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MEMBRANE-ASSOCIATED COMPONENTS OF THE BACTERIAL FLAGELLAR APPARATUS

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Summary

At the position of insertion of the flagellum into the Gram-negative bacterial cell envelope, a specialized membrane differentiation has been observed by electron microscopy. This structure, termed concentric membrane rings, is harboured on the under-side of the outer membrane of *Spirillum serpens*, and forms a plate-like array of up to seven rings (diameter 90 nm) and an interior supporting collar. The concentric membrane rings are sensitive to proteolytic digestion, but are lysozyme and phospholipase resistant. The structures are disrupted by ionic detergents, yet resistant to the action of non-ionic detergents. A model integrating the basal organelle of the bacterial flagellum and the outer membrane of the cell wall is presented.

Introduction

Much of the present understanding of the structural arrangement of the insertion of the flagellum into the bacterial cell envelope has been derived from the isolation of this organelle [1]. Three morphologically recognizable components of the flagellum may be distinguished: the filament of about 15 nm in diameter and extending up to several μm in length; a serologically distinct hook which is generally set off at an angle; and a basal complex first recognized in thin sections as a specialized structure in the polar cap of *Spirillum serpens* [2]. The retention of membrane and wall components confused the interpretation of the structure at the insertion site, which used to be called a basal body. The dilemma was partially resolved by removal of the two membranes that insert into the basal complex, leaving it more or less naked for study in negative stains [3]. The basal organelle for flagella of Gram-negative bacteria was further resolved into two pairs of stacked discs of outer diameter 22.5 nm and inner diameter 10 nm. Each of these discs was presumed to link with a corresponding layer in the Gram-negative cell envelope, thus defining the L, P, S, and M discs.

The L ring was positioned as inserting directly into the outer membrane, the P ring into the peptidoglycan, the S ring was superficial to the cytoplasmic membrane, while the M ring was in intimate association with the plasma membrane. Connecting the four rings and attaching to the hook was a central rod of 7 nm diameter, acting as a rigid component at the basal end of the flagellum. This whole basal complex, although well substantiated, is conceptually misleading because the process of cleaning the complex may remove neighbouring structures or associations of macromolecules residing in the membranes.

While these studies have been of importance in stimulating thoughts about the mechanism of motility and chemotaxis in bacteria, anatomical discrepancies are evident in comparing models and assessing structural associations in a variety of preparations for electron microscopy. Evidence for the anatomical association of the four discs thus far has been indirect.

We have explored the possibility that there are specialized differentiations of membrane lateral to the contact between basal organelle and membranes, which might ultimately contribute to functional aspects of the flagellar apparatus. This paper describes "concentric membrane rings", a previously unresolved component of the bacterial flagellar apparatus in the bipolar, multitrichously flagellated Gram-negative bacterium *Sp. serpens*, and attempts to clarify the associations of this structure.

Materials and Methods

Bacterium, medium, and growth conditions. Sp. serpens VHL was obtained from the Culture Collection of the University of Western Ontario (U.W.O. No. 368). Previous work has described the cell envelope of this organism [4] and it is devoid of a superficial protein layer; this strain therefore displays a typical Gram-negative cell envelope as observed by electron microscopy [5]. It was grown in liquid medium consisting of 1.0 g/l yeast extract (Difco), 1.0 g/l Bacto-tryptone (Difco), 0.5 g/l anhydrous sodium acetate, 0.25 g/l $MgSO_4$, pH adjusted to 7.6. After autoclaving, $CaCl_2$ was added to 0.25 g/l. The generation time of *Sp. serpens* when grown at 30° C in a reciprocal shaking water bath (New Brunswick) at 80 rev./min or in a rotary shaker-incubator (Psychotherm) was 80 min.

Preparation of spheroplasts. Cells grown in 1 l of culture medium in a 6-l flask were harvested in mid-logarithmic phase at a $Klett_{54}$ reading not exceeding 75 (stationary phase, $Klett_{54} = 145$). After centrifugation (Sorvall SS-3) at room temperature for 10 min at $1085 \times g$, the cells were immediately resuspended in buffer (30° C) containing 3.3 mM Tris · HCl, pH 7.6, and 0.30 M sucrose. The absorbance of the resuspended cells (Bausch and Lomb Spectronic 20 at 660 nm) was not allowed to exceed 3.0. A stock solution of 150 mM EDTA (pH 7.6) was added to give a final concentration of 0.5 mM in the diluted cell suspension. Within 5 min, a lysozyme solution was added slowly and beneath the surface of the suspension to a final concentration of 50 μg/ml. Rapid addition of lysozyme or higher concentrations of the enzyme resulted in massive and visible clumping of the cells. The mixture was incubated at 30° C with slow stirring to avoid settling of the cells. Phase contrast microscopy showed that approx. 95% of the cells formed spheroplasts in less than 90 min,

but there was considerable lysis and the $A_{660\ nm}$ fell to 50—60% of the original value. Increasing the tonicity of the suspending medium with sucrose was only moderately effective in reducing lysis and interfered with spheroplast formation.

Separation of outer and inner membranes. The spheroplast suspension was cooled in an ice-salt bath and sonicated at 20 kcycles (M.S.E. ultrasonic power unit) in bursts of 1 min with 1-min intervals. This was continued until the absorbance reached 10% of the starting value, and required usually four such bursts. To the cleared suspension were added DNAase (10 μg/ml), RNAase (20 μg/ml), and $MgCl_2$ (final concentration 1.5 mM); the mixture was incubated for 1 h at 30°C with slow stirring. The lysate was then centrifuged (Sorvall RC-2B) at 1085 × *g* for 20 min at 4°C to remove unlysed spheroplasts and whole cells. The supernatant containing membrane vesicles was centrifuged at R_{av} = 54 500 × *g* (Beckman Model L-2 ultracentrifuge, 30 rotor) for 1 h at 4°C; the volume of suspension at this step was usually 200 ml. Membranes were gently resuspended in cold (4°C) 3.3 mM Tris · HCl, pH 7.6, 0.30 M sucrose, and again recovered by ultra centrifugation under conditions identical to those above. The washed pellets, resuspended in 1.0 ml cold 30% (w/w) sucrose in 5.0 mM EDTA, pH 7.6, were layered on an 11.5 ml linear gradient of 30—55% (w/w) sucrose also made in 5.0 mM EDTA, pH 7.6, with 0.5 ml of 60% (w/w) sucrose cushion. Membranes were centrifuged to equilibrium in 16 h using a SW 41 rotor at R_{av} = 149 800 × *g*, 4°C. Fractionation of the gradient was by upward displacement (Isco Model D density gradient fractionator) and 40 fractions of 0.35 ml were collected on ice. Each fraction was analyzed for enzymatic markers and the major peaks were identified by continuous monitoring of the effluent at A_{280nm}. Membranes were recovered from pooled fractions as described above, twice washed in distilled water, resuspended in identical volumes of not more than 1.0 ml of distilled water, and assayed for total carbohydrate, heptose, protein, and organic phosphorous. This procedure is similar to that described for the separation of inner and outer membranes from *Escherichia coli* [6] as further elaborated by Osborn et al. [7] for *Salmonella typhimurium.* Inherent differences in membranes derived from *Sp. serpens*, however, necessitated introduction of several modifications in order to achieve good separation (see Results).

Enzymatic digestion of membrane preparations. Aliquots of membranes from a region of the sucrose density gradient enriched in concentric membrane rings were incubated at 37°C in the presence of three different enzymes. At intervals up to 11 h, samples were withdrawn and prepared for electron microscopy. Chloramphenicol was added at 10 μg/ml when prolonged digestion was required. The enzyme: envelope proteins ratio was maintained at 10 : 1 as recommended by Mescher et al. [8]. Myxobacter AL-1 protease, a homogeneous preparation known to cause lysis of whole cells of *Sp. serpens* (ref. 9, kindly supplied by R.S. Wolfe, University of Illinois) diphenyl carbamyl chloride (DCC)-inhibited trypsin, and phospholipase C were used in this study.

