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LA THÈSE A ÉTÉ MICROFILMÉE TELLE QUE NOUS L'AVONS REÇUE

CELL ENVELOPE ASSOCIATIONS OF BACTERIAL FLAGELLA

by

James William Coulton

Department of Bacteriology and Immunology

Submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

Faculty of Graduate Studies
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ABSTRACT

The anchoring device of the flagellum of the Gram-negative bacterium, Spirillum serpens, has been investigated. In the course of attempts to elucidate the properties of specific regions of the envelope which support the flagellum, the separation and biochemical identification of the outer, lipopolysaccharide-containing membrane and of the plasma membrane has been achieved. A specialized differentiation, termed concentric membrane rings (CMR's), was discovered to be harboured on the under-side of the outer membrane. This structure could be digested with proteases, but the outer membrane proved to be unusually resistant to detergents and chaotropic agents. It seems probable that CMR's confer structural rigidity at the flagellar insertion.

A protein responsible for CMR's was identified as follows: No components of the flagellar apparatus, including filament, hook, basal complex, or CMR's, may be observed by electron microscopy when cultures are grown at 42°C. All of these structures are present, however, in cells grown at 30°C. Autoradiography of outer membrane proteins after labelling with ^{35}S-methionine revealed the presence of four polypeptides in the membranes of cells grown at the permissive temperature. Antisera were raised against these four

candidates for CMR protein(s), and the respective gamma globulin fractions were coupled to ferritin to produce immuno-reactive conjugates. Only one of these conjugates, directed against the 72k outer membrane protein, was found to specifically label CMR's.

The rigid mucopeptide layer of the cell envelope was isolated free of contaminating membranous material. At the polar regions of the sacculus, perforations were found, indicative of the former insertions of flagella.

Electron microscopic examination of autolyzed preparations of *Spirillum* did not adequately resolve the orientation of the lower pair of rings of the basal organelle. Freeze etching, however, revealed depressions on the convex fracture face which correlated with the level of insertion of the basal organelle into this membrane. In thin sections, blebs beneath the plasma membrane were seen, which appear directly associated with the flagellar apparatus.

Matching the dimensions of the basal organelle with the dimensions of the Gram-negative cell envelope clearly necessitated revisions to currently accepted ideas concerning flagellar insertions. The structural features derived from the study of *Spirillum serpens* have been incorporated into a generalized anatomical model of cell envelope associations of bacterial flagella.

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CHAPTER I

INTRODUCTION

The property of movement and the mechanisms of motion of all forms of life are intriguing and taxing problems for biologists. Antony van Leewenhoek's descriptions (see Dobell, 1960) of little animalcules were predicated on the upwards and downwards motions of the organisms in aqueous suspension. No doubt his attention was directed as much towards their tumbling as to their morphological appearances, and one wonders whether his observations would have been as astute if the motility of the cultures had been less pronounced.

In the microbial world, frequent consideration is given to the swimming motion produced by the action of flagella. Bacterial cells, for example, may be observed in wet mounts to move individually and at random throughout aqueous medium. Their motion is an alternating sequence of intervals, interrupted by either gradual or abrupt changes in direction (Berg and Brown, 1972; Adler and Dahl, 1967). On solid or semi-solid surfaces, by comparison, other types of translocation have been recognized (Henrichsen,

1972) and these include swarming, gliding, twitching, sliding, and darting. The latter four kinds of spreading phenomena have been attributed to motive forces generated either by individual cells or a cell mass, but have no direct correlation with bacterial flagella. These observations may be important in a re-evaluation of taxonomic criteria, even as diverse types of flagellation have been a key to bacterial classification based on techniques for their specific staining (Rhodes, 1965).

Bacterial flagella were among the first biological specimens to be studied with the electron microscope (Mudd and Anderson, 1942) and from these observations the existence of this organelle as deduced by light microscopy of living cells (reviewed by Pijper, 1957) and stained bacteria (reviewed by Leifson, 1960) was confirmed. Diversity and complexity of the cytological arrangements of flagella was soon to be noted. The flagellar bundles of Spirillum serpens described by van Iterson (1947) in conjunction with C. F. Robinow, the sheathed flagellum of Bacillus brevis (DeRobertis and Franchi, 1951) and of Vibrio metchnikovii (van Iterson, 1947), and the more common peritrichous flagellation (Houwink and van Iterson, 1950) exemplified such diversity. It was generally agreed at that time that the flagellum passed through the cell wall and terminated in some structure in the cytoplasm. Such "basal granules" were seen in autolyzed cultures and were described as up to 200 nm in

diameter (Grace, 1954). Others however doubted the existence of such structures, and dismissed them as artifacts of autolysis.

