A high sterol concentration would ensure that the shear zone membrane remains sufficiently fluid to permit head rotation—whatever the reason for this motility—over a wide range of environmental temperatures. However, protozoa living in the hindgut of colonial insects from the neotropics would probably never experience extreme fluctuations in temperature, so other explanations must be sought.

Bacterial Junctions and Limitations of Cytochemical Probes

The absence of filipin- and digitonin-induced perturbations from the junctional membranes of cells treated conventionally with these agents could at first sight be taken to indicate that these regions of the plasma membrane are sterol-poor. However, other explanations are also plausible. Most membrane domains showing negative responses to filipin or digitonin, such as coated pits and vesicles, intercellular junctions, cell-

substrate contact sites, annulus and zipper regions of sperm, and the ciliary necklace, are characterized by electron-dense material or cytoplasmic filaments directly adjacent to the membrane, and/or by a high density of integral membrane proteins (4–7, 12–15, 24, 26, 29, 30, 32, 33, 35, 38). Similarly, the junctional membrane areas of the devescovinid bear a dense cytoplasmic coat, and contain a high concentration of intramembrane particles (43). These membrane-associated

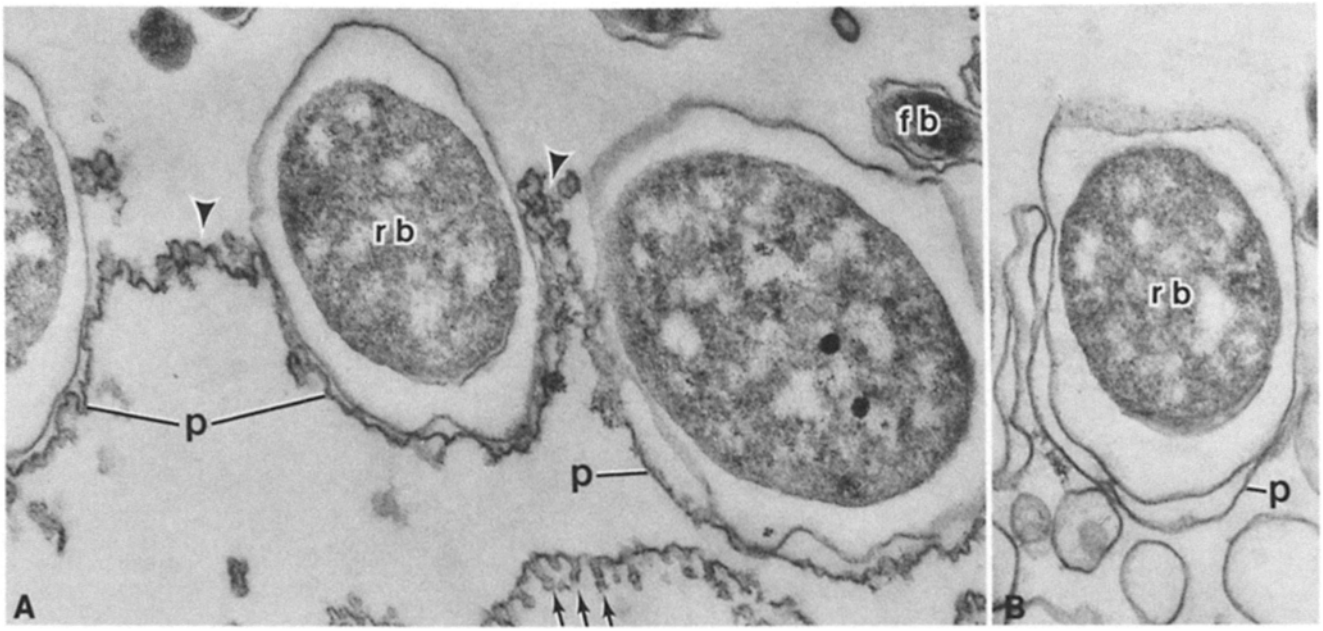

FIGURE 5 Transverse thin sections through junctional complexes of cells incubated in pepsin before labeling. Pepsin has removed the dense cytoplasmic coat from the junctional membranes, and digested most of the cell cytoplasm. Fusiform bacteria (*fb*) are detached from the surface ridges that are no longer distinguishable from the extra-junctional plasma membrane. (*A*) Filipin-treated cell. Filipin corrugates the denuded pocket membranes (*p*) enclosing the rod bacteria (*rb*), as well as the former membrane ridges, thus demonstrating the presence of filipin-sterol complexes