Incubation with chaotropic agents. Dissociative effects of several different agents on outer membrane vesicle preparations were examined by electron microscopy. A 50 μl aliquot of purified membranes was dialyzed against 2000 ml of 5.0 mM EDTA, pH 7.6, for 20 h at 4°C to demonstrate the action of a

metal-chelating agent. Alternatively, membranes were incubated directly with the non-ionic detergents Triton X-100 (final concentration 1%), Sarkosyl (1%), or Brij-58 (2%), or with the ionic detergent sodium dodecyl sulphate (1%) at 37°C for 1 h. Extensive dialysis was necessary to remove the detergent prior to ultrastructural observation. In a similar manner, *n*-butanol [10] or chloroform/methanol (3 : 1, v/v) [11] were added to preparations of membranes, mixed vigorously, incubated at ambient temperature for up to 1 h, and the organic and aqueous phases withdrawn for dialysis. Aqueous phenol (80%, w/w) was also added to small volumes of membranes and incubated at either 37 or 70°C [12]; the two phases were separated and the solvent was removed before electron microscopic examination.

Chemical and enzymatic analyses. Proteins were determined by the method of Lowry et al. [13] using bovine serum albumin as standard. Total carbohydrate content was estimated by the phenol-sulphuric acid procedure of Dubois et al. [14]; when L-rhamnose was employed as standard, the limit of detection was 5 μg. The assay for heptose was that of Dische [15] as modified by Osborn [16] using D-glucoheptose as standard. Total inorganic phosphorous was measured with a sensitivity of 1 μg by the method of Chen et al. [17].

Analyses of the activity of succinate dehydrogenase and D-lactate dehydrogenase in fractions of the sucrose density gradient was carried out as follows: For succinate dehydrogenase, the reagents used were 60 mM sodium phosphate buffer, pH 7.5, 30 μg/ml 3-(4,5-dimethyl thiazolyl-2)-2,5-diphenyl tetrazolium bromide, 10 μg/ml phenazine methosulphate, 25 mM succinate, 10 mM NaCN, and 10 ml sample in a total volume of 1.0 ml. Alternatively, for lactate dehydrogenase the reaction cuvette contained all of the above reagents but substituted 2.5 mM D-lactate for succinate. The change in absorbance at 550 nm was recorded on a Gilford 2400 Spectrophotometer over an interval of 5 min, and activity expressed as ΔA/min per 0.1 ml sample.

Electron microscopy. Negatively stained preparations were routinely made by allowing a 5 μl sample to partially dry on the surface of a Formvar-coated, carbon-stabilized 200 mesh copper grid. The excess liquid was then withdrawn with bibulous paper, and a thin loopful of stain was broken over the surface of the grid. Stains were either 1% ammonium molybdate in 0.05 mM ammonium acetate, pH 7.5, or 1% potassium phosphotungstate, pH 7.5. Methods for fixation, dehydration, and embedding in Epon 812, as well as those for freeze-etching of membranes samples, were identical to those previously described [18].

Chemicals. The following enzymes were purchased from Sigma Chemical Co., St. Louis, Mo.: bovine pancreas trypsin, DCC inhibited; phospholipase C from *Clostridium welchii;* egg white lysozyme; bovine pancreas deoxy-ribonuclease I; and bovine pancreas ribonuclease, type X-A. Sources of other reagents were: disodium ethylene diaminetetraacetate, Fisher Scientific Co. (Fair Lawn, N.J.); Triton X-100 and density gradient grade sucrose, Schwarz/Mann (Orangeburg, N.J.); Sarkosyl NL-30, Geigy Chemicals (Toronto, Canada); Brij-58, Sigma; sodium dodecyl sulphate, Fisher; *n*-butanol, Mallinckrodt (Toronto, Canada); phenol, B.D.H. Chemicals (Poole, England). Other compounds obtained from Sigma included phenazine methosulphate, MTT, sodium succinate, D-lactate, and Trizma base. In constructing standard curves for the

assays given above, the reference compounds were bovine serum albumin, Calbiochem (La Jolla, Calif.); α-L-rhamnose, Singma; D-glucoheptose, Sigma; and potassium phosphate monobasic, Fisher. All chemicals were of the high grade commercially available.

Results

Initial indications of an additional component of the flagellar apparatus were obtained from negatively stained preparations of whole cells that had been lysed by sonic oscillation or by brief treatment with a proteolytic enzyme (Myxobacter AL-1 protease). At the polar cap of the cells was observed the outline of a halo lateral to the basal organelle. Were the specimen to lie flat on the grid, a collar-like disc was recognizable (Fig. 1). Brief digestion with protease resulted in the loosening of the envelope structures in the immediate vicinity of the basal complex (Fig. 2). The objective of further study was to assign this structure made up of concentric membrane-associated rings to one of the layers of the Gram-negative cell envelope: inner membrane, outer membrane, or mucopeptide.

Confirmation that this structure was associated with one of the two mem-

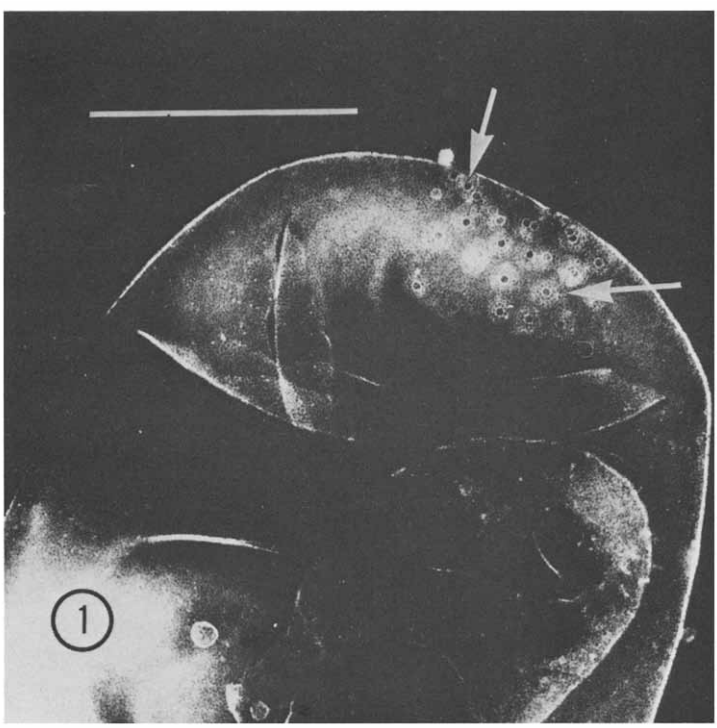


Fig. 1. The polar cap of *Sp. serpens* reveals by negative staining (1% phosphotungstic acid) an array of disc-like components (arrows) at the insertion sites of flagella. The flagellar filaments have been removed by sonic oscillation, which additionally permits penetration of the stain into the cell envelope. Bar equals 1 μm.

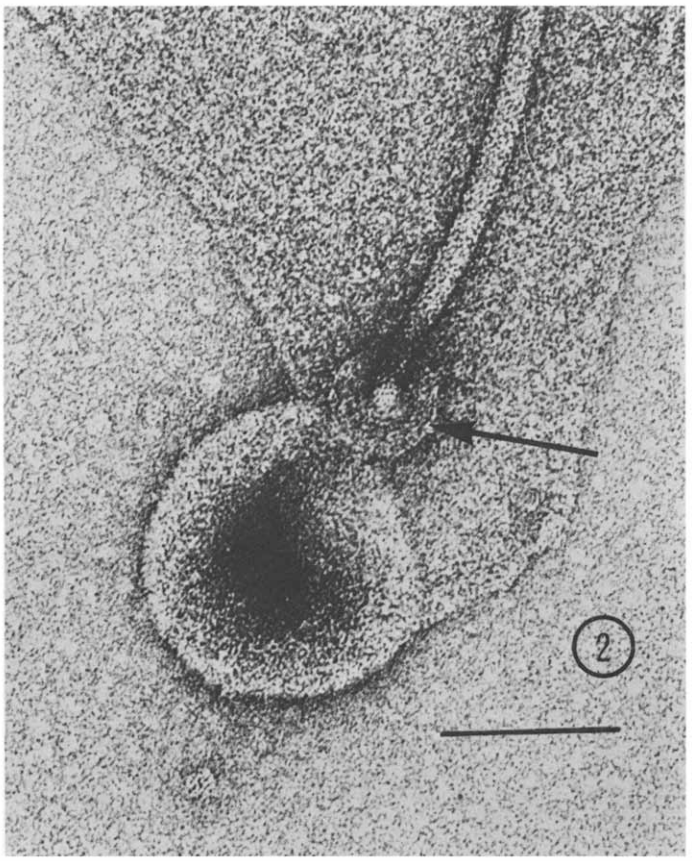


Fig. 2. Brief treatment (2 min) of whole cells of *Sp. serpens* by Myxobacter AL-1 protease yields fragments of the same discs (arrow) shown in Fig. 1, but still attached to the flagellar filament. Stained with 1% phosphotungstic acid. Bar equals 200 nm.

branes of this Gram-negative organism was obtained by lysing spheroplasts in the presence of Triton X-100 at a final concentration of 1%. When effective dialysis of the non-ionic detergent allowed electron microscopic observation, negative stain revealed that the concentric membrane rings had not been lost during spheroplasting (Fig. 3). Hence, the cells which had been treated with EDTA and lysozyme still maintained this accessory component intact.