A. Basal Structure of the Bacterial Flagellum

These early observations by electron microscopy were made on bacterial specimens which were metal-shadowed, and so did not provide an accurate representation of flagellar insertion into the cell envelope. But with the emergence of techniques for thin-sectioning and negative staining, new insights were possible into this unresolved problem. In 1963, Murray and Birch-Andersen described their interpretation of electron micrographs of sections of Vestopal embeddings stained with uranyl acetate. The flagella, which appeared as a polar tuft in *Spirillum serpens*, were postulated to originate as knobs just beneath the cytoplasmic membrane and pass through the cell envelope individually in a defined region. Cytoplasmic organization was recognized in fortunate sections which showed flagella passing through the cell wall and through the plasma membrane, to terminate as a small button. This bleb did not appear to have any direct association with specialized polar membrane, which could be identified as a columnar arrangement firmly attached to the plasma membrane. Polar membrane was disposed lateral to the polar cap, and not immediately beneath the region of flagellar insertion. Description of the constituent

layers of the cell envelope focused attention on the smoothness of the wall over the polar area, whereas the thin section profile usually revealed a "wavy" unit membrane. A delicate and taut dense layer, later identified as the mucopeptide (Murray, Steed, and Elson, 1965), would also be perforated by emerging flagella. All of these observations were summarized in a model which attempted to clarify the structures demonstrated in the electron micrographs (Figure 1).

In contrast with the study of thin sectioned materials which advanced the concept of flagellar insertions was the concurrent work of Glauert et al. (1963). Vibrio metchnikovii was chosen as the object of study since the flagellum is sheathed and therefore of larger diameter (30 nm) than most other peritrichous bacteria such as Escherichia coli with flagella of 15 nm diameter. Furthermore the sheath was determined as continuous with the outer membrane, having the same layered structure as this "double track" layer. Negative staining of autolyzed cells with potassium phosphotungstate showed that the flagella ended in basal structures associated with the plasma membrane. This component was described as a small disc or cup, 30 to 35 nm in diameter, with the end of the flagellum near the basal discs appearing cross-banded. This was the first indication of the complexity of the basal organelle which was to be resolved many years later.

Following Weibull's observation (1953) that in Bacillus

megaterium flagella remained attached to the naked protoplast membrane after digestion of the cell wall with lysozyme, it was generally agreed that the flagella terminated in the cytoplasmic membrane. But the question of basal bodies was still debated, and in 1966 van Iterson *et al.* addressed themselves to the fine structure of such "blepharoplasts" in *Proteus mirabilis*. Treatment of actively motile swarmers of *Proteus* with penicillin served to loosen the cell wall, thereby rendering cells osmotically fragile. The bacteria were then subjected to osmotic shock in water to release the cytoplasmic contents. Flagella seemed to emerge from rounded structures approximately 25 to 45 nm wide, as determined by thin sections. A portion of the flagellum extended between the cell wall and the plasma membrane, but the thickness of the sections as compared to the diameter of the basal granules precluded profitable study.

A complementary study (Hoeninger *et al.*, 1963) of negatively stained preparations of *Proteus* was more successful in demonstrating intact associations of basal bodies with cell envelope layers. "Brilliant dots" were described as being part of the basal granule, hooks were clearly distinguishable, and in one instance two pairs of discs were apparent. However the authors misinterpreted the micrograph, suggesting that the uppermore pair of discs was a collar of wall material and not related to the basal organelle. The lower pair was frequently seen

attached to a disc-shaped area, apparently more solid than the rest of the structure. It is apparent, with hindsight, that the "disc" was formed by cytoplasmic membrane enveloping the inner end of the complex and not necessarily an integral part of the complex. One of the chief difficulties in interpretation of the electron micrographs seemed to be the inability to obtain flagella free from membranes. While autolysis of whole cells or penicillin treatment sometimes clarified or lightened the electron density of negative stains, it was readily apparent that the presence of the complex cell envelope was hampering the resolution of the flagellar insertion structure.

Abram, Koffler, and Vater (1965) disagreed with van Iterson _et al_. on interpretation of the origin of _Proteus_ flagella. Rather than autolyzed preparations, they chose to examine ghosts of cells stored at low temperature (6 to 10°C) in which cell wall could be differentiated from cell membrane. The flagella were thought to originate from spherical structures of diameter 11 to 14 nm, associated with the cytoplasmic membrane and probably a part of this membrane. "The large bodies at the base of the flagella may not be real structural entities, but perhaps are artifacts resulting from the persistence of a part of the membrane after the rupture of the wall." This description applied even to a micrograph of an isolated flagellum and basal complex, since resolved as four stacked rings. The upper two discs were

described by Abram *et al.* as a "constricted region" where the hook was connected to the basal unit, and the lower two discs were an unresolved "spherical structure". It is interesting that these authors postulated various functions for the basal structure: a site at which flagellin may be synthesized or assembled; an anchorage site; an undefined role in motility due to known localization of energy-yielding reactions in the cytoplasmic membrane.

While the precise mode of insertion remained uncertain, the contribution of the cell wall and membranes towards flagellation and functional motility was recognized by Vaituzis and Doetsch (1965). These workers examined penicillin-induced spheroplasts of *Salmonella* under the light microscope and the electron microscope. Flagella were confined to those areas of the spheroplast where cell wall fragments persisted, and eventually disappeared as the spheroplasts aged in isotonic solution. If spheroplasts were formed from cells non-flagellated under physiologically restrictive conditions and then allowed to revert to rod forms, no flagella were assembled. But if penicillin was removed from a culture of induced spheroplasts, then the reverting rod forms generated flagella as if there had been no lesion induced by the antibiotic. These results clearly showed that the lack of a rigid cell wall or mucopeptide layer influenced the ability of a Gram-negative bacterium to assemble flagella *de novo* or to maintain motility, as Weibull had shown for

Gram-positive cells. Support was obtained for the idea (Stocker, 1956) that a rigid or semi-rigid structure such as the cell wall may be a prerequisite for a flagellum to exert its "thrust". The manipulation of the integrity of the cell wall led to speculation that the flagellin-aggregating mechanisms might be associated with the wall; the suggestion is intriguing but perhaps premature, as a more reasonable "nucleating centre" for such assembly might reside in or near the cytoplasmic membrane.