in the junctional membrane regions. Comparison of the three membrane pockets visible here shows that the degree of filipin labeling varies, but is usually not as strong as in the extra-junctional regions (arrowheads). Note the characteristic filipin-induced protuberances (arrows) in an internalized part of the plasma membrane. The bacterial membranes are not affected by filipin. (*B*) Control without filipin. The devescovinid pocket membrane (*p*) appears uniformly smooth and well preserved. × 61,900 (*A*); × 57,500 (*B*).

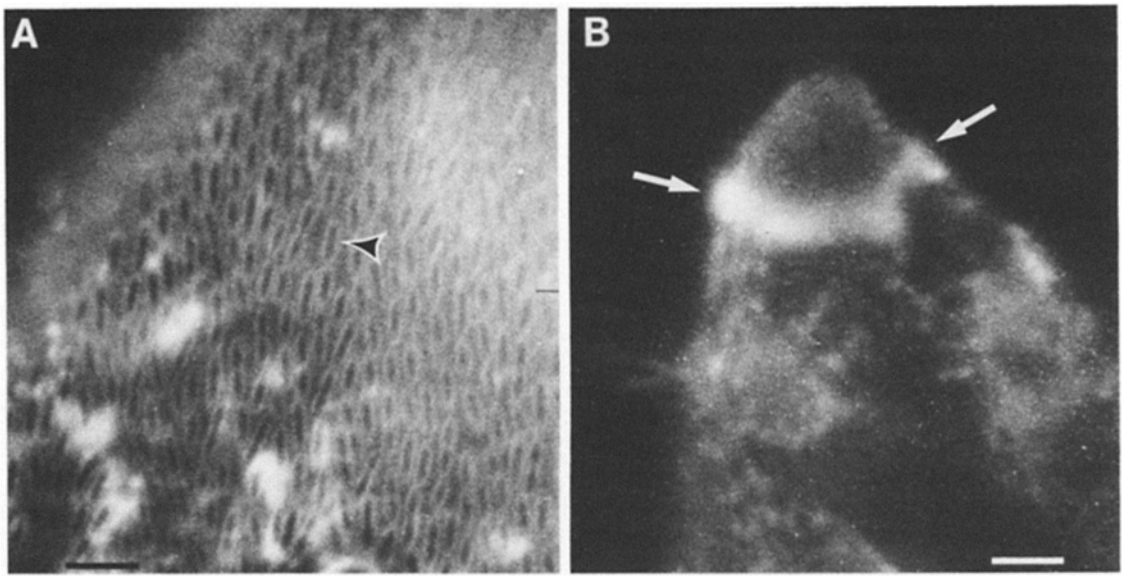

FIGURE 6 Fluorescence micrographs of cells in filipin/Ringer's. (*A*) Honeycomb fluorescence pattern of the body surface. Each rod bacterium is surrounded by a brightly fluorescent envelope (arrowhead) representing the sides of the membrane pocket holding the bacterium. The pocket membrane directly under the rod bacteria appears dark like the background, while the surface membrane between the bacteria is weakly fluorescent. (*B*) Anterior part of cell. An intensely fluorescent cytoplasmic collar encircles the neck of the cell (arrows), corresponding to the location of densely-packed membranous tubules that extend from the Golgi to the shear zone membrane (cf. Figs. 1 and 2). The surface fluorescence pattern is not in focus here. Bar, 5 μm. Ultraviolet excitation, × 100/1.3 NA objective.

proteins may limit the ability of filipin to penetrate the membrane, and hence to interact with sterols present in the bilayer (5, 12, 13, 38). Alternatively, membrane densities or membrane-cytoskeleton linkages may hinder the aggregation of primary filipin/sterol complexes necessary for formation of visible membrane lesions (9, 34). It is also possible that closely-packed protein subunits within or directly under the membrane could confer sufficient rigidity, especially after glutaraldehyde cross-linking, to prevent deformation of the membrane even in the presence of filipin-sterol aggregates (5, 13, 38).