Effective separation and identification of the two membranes of the cell envelope was needed to assign the concentric membrane rings to one or the other membrane. No reproducible separation was obtained using published procedures [6,7]. Therefore, several modifications were derived empirically, and these included conducting the spheroplasting procedure at 30°C, a temperature deemed to exceed the temperature of membrane fluidity [19], use of low ionic strength buffers in order to avoid aggregation, absence of any divalent cation in the washing or resuspending buffers, maintenance of sucrose levels at 0.30 M throughout the pre-gradient steps, and the use of unbuffered 5.0 mM EDTA, pH 7.6, for the linear sucrose density gradient of 30–55% (w/w) with

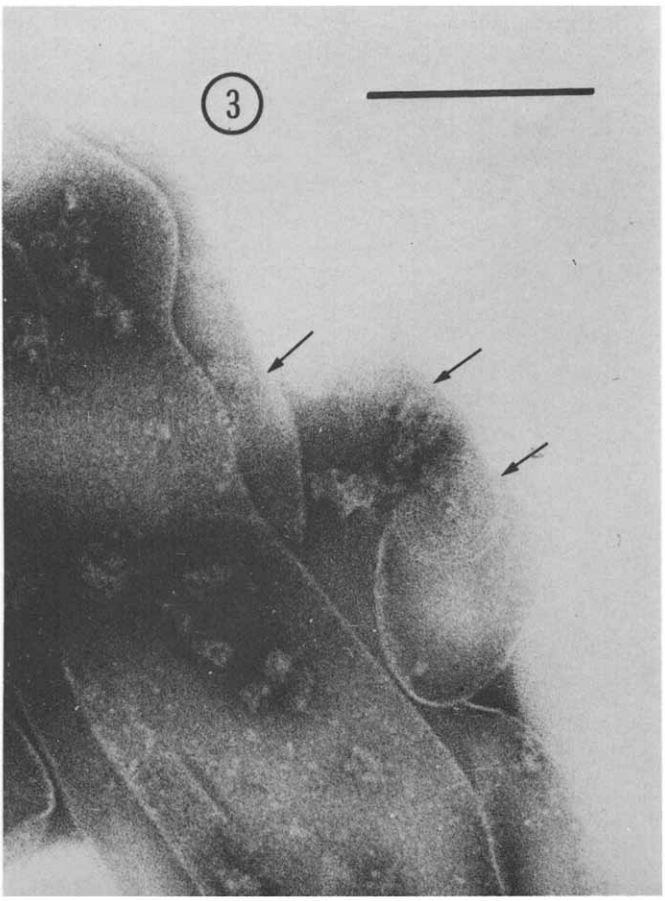


Fig. 3. Vesicles of outer membrane of *Sp. serpens* obtained by lysis of spheroplasts with 1.0% Triton X-100. Concentric membrane rings are overlying the lamellae in three discreet regions in this preparation (aarrows). Aggregates of densely staining (1% ammonium molybdate) material are cytoplasmic membrane. Bar equals 200 nm.

a 60% (w/w) sucrose cushion. The procedure is described in Materials and Methods, and in our hands yielded effective and reproducible separation of inner and outer membranes of *Sp. serpens.*

A typical elution profile from the sucrose density gradient is shown in Fig. 4. A broad band of material absorbing at 280 nm eluted near the middle of the gradient with a buoyant density of 1.17 as determined by an Abbé refractometer. Towards the bottom of the tube was a second band of heavier material close to the interface of the 55 and 60% sucrose cushion. The trough between fractions 23 and 32 in the A_{280nm} scan in Fig. 4 is indicative of a minimum of hybridized membranes. Identification of the two peaks was based upon the localization of the enzymes known to be associated with the cytoplasmic membrane and thought to be absent from the outer membrane [7]. Each fraction of the gradient was assayed for succinate dehydrogenase and D-lactate dehydrogenase, and the results are also given in Fig. 4. In both instances, the markers were predominantly localized to the material in the lighter band, which was

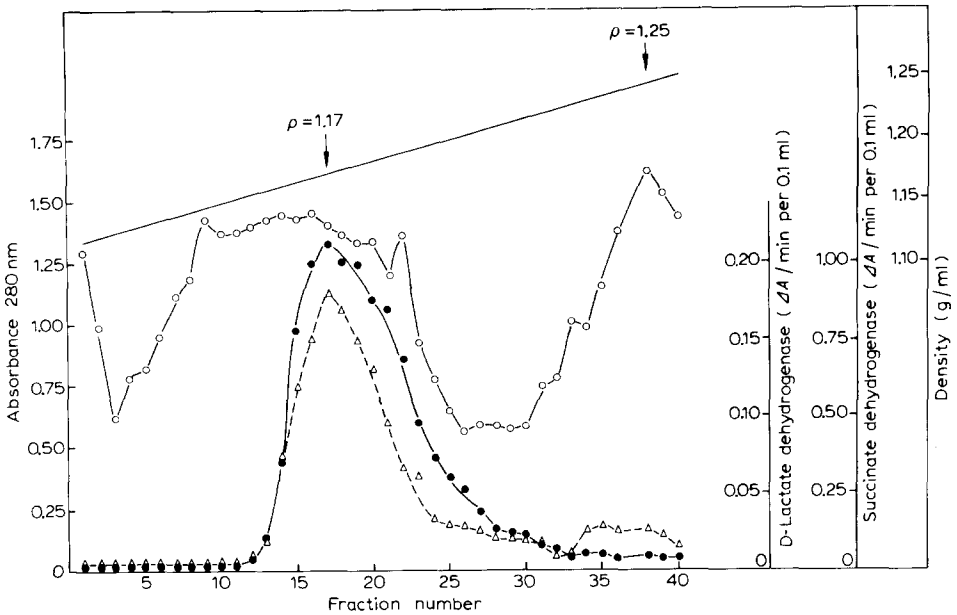


Fig. 4. Elution profile of sucrose density gradient centrifugation of total membranes preparation. The buoyant density of membranes in sucrose is shown in g/ml by the diagonal line. The effluent was scanned for absorbance at 280 nm (○———○). Enzymatic assays of individual fractions were carried out as described in the text: △———△, lactate dehydrogenase and ●———●, succinate dehydrogenase. Two peaks of membranes were identified: cytoplasmic membrane (ρ = 1.17) and outer membrane (ρ = 1.25).

therefore presumed to be inner membrane. By comparison, very little enzyme activity was observed in the heavier band, the presumptive outer membrane.