Cohen-Bazire and London (1967) recognized the clarity of the electron micrographs of Proteus basal organelle published by Abram et al. in 1965. Two pairs of discs were resolved (each pair 20 nm wide and 10 nm long), separated by a thin strand of connecting material. The total length of the structure was 30 nm. Both Abram et al. and Hoeninger et al. (1966) had interpreted these rare objects as due in part to cytoplasmic debris. But when the flagella of Rhodospirillum rubrum, R. molischianum, and R. fulvum were liberated by exposure to a cell wall lytic enzyme, Cohen-Bazire and London also found two pairs of discs closely adherent to the base of the hook region. Again membrane and wall fragments overlay the preparations, but in some instances an isolated basal organelle was observed. The dimensions of this specialized structure were given as: a narrow collar 8 to 9 nm in diameter connecting the rounded extremity of the hook to a pair of discs 25 nm wide and 14 nm thick; the upper pair of discs

was then attached by a second narrow collar to a second pair of the same dimensions. In all species of Rhodospirillum examined, the basal organelle was considered structurally homologous and of identical dimensions. A model (Figure 2) was presented to summarize not only the confirmatory findings of the two pairs of discs, but also to integrate those components relative to the cytoplasmic membrane and cell wall layers. They suggested that the flagellum was anchored to the cell envelope by the discs, all of which were positioned beneath the cytoplasmic membrane. The lowermost of the four discs was imagined to rest upon a "flagellar membrane" which in turn was connected to a "polar membrane". This model failed in two respects: (i) it required an unusually long central rod connecting the upper pair of discs to the hook, and penetrating all cell envelope layers; there was no evidence for such an extended connection; (ii) it attempted to integrate the thin section profile including "polar membrane" into the scheme by invoking a "flagellar membrane"; no evidence for those associations was presented.

Baumann et al. (1971) made detailed studies of 145 Gram-negative, flagellated, facultatively anaerobic, straight or curved rods of marine origin, and established by numerical data analysis eight groups which were separable by unrelated phenotypic traits. Six of these groups were classified under the genus Beneckea, and one was identified as Photobacterium fischeri. Members of

the former genus had single polar flagella when grown in liquid medium, but when grown on solid medium, four species became peritrichously flagellated. P. fischeri, by comparison, had flagella arranged in polar tufts. A continuation of the study of flagellation of these organisms using electron microscopy extended the information obtained from staining by Liefson's method (Allen and Baumann, 1971). Two micrographs of negatively stained polar tufts of P. fischeri clearly demonstrated basal discs, although their number and arrangement was unspecified. It is also possible to discern some membrane differentiation surrounding selected basal discs, but the authors do not identify this component. Attention was directed to "knobs" or "tubules" at the distal end of sheathed flagella in species of the genus Beneckea and P. fischeri; no explanation of their role was offered.

High resolution microscopy of three polarly flagellated bacteria showed that their flagella originated as one circular disc (25 nm diameter) at the level of the cytoplasmic membrane and showed a pair of discs attaching them to the cell wall (Vaituzis and Doetsch, 1969). Negative stains of V. metchnikovii, Sp. serpens, and P. aeruginosa were prepared from cells which had autolyzed in distilled water, leaving the lower single disc firmly attached and continuing with the plasma membrane. The distal discs were a composite pair which were either free or attached, depending upon the integrity of the

autolyzed cell wall. That the membrane disc was firmly attached contrasted with previous reports that basal structures terminated in the cytoplasm. One additional structure of the flagellar apparatus was noted, however: alkali-lysed *V. metchnikovii* showed flagella with "under side" of flagella discs, from which five or six peripheral projections were noted. Although the published micrographs are very dark, it is conceivable that these protrusions may extend into the cytoplasm.

These same authors also presented a model of the mechanism of flagellar motion, based upon circular wobbling of the disc inserted into cytoplasmic membrane. The upper discs would act as stabilizing components. The hook region would simply transmit an activating force from the basal structure to the flagellum. The result of such wobble would be a cone of revolution by an inert-rigid, or semi-rigid helix. This mechanism would be dependent on rigidity of the cell wall in the region at which a flagellum emerges through the envelope. Thus, in Gram-positive bacteria which lack the upper pair of discs, the cell wall might be sufficiently strong to impart structural rigidity. If membrane displacement were involved in motility, two mechanisms might be imagined: one would assign significance to the short radiating filaments which would act as contractile fibers, but more likely these serve as anchoring lines to the membrane; alternatively, the underlying polar membrane

might contract and relax, causing displacement of cytoplasmic membrane. This polar plate was implicated by Murray and Birch-Andersen (1963) as a source of energy for motility, and others (Keefer et al., 1969) suggested specific electron transport systems within the membrane responsible for motility. One elusive property of polar membrane, however, has been that it is not continuous over the entire polar cap; it has been observed lateral to the polar tip in thin section, and does not seem to be present in all sections of the end regions of the cells. Mention was also made of the "basal bulbs", rounded fragments of cytoplasmic membrane adhering to the flagellum, with varying diameters up to 200 nm. These bulbs were occasionally bounded by a double set of membranes, which presumably included the cell wall layer. It is difficult to determine whether the bulbs have any association with the flagellar apparatus, or represent a non-specific fragmentation of autolyzed cell membranes.