We used digitonin to confirm the insensitivity of junctional membranes to filipin, because it was recently reported that a membrane domain that is known biochemically to contain cholesterol is not perturbed by filipin, but does respond to digitonin (38). In this case, therefore, digitonin was less susceptible to the local physical constraints that operate on filipin action.

In an attempt to remove a possible barrier to filipin action, we pretreated cells with pepsin. Feltkamp and van der Waerden (15) recently found that retroviral membranes that do not react with filipin become sensitive to this agent after removal of an electron-dense coat by pepsin degradation. Our finding that pepsin treatment resulted in a positive reaction of the pocket membrane to filipin suggests that sterols may be present in these regions, but that membrane-associated proteins, visible as an electron-dense coat, prevent the formation of filipin-induced membrane lesions, and thus give rise to a spurious negative response.

However, it is also possible that protease digestion removes a barrier to lipid diffusion and allows sterols to flow into a previously restricted membrane area. It is known that lipids can still move within the membrane bilayer after glutaraldehyde fixation (18, 20, 34).

The fluorescence properties of filipin (2, 3, 11, 27, 36) provided an independent means to distinguish between these possibilities. For example, in certain cases where filipin is unable to cause ultrastructural lesions in a cholesterol-containing membrane domain, it can still bind to cholesterol to produce fluorescence (6, 16).

The brightly fluorescent halo observed around each rod bacterium probably represents the sides of the membrane pockets; assuming that these regions have a sterol content similar to that of the weakly fluorescent extra-junctional membrane, then the sides of the pockets would be expected to appear brighter by virtue of being viewed end-on. It thus seems likely that the absence of filipin-induced deformations in the junctional membranes—at least in the sides of the pockets—is not due to a low sterol content, but to local physical constraints that prevented deformation of the membrane in response to filipin-sterol interaction.

What, then, is the reason for the absence of fluorescence at the bottom of the pocket membrane directly under the rod bacteria? This result could be due to a low sterol content in this region of the pocket membrane. However, evidence against such microheterogeneity in sterol distribution within the junctional membrane comes from our finding that the bottom of the pocket membrane remains nonfluorescent even after pepsin treatment, despite the presence of sterol in this region as shown by electron microscopic labeling. The lack of fluorescence, like the negative ultrastructural response to filipin of nonproteolysed junctional membranes, is apparently not a sufficient criterion for ruling out the presence of sterols in a membrane area.

Thus, the nonfluorescence of the bottom of the pocket membrane may be due to some local constraint that prevented filipin from binding to sterols, or to a masking or quenching (11) of fluorescence in this region. Simple masking of fluorescent excitation or emission by the bacterial body itself is ruled out on the basis of our observation that the bottom of the pocket was nonfluorescent regardless of from which side it was viewed. Other possible reasons for the negative fluorescence response in this region are currently being investigated.

In conclusion, we have employed a combination of techniques to address some of the problems associated with the use of cytochemical probes such as filipin for mapping topographical variations in membrane sterol content. By correlating results of enzymatic removal of membrane-associated proteins with fluorescence microscopy of filipin-sterol interaction, we found that neither the absence of filipin-induced perturbations nor the lack of filipin-produced fluorescence in a membrane region is sufficient by itself to demonstrate a low sterol concentration. Therefore, the distribution of filipin-sterol complexes in conventionally-treated membranes may not reflect actual heterogeneity in membrane sterol content. As emphasized by others (5, 6, 15, 35, 38), reports of sterol heterogeneity based on negative responses to filipin and/or digitonin, particularly in membrane domains characterized by closely associated proteins, should be interpreted with caution.

We thank Dr. R. L. Conner, Bryn Mawr College, Bryn Mawr, Pennsylvania, for performing the sterol analysis, and Mr. Philip H. Presley, Carl Zeiss Inc., for helpful advice on interpretation of fluorescence images.

This work was supported by National Institutes of Health Grant GM 27903.

Received for publication 14 March 1983, and in revised form 17 June 1983.

Note Added in Proof: In a recent critical evaluation of the use of cytochemical probes for mapping membrane sterol distribution, Severs and Robenek (*Biochem. Biophys. Acta*, 1983, 737:373–408) have presented convincing evidence that negative responses of certain membrane domains to filipin are due solely to the presence of membrane-associated proteins and not to low sterol content, supporting the interpretation of our results given above.

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