Membranes recovered from the sucrose density gradient by ultracentrifugation of pooled fractions (14—22, and 33—40) were subjected to biochemical analysis after two high speed washes in distilled water to remove residual sucrose. Results of such analysis are given in Table I. The protein content of the outer membrane was always higher than that of the inner membrane fractions, perhaps indicating a more tightly bound arrangement of these proteins

TABLE I

ANALYSIS OF MEMBRANE VESICLES RECOVERED FROM SUCROSE DENSITY GRADIENT

	Outer membrane	Inner membrane
Succinate dehydrogenase (ΔA/min/mg protein)	0.08	2.58
D-Lactate dehydrogenase (ΔA/min/mg/protein)	0.04	1.00
Protein estimation	9.8 mg/ml	5.9 mg/ml
Total carbohydrate	1.35 mg/ml	0.40 mg/ml
Ratio carbohydrate (mg/mg) protein	0.138	0.066
Total heptose	317 μg/ml	80 μg/ml
Ratio heptose (μg/ml) protein	32.4	13.6
Organic phosphorous	815 μg/ml	543 μg/ml
Ratio phosphorous (μg/mg) protein	83	93

than those of the cytoplasmic membrane which may be more readily removed by washings. The carbohydrate content per volume of membranes sample was significantly higher in the outer membrane which is known to contain the bulk of the lipopolysaccharide. However, when this value was related to mg of protein, the ratio of carbohydrate to protein was only marginally higher in outer membrane as compared to inner membrane. Similar results were obtained in comparison of the heptose content of the two fractions. Phospholipid phosphorous per volume of membranes sample was not appreciably different in the two membranes, and the ratio of phosphorous to protein was almost the same. If pooled membranes were analyzed for the two enzymatic markers selected for initial identification of the membrane fractions on sucrose density gradient, then the specific activity of succinate dehydrogenase and lactate dehydrogenase associated with the outer membrane would reflect the efficacy of separation achieved free of inner membrane. As indicated in Table I, only 3—4% of the total recoverable activity of each of these markers is found in outer membrane preparations. The procedure employed for the separation of membranes is therefore satisfactory.

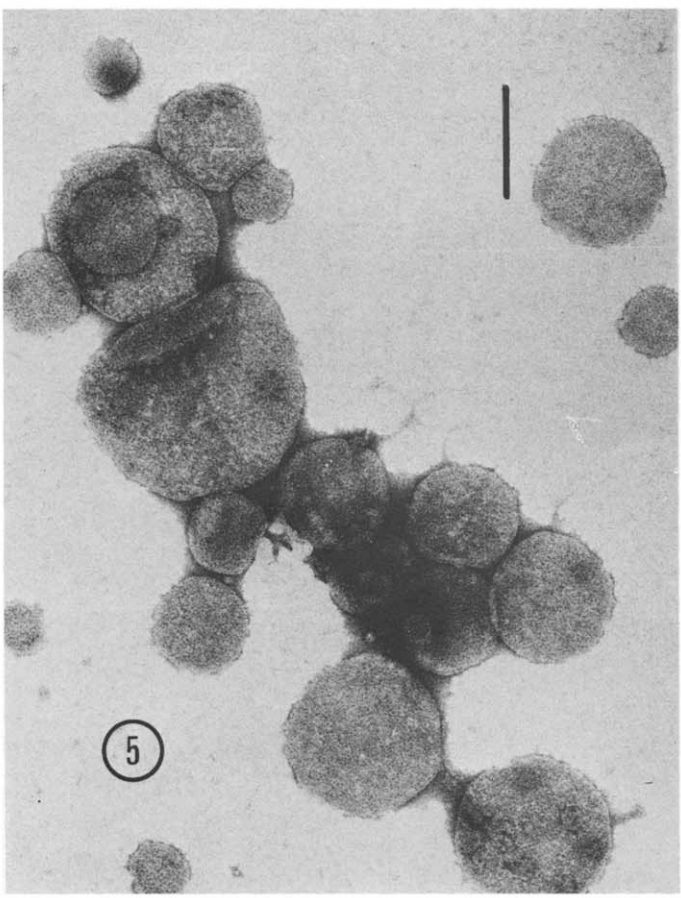


Fig. 5. Negatively stained preparation (1% ammonium molybdate) of outer membrane vesicles of *Sp. serpens* derived from the heavy region of the sucrose gradient. Bar equals 200 nm.

Examination of negatively stained preparations of the pooled membranes revealed differences in each of the two fractions. Outer membrane was composed of closed vesicles with some evidence of pebbling where the negative stain had penetrated the surface of the vesicle (Fig. 5). That these membranes were devoid of cytoplasmic contents may be confirmed by both thin section and freeze-etching (Figs. 6 and 7). Such samples revealed double track unit membranes which had sealed upon themselves and showed no evidence of material within the vesicle. Cytoplasmic membranes on the other hand were not as discretely formed into vesicles as the outer membrane preparation, and often appeared as sheets of membrane with irregular features of the surface, perhaps indicative of some state of disaggregation (Fig. 8). Any such morphological identification was dependent upon the conditions of preparation and the features may not always be recognizable immediately. Comparison of total membranes following spheroplasting but before extensive washings was often more conductive to recognizing such differences as demonstrated in Fig. 9. Here two distinctly different membranes are shown, but the identification of inner or outer membrane was ultimately dependent upon their effective separation.

Extensive scanning of the two membrane fractions was required to localize

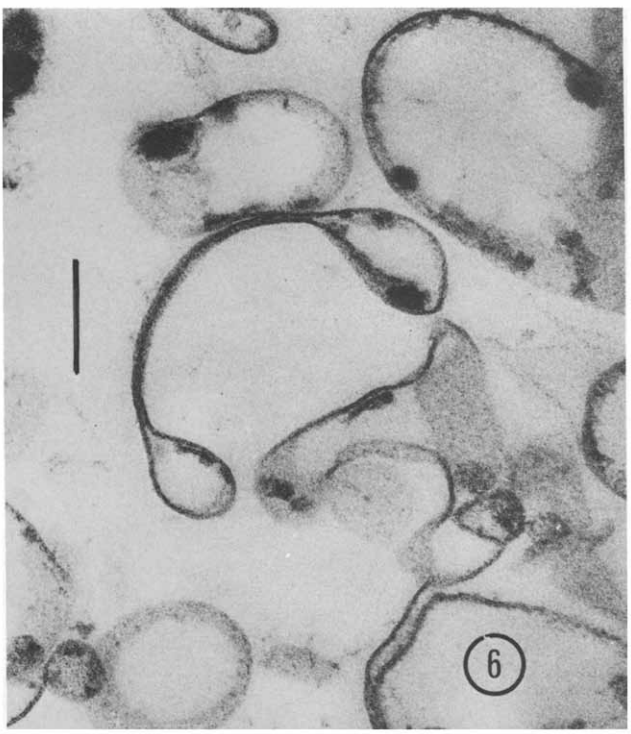


Fig. 6. Thin section of outer membrane vesicles of *Sp. serpens*. The collapsed spheres are devoid of cytoplasmic contents, and exhibit a "double track" membrane profile. Fixation employed acrolein-glutaraldehyde: the specimen was embedded in Epon 812, and stained with uranyl acetate and lead citrate. Bar indicates 200 nm.

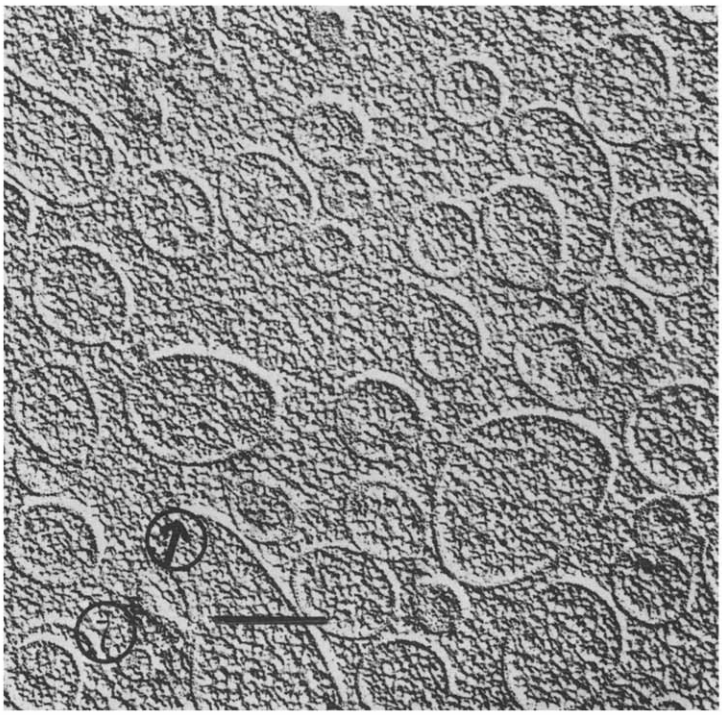


Fig. 7. Freeze-etching of outer membrane vesicles using glycerol as cryoprotectant. The membranes appear as closed spheres which have been cross cleaved to reveal unit membrane structure. The arrow indicates direction of shadowing. Bar equals 200 nm.