Another model for flagellar insertions was derived from a study of the fine structure of Ectothiorhodospira mobilis (Remsen et al., 1968). This photosynthetic Gram-negative vibrioid organism is motile by means of a flagellar tuft. Structural details of the flagella were determined on osmotically lysed cells (Figure 3). At the base of each flagellum was a pair of discs spaced 15 to 20 nm apart; the upper disc was 20 to 25 nm diameter and 20 nm thick, but on one occasion could be resolved

into two discs of 10 nm thickness separated by a space of 5 nm; the lower disc was always observed as a single structure, also about 20 nm in diameter and 10 nm in thickness. The basal organelle was then considered to insert into a polar disc, 40 to 50 nm diameter, by means of a central pore. Eight to ten of these polar discs were then seen as a polar plate, a large structure of up to 250 nm in diameter. All of these relationships were based on negatively stained material, where an aggregate of flagella could be construed as forming a plate. In an attempt to relate the polar membrane of E. mobilis to the polar plate, the authors suggested that the latter occupies a "gap" position left by the discontinuous polar membrane, lying on the outer surface of the plasma membrane. These arrangements, however, should not be interpreted as discrete structural entities differing in membrane composition.

In 1971, DePamphilis and Adler reported a procedure for purification of intact flagella from Escherichia coli and Bacillus subtilis, free from detectable cell wall, plasma membrane, or cytoplasmic material. The cells were first converted to spheroplasts using lysozyme and ethylenediamine tetraacetic acid (EDTA), lysed with the non-ionic detergent Triton X-100, and then flagella were precipitated by ammonium sulphate fractionation. Differential and then equilibrium centrifugation gave a single translucent band which contained 98% of the flagella in

the tube, with an overall yield of 14 to 40%. The method was developed specifically for E. coli, and many of the empirically-derived steps were critical to obtaining satisfactory yields. Electron microscopy of the preparations revealed a hook and a basal body at one end of many of the flagella; the loss of these terminal structures was considered to be low. While the microscopy showed such "clean" preparations, it should be recorded that chemical and spectral analysis revealed less than 0.15% inorganic phosphorous and 1.8 to 2.2% carbohydrate; these values would suggest some contamination by the lipopolysaccharide- (LPS) containing outer membrane, and so perhaps the electron microscopy was overly selective. Their accomplishment clearly reflected the increased understanding of the biochemical composition of the bacterial cell wall and of conditions necessary to selectively dissociate the constituent layers. The method employed was also a great advance over the simple observational approach of viewing autolyzed preparations in the electron microscope.

It was evident from all of the work describing the shapes and dimensions of the basal body that many of the variations could be ascribed to the presence of cell wall or membrane fragments which either masked the structure or showed artifacts that had no genuine association with the flagellum. The confusion was much resolved in the second paper of their series (DePamphilis and Adler, 1971b)

which described the fine structure and isolation of the hook-basal complex in *E. coli* and *B. subtilis*. High resolution electron microscopy of the intact basal complex and of partly damaged structures led to the proposal of a model of the base of the flagellum (Figure 4). Four discs were clearly identified, arranged as two pairs: an upper pair (L and P rings) appeared connected near their periphery because of a lack of penetration of negative stain; the lower pair (S and M rings) were in closer proximity to each other. All discs were named according to their presumed attachments, from "top" to "bottom": the L ring for its attachment to the lipopolysaccharide-containing membrane; the P ring for its association with the peptidoglycan layer; the S ring for "supramembrane"; and the M ring for its attachment to the cytoplasmic membrane.

A critical question as to whether an essential component of the apparatus was lost during the preparation cannot be satisfactorily answered. No flagellum has ever maintained activity *in vitro* and so the possibility exists that a fragile component at the flagellar base was destroyed in the isolation procedure. But the proposed model seems convincing for a variety of reasons: first, variations in the procedures for spheroplast formation or lysis gave the same structure; second, the basal complex appeared identical when negatively stained with phosphotungstic acid or with uranyl acetate; third, an analogous structure had been reported in a wide variety of flagellated bacteria,

despite all previous attempts having failed to isolate flagella plus basal organelle.

To determine the specific attachments of each of the L, P, S, and M rings to the cell envelope, DePamphilis and Adler (1971c) purified the outer membrane of *E. coli* and observed that the ring closest to the hook region was attached to this membrane. Confirmation of this observation was also obtained when spheroplasts were osmotically shocked: the L membrane still attached to the basal bodies via the L ring. The lowermost M ring, on the other hand, was seen by electron microscopy of lysed spheroplasts to specifically attach to the cell envelope's inner membrane. Hence the evidence for the association of these two rings' attachments was direct. The second ring from the top (P ring) was considered by DePamphilis and Adler to be in register with the peptidoglycan layer to which it was presumably attached. This postulation was made by matching the dimensions assigned to the layers of the cell envelope (dePetris, 1965, 1967) with the dimensions of the basal complex (Figure 5). The supramembrane (S) ring did not appear to have any material attached to it, and was considered not in register with any known cell envelope structure.

In assessing frequent references to granules or large cytoplasmic membranes that might be attached to the flagellar apparatus, DePamphilis and Adler dismissed such basal body structures as being artifacts from vesiculation

of fragments. Their observations indicated that flagella did not have any structure penetrating the cytoplasm, but that they terminated at the junction of M ring with plasma membrane. Due to differences in the locations of the basal body rings and their respective attachments, it was hypothesized that each ring was a chemically distinct component; the chemistry of the basal organelle was not established.

B. Structure of the Flagellar Filament

Although much of our attention has focused on the insertion of the flagellum into the bacterial cell envelope as interpreted by electron microscopy, it should be noted that the understanding of this "simple" organelle was advanced in several other directions. Among the many reviews of this subject, the following are particularly valuable: Newton and Kerridge (1965), Smith and Koffler (1971a), Doetsch (1971), Bode (1973), and Adler (1975).

The flagellum consists of three morphologically distinguishable regions: a filament external to the cell surface; a hinge region termed the hook, generally set off at an angle; and the basal complex which has already been discussed. The filament is clearly the most conspicuous of these three elements and so was readily isolated by mechanical shearing free from the cell body. Purification has generally been achieved by repeated differential centrifugations, ammonium sulphate precipi-

tation, or ion-exchange chromatography on diethylaminoethyl-(DEAE) cellulose. For a review of the isolation methods, see Smith and Koffler (1971b).

The filament is now considered to be a homopolymer of the protein flagellin, although it is difficult to exclude the possibility that loosely bound non-protein material may be released during purification. Martinez (1963) reported small amounts of carbohydrate tightly bound to flagella purified by column chromatography. Flagellins have been isolated from a large number of both peritrichously and polarly flagellated bacterial species, and are considered to have a molecular weight in the range of 33,000 to 40,000 daltons (Martinez et al., 1967). The amino acid compositions of numerous flagellins show an absence of tryptophan and half-cystine residues, a paucity of aromatic amino acids, and in some cases an unusual amino acid, ε-N-methyl lysine. Such similarity of composition undoubtedly reflects a common ancestry of this class of protein molecules. A partial amino acid sequence of the 304-residue flagellin polypeptide from Bacillus subtilis has recently been reported (Delange et al., 1973). Comparison of very limited sequence information from flagellins of Salmonella, Proteus, and B. subtilis showed remarkable homology at the carboxy terminal residues.

Many models for the arrangement of flagellin molecules into the filament have been proposed, including those for Salmonella typhimurium (Kerridge et al., 1962; Lowy and

Hanson, 1965; Finch and Klug, 1973), for Proteus vulgaris (Lowy and Hanson, 1965), and for Proteus mirabilis (Bode et al., 1972). The arrangements were originally proposed as a structure of eight rows of globules with diameter 5 nm running parallel to the axis of the filament; this information was consistent with electron microscopic and X-ray data. But small angle X-ray scattering showed that the shape of the monomeric flagellin was not spherical, but very elongated, with its largest dimension about 16 nm. This shape was maintained when the protomers were incorporated into flagella, such that a model constructed from wedge-shaped protomers would be consistent with X-ray and electron microscopic investigations. The number of rows of protomers running parallel to the cylinder axis was estimated as nine. In a model showing the longitudinal cross-section of the flagellum, Bode et al. (1972) demonstrated how the packing of protomers could give rise to V-shaped arrow tips and tail ends, a phenomenon shown many years earlier.

Finch and Klug (1973) have ~~re-examined~~ micrographs of flagella of S. typhimurium, P. fluorescens, and B. subtilis, all negatively stained with uranyl acetate. Using optical diffraction and computer-generated projections, they concluded that the number of longitudinal strands was eleven for each of the species examined. These strands would be twisted into a helix which was also hollow, in agreement with the freeze etching of a

filament which revealed the empty core (Sleytr and Glauert, 1973).

C. The Hook Region of the Bacterial Flagellum

The presence of the hook region of the flagellum has been described for many bacterial species (see review of Smith and Koffler, 1971a). The structures are usually resistant to disruption by acid, alcohol, and elevated temperatures, and so may be purified free from flagellar filaments and membrane vesicles (Dimmitt and Simon, 1971). Antisera specifically directed against a preparation of hooks from *Salmonella* was shown by Kagawa *et al.* (1973) to (i) cross react with a large number of *Salmonella* strains, including flagella-shape mutants, paralyzed (non-motile) mutants, and mutants with different H- and O-serotypes; (ii) show immunological homology with hooks of an *Escherichia coli* strain; (iii) appear serologically unrelated to *Serratia*, *Proteus*, *Aerobacter*, and *Klebsiella*; (iv) have little effect on motility when added to the medium. It was also important to note that purified hooks could not act as seeds for polymerization of flagellin, whereas *in vitro* polymerization of flagellin was shown to proceed in the presence of short fragments of the flagellar filament (Asakura *et al.*, 1964). Such a result may have been due to the severe conditions of purification, since the *in vivo* situation clearly involves the hook serving as the initiation site for flagellin assembly into the

filament.