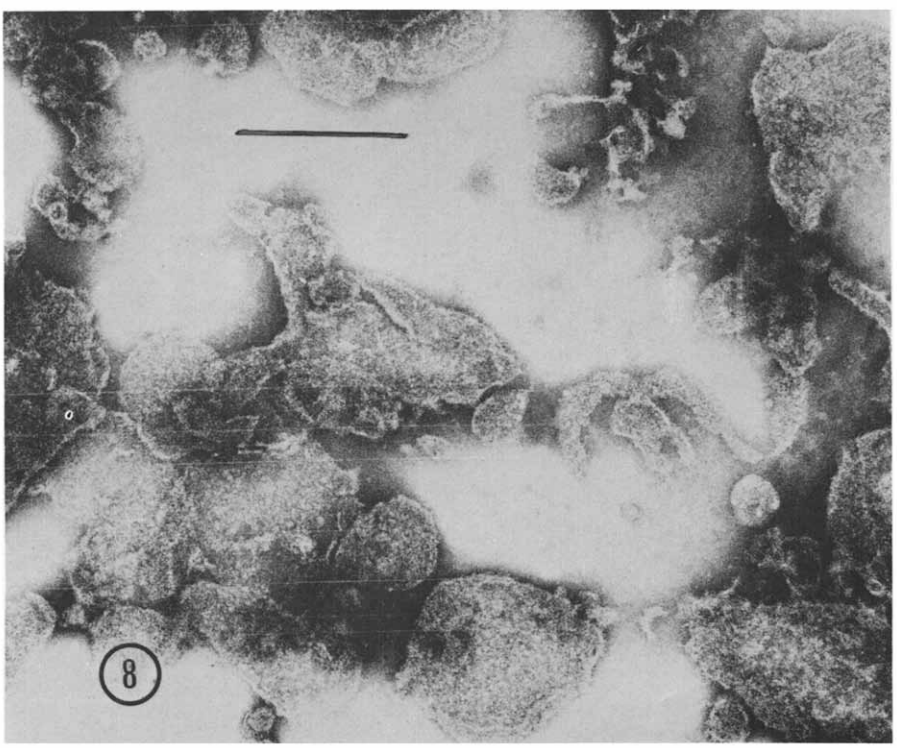


Fig. 8. Negatively stained preparation of cytoplasmic membranes harvested from the light region of the sucrose gradient. Membranes are irregular flat sheets, not discretely collapsed spheres as shown in Fig. 5. Stained with phosphotungstic acid, 1%. Bar equals 200 nm.

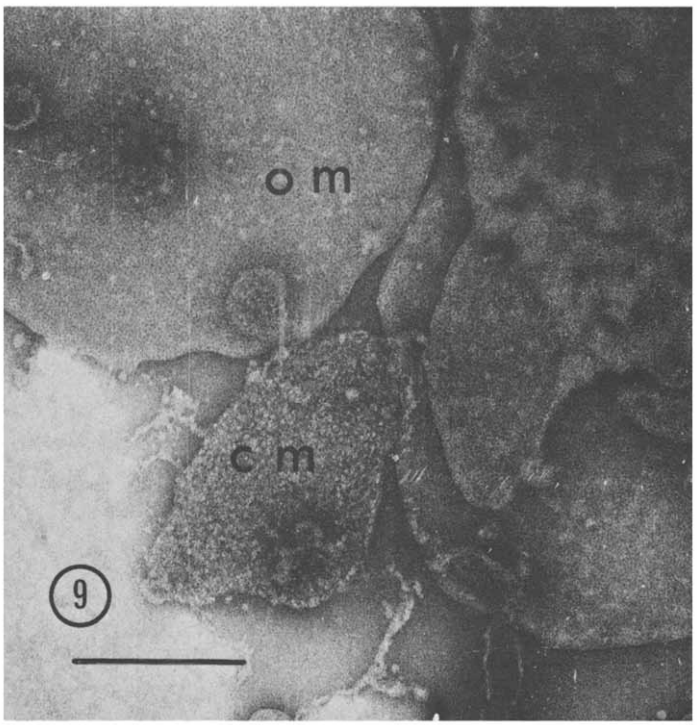
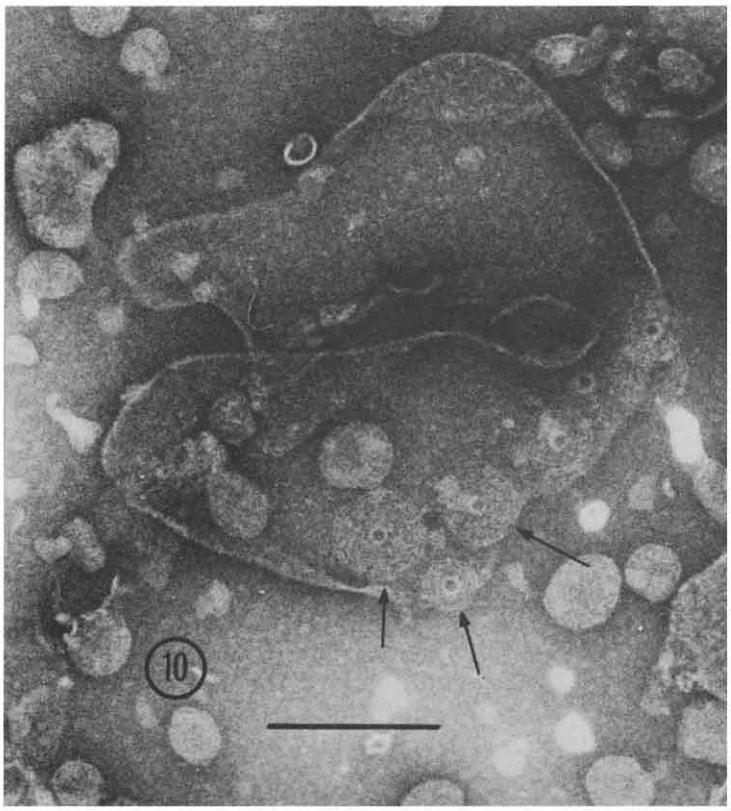
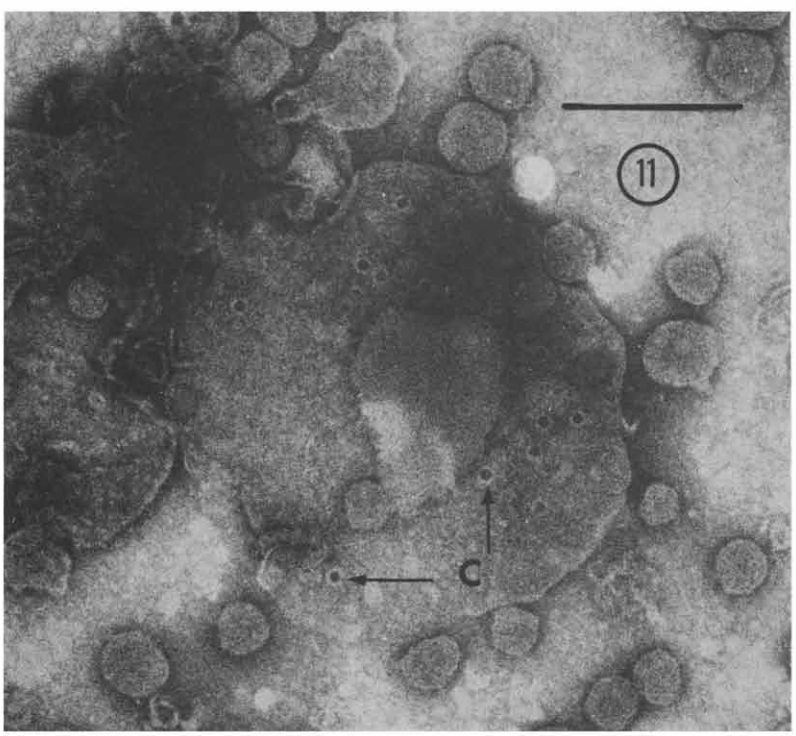


Fig. 9. Total membranes preparation of *Sp. serpens*, obtained following lysis of spheroplasts (see Materials and Methods). Cytoplasmic membrane (cm) and outer membrane (om) may be differentiated by their surface staining properties (1% ammonium molybdate). Bar equals 200 nm.