D. Genetic Analysis of Bacterial Flagellation

A comprehensive review on the genetics of flagellar formation appeared several years ago (Iino, 1969). Interest in genetic analysis stemmed primarily from the variation in the antigenic characteristics of *Salmonella* (Joys and Stocker, 1966), but this has now been expanded to include a more comprehensive understanding of many bacteria. In the *Salmonella* system, ten *fla* cistrons within two gene clusters have been described, such that a mutation in any one of these regulatory genes from fla^+ to fla^- resulted in loss of ability to produce flagella. H_1 and H_2 are the structural genes for the protein flagellin; AH_1 and AH_2 are genes which determine the activity of their adjacent structural genes. This analysis has been carried out using the complementation behaviour of flagellar mutants by P22-mediated abortive transduction (Yamaguchi *et al*., 1972). Genes designated *mot* regulate motility; *mot* mutants are paralyzed (non-motile) despite being flagellated.

In *E. coli*, Silverman and Simon (1972, 1973) have described the selection of flagellar mutants which were assigned to three regions of the chromosome, I, II, and III. Region III included at least six cistrons, of which lesions in one cistron gave rise to "polyhooks" and in another to loss of ability to produce flagellin. Region II

was comprised of at least four cistrons; mot mutants mapped within this region. Strains carrying mutations in all of the other cistrons had no observable flagellar filament structures. Because of the multiplicity of genes for morphologically complete and functional flagella, one can only surmise that such genes might contribute to the structural complexity of the organelle and to the control mechanisms of motility (Silverman and Simon, 1974).

CHAPTER II

MATERIALS AND METHODS

A. Bacterium, Medium, and Growth Conditions

Spirillum serpens VHL was obtained from the Culture Collection of the University of Western Ontario (U.W.O. #368). Previous work has described the cell envelope of this organism (Murray, 1963). VHL, chosen as representative of *Spirillum* species, lacks the superficial protein arrays (Buckmire and Murray, 1970, 1973, and 1976) which would have added to the problems of outer membrane isolation and characterization required in this study. This strain therefore displays a typical profile of a Gram-negative cell envelope as observed by electron microscopy (Murray, Steed and Elson, 1965; dePetris, 1965 and 1967). Cells were grown in liquid medium consisting of 1.0 g/l yeast extract (Difco), 1.0 g/l Bacto-tryptone (Difco), 0.5 g/l sodium acetate anhydrous, 0.25 g/l magnesium sulphate, pH adjusted to 7.6. The generation time of *Sp. serpens* when grown at 30°C, 37°C, or 42°C in a reciprocal shaking water bath (New Brunswick) at 80 rpm or in a rotary shaker-incubator (Psychrotherm) at 100 rpm

was sixty minutes.

Spirillum volutans (Wells strain) was also examined in pure culture, as reported by Wells and Kreig (1965). This organism has a requirement for microaerophilic growth conditions, and so was grown in the same medium as Sp. serpens but in an atmosphere of 6% oxygen, 94% nitrogen, at 30°C.

B. Preparation of Spheroplasts

Sp. serpens grown in one-litre of culture medium in a six litre flask was harvested in mid-logarithmic phase of growth at a $Klett_{54}$ reading not exceeding 75 (stationary phase, $Klett_{54}$ = 145). After centrifugation (Sorvall SS-3) in a pre-warmed rotor (30°C) for ten minutes at 1085 xg, the cells were immediately resuspended in buffer (30°C) containing 3.3 mM Tris-HCl, pH 7.6 and 0.30 M sucrose. The optical density of the resuspended cells (Bausch and Lomb Spectronic 20 at 660 nm) did not exceed 3.0. A stock solution of 150 mM EDTA (sodium salt) pH 7.6 was added to give a final concentration of 0.5 mM in the diluted cell suspension. Within five minutes, a lysozyme solution of 2.0 mg/ml prepared in suspending buffer 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 approximately 95% of the cells formed spheroplasts in less than ninety minutes, but that there was considerable lysis and the $A_{660\ nm}$ fell to 50 to 60% of the original value. Increasing the tonicity of the suspending medium with sucrose was moderately effective in reducing lysis and interferred with spheroplast formation. It is important to note that all operations up to this point were conducted at 30°C; any cold shock or reduction below growth temperature was carefully avoided until completion of spheroplasting.