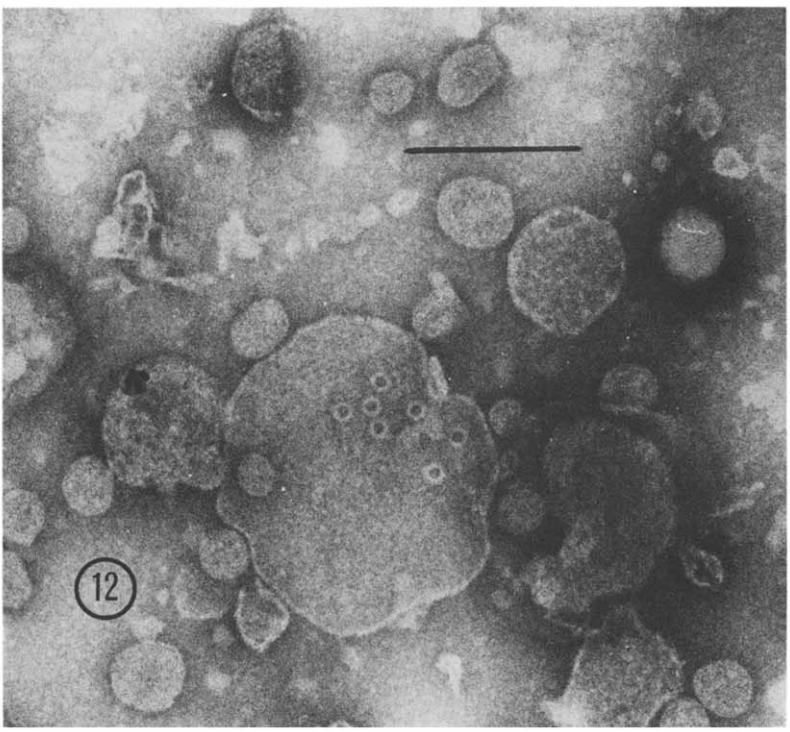
the concentric membrane rings. Outer membrane was very much enriched for these structures by comparison with inner membrane, where the concentric membrane rings were virtually absent. This agreed with the evidence cited above that Triton X-100-resistant material, demonstrated to be outer membrane [20], also possessed concentric membrane rings overlying the vesicles.

Indirect determination of the nature of these structures was undertaken in an attempt to identify some of their biochemical properties. A series of enzymatic digestions with proteases or phospholipase C was carried out on preparations of outer membrane vesicles and samples prepared for electron microscopy after various times of incubation. When vesicles were incubated with proteases for a long time, the concentric membrane rings were degraded to such an extent that the discrete rings were no longer recognizable, but an inner collar surrounding the insertion of the basal complex persisted as a remnant of the structure. If samples were taken after only brief exposure to either trypsin or AL-1 protease, then the concentric membrane rings seemed to be accentuated on the surface of the vesicle, reflecting preferential digestion of membrane proteins in the immediate vicinity of the rings. A sequence showing the effect of digestion with AL-1 protease is shown in Figs. 10, 11, and 12. The vesicle itself remained morphologically intact, but the rings of the concentric membrane rings became progressively less distinct. The inner collar released showed unusual lateral mobility over the surface of the vesicle. Where formerly the





Figs. 10, 11. For legend see opposite page.

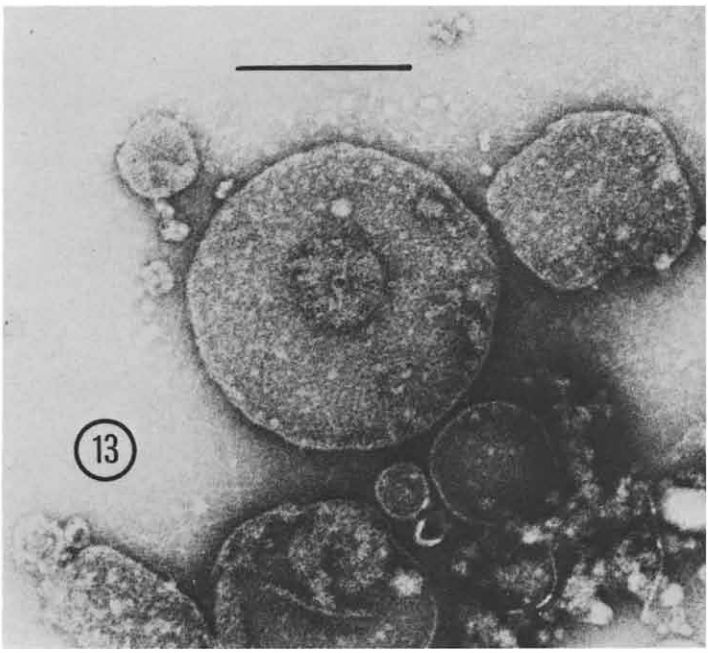


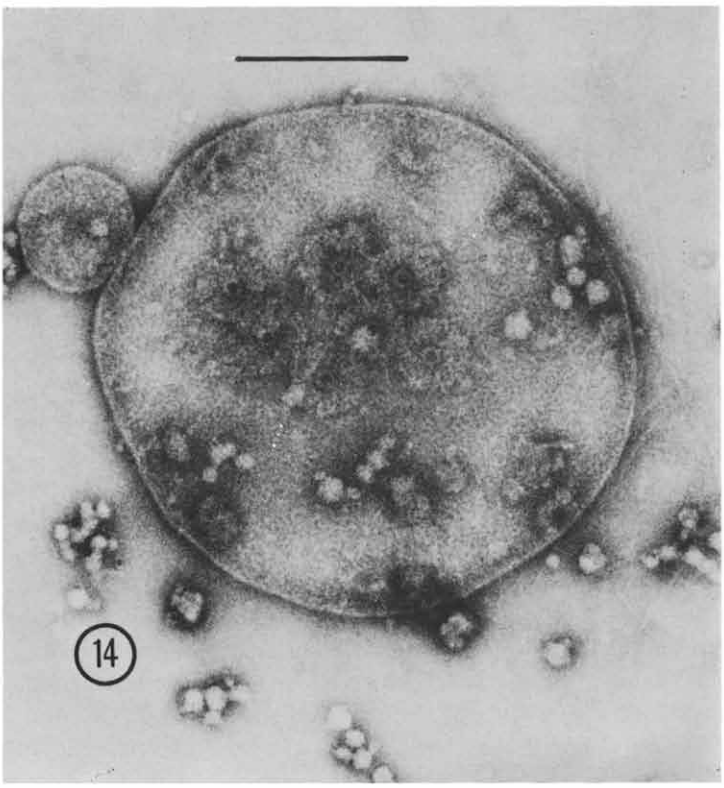
Figs. 10, 11 and 12. A series of electron micrographs showing outer membrane vesicles and concentric membrane rings after digestion with Myxobacter AL-1 protease. Some concentric membrane rings (arrows) in Fig. 10 appear accentuated on the vesicle after 15 min digestion. Prolonged incubation of vesicles for 75 min completely digests the concentric rings, but the electron translucent collar (c) is not susceptible to proteolysis (Fig. 11). Aggregation of the collar remnants (Fig. 12) indicates possible redistribution of these structures over the surface of the vesicle. Bars equals 200 nm.

dimensions of the concentric membrane rings had defined an "inter-flagellum distance" by a centre-to-centre spacing of 90 nm, it now appeared that the collar remnants had slipped into closer proximity to each other.

Not all concentric membrane rings in any given preparation were sensitive to protease digestion, however, and this was considered to be due to the presence of concentric membrane rings on the inside surface of the vesicle. Were the vesicle to evert to an inside-out orientation during the preparative procedures, such a closed sphere would show a different distribution of macromolecular components as compared to the native orientation of right-side out. It was, therefore, of interest to locate anatomically the concentric membrane rings on one or the other aspect of the bimolecular leaflet of the outer membrane. Enzymatic digestion of whole cells with trypsin or AL-1 protease, followed by electron microscopic examination for the presence of concentric membrane rings demonstrated that the structures in the intact cell were resistant to such treatments. This suggested again that the membrane was interposed, preventing the access of enzyme.

Phospholipase C was employed in similar fashion to determine whether the underlying membranes could be loosened sufficiently to allow selective release





Figs. 13 and 14. Treatment of outer membrane vesicles of *Sp. serpens* with phospholipase C. Concentric membrane rings are not digested by such treatment, but lose the integrity of the concentric rings and seem to be in relief on the vesicle surface. Stained with 1% ammonium molybdate. Bars equals 200 nm.

of the rings. Short digestions of vesicles accentuated the morphological appearance of the concentric membrane rings (Fig. 13) whereas prolonged incubations demonstrated loss of the discrete aspects of the structures without the complete digestion observed for proteases (Fig. 14).

Direct biochemical analysis of the concentric membrane rings would only be possible if the structures could be isolated in their native form. Attempts to achieve this were initiated with the use of agents disruptive to intrinsic and to extrinsic membrane proteins [21] in anticipation of being able to slip the structures off the underlying membrane. Incubation of outer membranes in 1% sodium dodecyl sulphate severely disrupted the vesicular shape of the membrane (Fig. 15) and the concentric membrane rings were not observable following such treatment. Use of non-ionic detergents Brij-58 and Sarkosyl (final concentrations of 2 and 1%, respectively) yielded vesicle preparations which were very smooth in their electron microscopic appearance (Fig. 16) but the concentric membrane rings were not recovered. Partitioning of the membrane in a two phase system of *n*-butanol/water yielded effective separation of protein into the aqueous phase and lipids into the organic phase with ox erythrocytes [10], but when the method was repeated on outer membrane preparations of *Sp. serpens*, visible aggregates were observed at the interface of the two phases and the concentric membrane rings were not observed by electron microscopy.

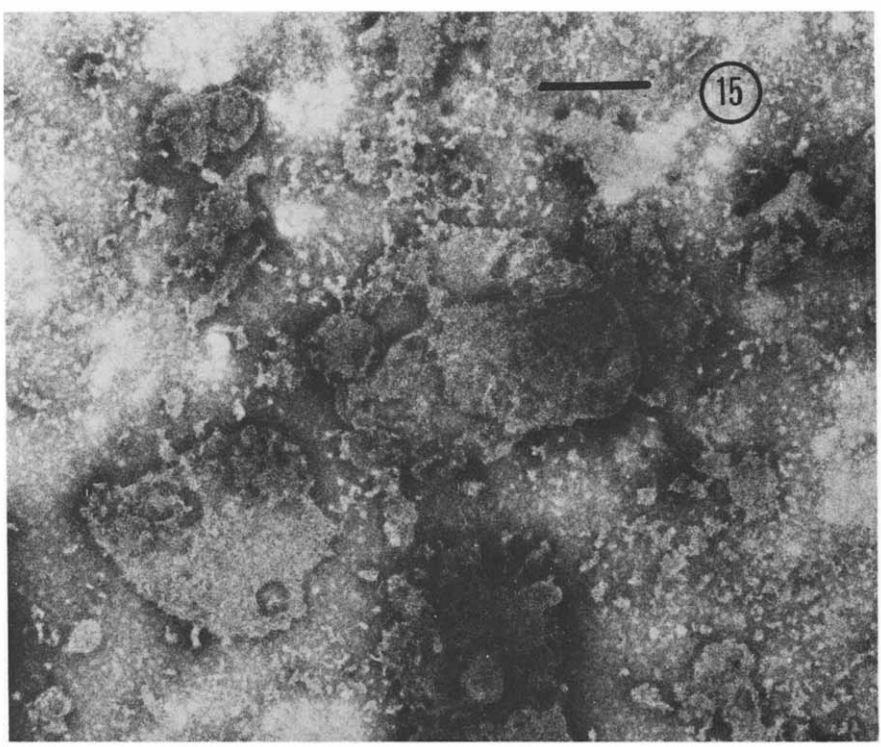


Fig. 15. Sodium dodecyl sulphate (1%) severely disrupts the vesicular morphology of outer membranes, giving rise to amorphous material. Concentric membrane rings are no longer recognizable following such treatment. Stained with 1% ammonium molybdate. Bar equals 200 nm.

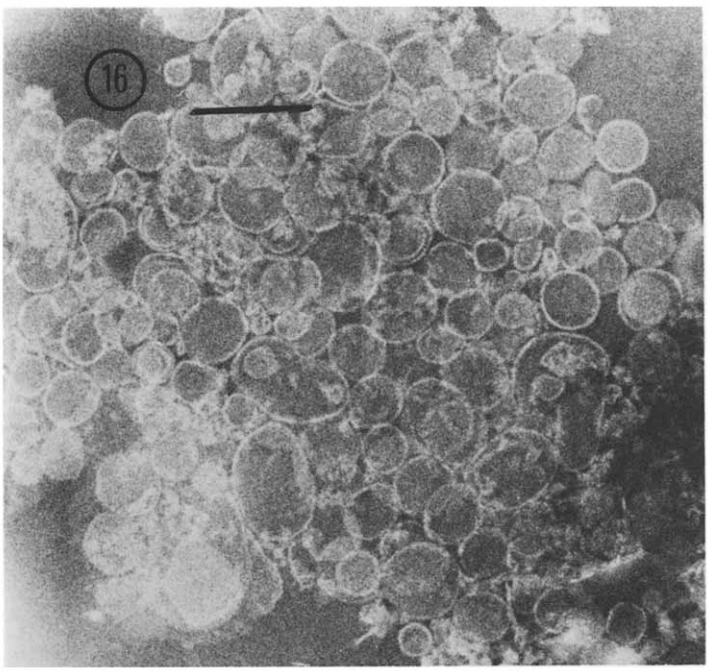


Fig. 16. Non-ionic detergents (Brij-58), when added to membranes at a concentration of 2%, caused the vesicles to reseal into smaller diameter spheres, and no concentric membrane rings were recovered. Stained with ammonium molybdate. Bar equals 200 nm.

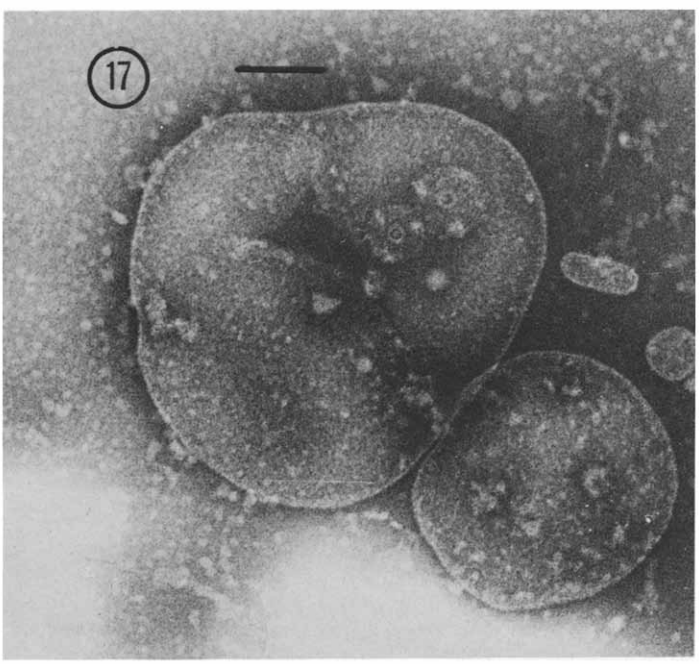
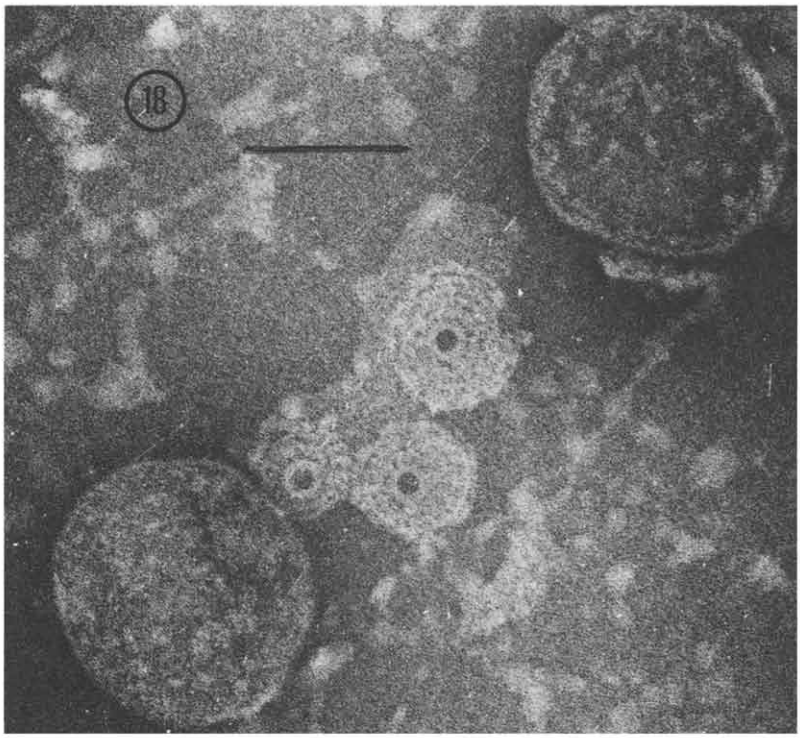
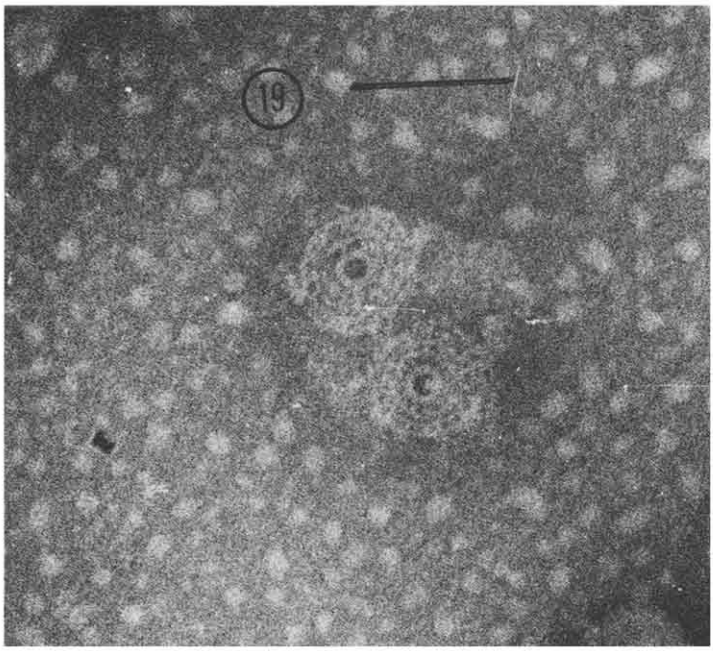