C. Separation of Inner and Outer Membranes

The spheroplast suspension was cooled in an ice-salt bath and sonicated at 20 KC (M.S.E. ultrasonic power unit) in bursts of one minute with one minute intervals. This was continued until the optical density reached 10% of the starting value, and required usually four such bursts. To the cleared suspension were added DNase at 10 µg/ml and RNase at 20 µg/ml (both from Sigma Chemical Co., St. Louis, Mo.) and $MgCl_2$ to a final concentration of 1.5 mM; the mixture was incubated for one hour at 30°C with slow stirring in order to reduce viscosity. Following this step, membranes were always handled at 4°C to minimize the action of endogenous phospholipases and proteases. The lysate was centrifuged (Sorvall RC-2B)

at 1085 xg for twenty minutes to remove unlysed spheroplasts and whole cells. The supernatant containing membrane vesicles was centrifuged at R_{av} = 54,500 xg (Beckman Model L-2 ultracentrifuge, 30 rotor) for one hour; the volume of suspension at this step was usually 200 ml. Pelleted membranes were gently resuspended in cold (4°C) 3.3 mM Tris-HCl, pH 7.6, 0.30 M sucrose, and again recovered by ultracentrifugation 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 to 55% (w/w) sucrose also made in 5.0 mM EDTA, pH 7.6, with 0.5 ml sucrose cushion of 60% (w/w). Membranes were then centrifuged to equilibrium in sixteen hours using a SW 41 rotor at R_{av} = 149,800 xg, 4°C.

Fractionation of the gradient was by upward displacement (Isco Model D density gradient fractionator) and forty fractions of 0.35 ml were collected on ice. Each fraction was analyzed at ambient temperature for enzymatic markers and the major peaks were identified by continuous monitoring of the effluent at $A_{280\ nm}$. Membranes were recovered from pooled fractions by ultracentrifugation, twice washed in 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 <u>Escherichia</u>

coli (Miura and Mizushima, 1968, 1969) as further elaborated by Osborn et al. (1972, 1974) 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).

D. Enzymatic Digestions of Membrane Preparations

Aliquots of membranes from a region of the sucrose density gradient of buoyant density 1.25 g/ml were incubated at 37°C in the presence of three different enzymes. At intervals up to eleven hours, 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 (µg:µg) was maintained at 1:10 as recommended by Mescher et al. (1974). Myxobacter AL-1 protease, a homogeneous preparation known to cause lysis of whole cells of Sp. serpens (Ensign and Wolfe, 1965) and kindly supplied by R. S. Wolfe, University of Illinois; DCC-inhibited trypsin from bovine pancreas (Sigma); and phospholipase-C from Clostridium welchii (Sigma) were used in this study.

E. 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 twenty hours 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 (Schwarz/Mann, Orangeburg, N.J.) at a final concentration of 1%, Sarkosyl NL-30 (Geigy Chemicals, Toronto, Canada) at 1%, or Brij-58 (Sigma) at 2%. The ionic detergent sodium dodecyl sulphate ($NaDodSO_4$, Fisher Scientific Co., Fair Lawn, N.J.) was also used at 1% to disrupt membranes. Extensive dialysis was necessary to remove the detergent prior to ultrastructural observation. In a similar manner, n-butanol (Maddy, 1966) or chloroform:methanol (3:1) (Bligh and Dyer, 1959) were added to preparations of membranes, mixed vigorously, incubated for up to one hour, and the organic and aqueous phases withdrawn for dialysis. Aqueous phenol (B.D.H. Chemicals, Poole, England) at 80% (w/w) was also added to small volumes of membranes and incubated at either 37°C or 70°C (Westphal and Jann, 1965); the two phases were separated and the solute was removed before EM examination.

F. Chemical and Enzymatic Analyses

Proteins were determined by the method of Lowry *et al.* (1951) using bovine serum albumin (Calbiochem, LaJolla, Calif.) as standard. Total carbohydrate content was estimated by the phenol-sulphuric acid procedure of

Dubois et al. (1956); when L-rhamnose (Sigma) was employed as standard, the limit of detection was 5 µg. The assay for heptose was that of Dische (1962) as modified by Osborn (1963) using d-glucoheptose (Sigma) as standard. Total inorganic phosphorous was measured with a sensitivity of 1 µg by the method of Chen et al. (1956); the reference compound was K_2HPO_4 from Fisher Scientific Co., Fair Lawn, N.J.

Analyses of the activity of succinate dehydrogenase (SDH) and d-lactate dehydrogenase (d-LDH) in fractions of the sucrose density gradient were carried out as follows: For SDH, 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 (MTT tetrazolium), 10 µg/ml phenazine methosulphate (PMS), 25 mM succinate, 10 mM sodium cyanide, and 0.10 ml sample in a total volume of 1.0 ml. Alternatively, for the d-LDH reaction, the cuvette contained all the above reagents but substituted 2.5 mM d-lactate for succinate. All chemicals for enzyme assays were obtained from Sigma. The change in absorbance at 550 nm was recorded on a Gilford 2400 Spectrophotometer over an interval of five minutes, and activity expressed as Units/0.1 ml sample. One Unit is defined as the change in absorbance at 550 nm of 1.0 per minute.