Fig. 17. For legend see opposite page.





Figs. 17, 18 and 19. Dialysis of outer membranes against 5.0 mM EDTA, pH 7.5, usually left concentric membrane rings overlying the vesicles (Fig. 17). In some instances (Figs. 18 and 19) this treatment is effective in removing the structures from the membrane, allowing them to float free in suspension. Stained with ammonium molybdate. Bars equal 100 nm.

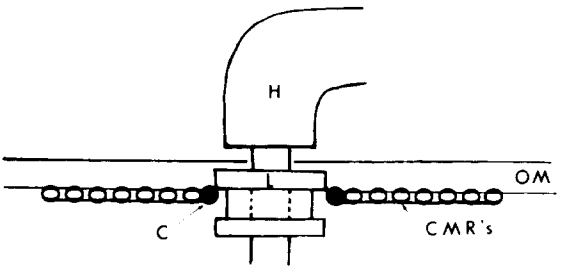


Fig. 20. A model of the anatomical association of the flagellum of *Sp. serpens* and the outer membrane (OM). Concentric membrane rings (CMR's) are shown in cross-section by oval shapes, and are represented as a plate adposed on the inner leaflet of the membrane. Circles immediately lateral to the basal organelle are the supporting inner collar (c). The uppermost (L) ring of the basal organelle inserts into the outer membrane as shown, and is connected to the ring below by a central pin. The hook region (H) of the flagellum is set off at an angle, and is attached to the basal organelle by an extension of the pin.

Both chloroform-methanol extraction and treatment of vesicles with saturated aqueous phenol gave a similar result to *n*-butanol extraction.

The most effective procedure for removal of concentric membrane rings from membrane was accomplished with the use of extensive dialysis against a large excess volume of 5.0 mM EDTA, pH 7.5. While most of the structures did not lose their intimate association with the membrane (Fig. 17) some concentric membrane rings were found to have become detached from underlying material (Figs. 18 and 19). Vesicular morphology was maintained and some substructure material was also observed as a result of dissociation of macromolecular components of the membrane. Complete dissociation under mild conditions was not achieved either with the use of EDTA alone, or by the concerted action of EDTA and Triton X-100. A decrease in the absorbance ($A_{400\,nm}$) to 88% of the original value was observed when membranes were dialyzed against EDTA under the above conditions, but subsequent addition of Triton X-100 lowered this value to only 75%.

Discussion

This communication describes the morphological recognition of an auxillary component of the bacterial flagellar apparatus of *Sp. serpens*. The structure appears as a series of up to seven concentric rings and an inner collar which form a plate of maximum diameter 90 nm in the immediate vicinity of the insertion of the flagellum into the cell envelope. That concentric membrane rings are associated solely with the flagellum was verified by their absence from any region of the lateral walls of this bipolarly flagellated Gram-negative bacterium.

Effective separation of the inner and outer membrane of this organism has been achieved, following lysis of spheroplasts and fractionation on sucrose density gradient centrifuged to equilibrium. Analysis of individual fractions on the gradient and of pooled membranes harvested from the gradient has demonstrated that isolated fractions may be obtained. Localization of the concentric membrane rings was then made by direct observation under the electron microscope of the isolated membranes, and evidence was obtained supporting the

positioning of the structures on the outer membrane. No morphological components at the former insertion of the flagellum have been detected on the cytoplasmic membranes of this organism.

Characterization of the concentric membrane rings has been carried out using indirect techniques. Proteolysis destroyed the seven rings but left an inner collar as the remnant following digestion. Phospholipase C by comparison weakened the contrast and visibility of the rings in the electron microscope, without digesting them to completion. Remarkable resistance was observed when outer membrane was extracted with non-ionic detergents such as Triton X-100.

Methods applicable to the isolation of membranes from one Gram-negative species are not necessarily pertinent to the isolation of those from another organism. In our hands, those protocols developed for *S. typhimurium* [7], for *E. coli* [6], and for *Proteus mirabilis* [23] were not entirely effective; variations from each of these procedures were selected to yield reproducible preparations.

Intrinsic differences in membrane composition undoubtedly account for the marked dissimilarity in behaviour of bacterial membranes treated with EDTA. Reports [22] of the dissociation of Mg^{2+}-depleted membranes by the concerted action of 5 mM EDTA and of 0.2% Triton X-100 into what were termed "substructures A and B" prompted investigations into the possibility of a similar phenomenon in *Sp. serpens*. No analogous behaviour was observed. It is also noteworthy that optimum separation of membranes was accomplished by including 5.0 mM EDTA in the sucrose density gradients. Leive et al. [24] have reported the loss of 50% of the lipopolysaccharide from intact cells of *E. coli* upon treatment with EDTA, with the remaining lipopolysaccharide still found within the membrane. If a similar phenomenon were applicable to *Sp. serpens*, the observation might explain why recovery of total carbohydrate is only 3-fold enriched over the value obtained for carbohydrate in the inner membrane of *Sp. serpens*. One might have anticipated an appreciably higher value since lipopolysaccharide is thought to be translocated to the outer face of the outer membrane.

In the absence of phenotypic mutants showing defects in the synthesis or assembly of the flagellum, we can only surmise on the possible role of the concentric membrane rings. No migration of flagella occurs over the surface of the cell, since all of the flagella are found in bundles at both poles. Similarly in *Bacillus subtilis*, Ryter [25] has demonstrated that there is no redistribution of these organelles once they have appeared through the cell envelope. We propose that concentric membrane rings act as a stabilizing plate about the insertion of the flagellum such that it remains rigidly inserted into the cell envelope. Alternatively, the structure may act to exclude the fluid lipid phase in which other proteins are floating [19] but which would be undesirable in juxtaposition to a semi-rigid helix. Concentric membrane rings might minimize lipid interactions in the vicinity lateral to the flagellar insertions.

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