G. Preparation of Murein Sacculi

The basis for isolation of murein sacculi from Spirillum serpens and from Spirillum volutans was the elaborate procedure first described by Weidel, Frank, and Martin (1960) which was used to identify the rigid layer of the cell wall of Escherichia coli B. Stationary phase cells were harvested by centrifugation (Sorvall SS-3) for ten minutes at 1085 xg, room temperature, and twice washed in distilled water. The pelleted material was then resuspended in a solution of 4% sodium dodecyl sulphate in distilled water, and immersed in a boiling water bath for thirty minutes. The sacculi were recovered by centrifugation (Sorvall RC-2B) for fifteen minutes at 27,000 xg, 4°C, and washed six times in distilled water. The washed pellet was then extracted with an aqueous solution of 80% (w/w) phenol for two hours at ambient temperature, and again washed as described above. Finally, sacculi were incubated in 0.10 M Tris-HCl, pH 7.6, containing 250 µg/ml trypsin for twelve hours at 37°C. After three washes in distilled water, the sacculi were negatively stained and examined by electron microscopy.

H. Isolation of Intact Flagella

Intact flagella from Spirillum serpens were isolated by the procedures of DePamphilis and Adler (1971a). If all steps in their method were faithfully reproduced,

the yield of basal organelle plus hook plus filament for *Spirillum* was very low by comparison to their value of up to 40% intact flagella. Preparations of *Sp. serpens* spheroplast membranes do not display the same sensitivity to disruption by the concerted action of Triton X-100 and EDTA, as do the spheroplast membranes of *E. coli*. For this reason, membrane vesicles appeared as contaminating material in the flagellar preparations. This did not preclude, however, the finding of intact basal organelles which were suitable for accurate measurements of the fine structure of the four rings, connecting rod, hook, and filament.

I. Polyacrylamide Gel Electrophoresis

Two methods of polyacrylamide gel electrophoresis were employed: the first was that of Inouye and Guthrie (1969) for doubly radioactively labelled membrane proteins, and the second was a modified slab gel procedure, similar to the discontinuous gel system of Laemmli (1970). Since modifications to each of these procedures were introduced, the systems will be described briefly.

The composition of the gel for analysis of membrane proteins which were radioactively labelled with ^{14}C-Asp and ^{3}H-Asp was as follows: 7.5% (w/v) acrylamide (electrophoresis grade, Eastman Kodak Co., Rochester, N.Y.), 0.13% (w/v) ethylene diacrylate as cross-linking agent (Electron Microscopy Sciences, Fort Washington,

Pa.), 0.1% $NaDodSO_4$, in a buffer containing 0.10 M NaH_2PO_4/Na_2HPO_4, pH 7.1. Polymerization was initiated by the addition of 0.6 μg/ml N,N,N',N'-tetramethyl ethylenediamine (TEMED, Eastman Kodak) and 1.2 mg/ml ammonium persulphate, and was completed in the presence of fluorescent light for a minimum of one hour. The gels were prepared in tubes of 150 x 4.5 mm. Electrophoresis was carried out in the $NaDodSO_4$-phosphate buffer described above at 4 mA constant current per gel for ten hours at room temperature, using a Shandon Vokam power supply. When the tracking dye had moved to within 15 mm of the end of the tube, the run was terminated. Gels were removed from the tubes and immediately frozen in liquid nitrogen. A Joyce Loebl gel slicing apparatus was then used to section the frozen gel into approximately 140 x 1.0 mm sections, each of which was transferred to a separate liquid scintillation vial. The slices were solubilized by addition of either 1.0 ml of 1.6 M NH_4OH, 37°C, sixteen hours (Goodman and Matzura, 1971), or 0.5 ml NCS Tissue Solubilizer (Amersham-Searle), 50°C, sixteen hours. Insta-Gel (Packard) was used as the scintillation cocktail, and samples were counted in a Beckman LS-250 liquid scintillation counter.

The use of slab gels for improved resolution of bacterial proteins has been described in detail by Ames (1974). Multiple samples from different preparations may be run simultaneously, giving patterns of perfect

correspondance. When coupled with a regimen of labelling membrane proteins with ^{35}S-methionine, maximum resolving power for sensitive detection of nanogram quantities of material may be expected.

The composition of the separating gel was 7.5% (w/v) acrylamide, 0.20% (w/v) bisacrylamide (Eastman Kodak), 0.1% $NaDodSO_4$, in 0.375 M Tris-HCl, pH 8.9; the polymerizing catalysts were TEMED, 500 μg/ml, and ammonium persulphate, 500 μg/ml. The spacer gel and the finger gel were each prepared in 60 mM Tris-H_3PO_4, pH 6.7, and contained respectively 4% acrylamide, 0.11% bisacrylamide, 0.1% $NaDodSO_4$, and 20% acrylamide, 0.54% bisacrylamide, 0.1% $NaDodSO_4$. The running buffer in both the upper and lower chambers was 0.05 M Tris-glycine, pH 8.4, 0.1% $NaDodSO_4$. Electrophoresis was initiated at a constant current of 20 mA, giving a potential difference of 63 V. After four hours and fifteen minutes, the tracker dye (pyronin Y) had migrated 140 mm and the voltage had increased to 160 V. The slab was fixed in 10% trichloroacetic acid, 25% isopropanol for thirty minutes, 37°C; washed in 25% isopropanol, 10% acetic acid for thirty minutes, 37°C; and stained overnight at 37°C in a solution of 0.10% Coomassie brilliant blue (Schwarz/Mann), 25% isopropanol, 7% acetic acid. Destaining was achieved by repeated changes of 10% isopropanol, 7% acetic acid.

To prepare a gel for autoradiography, the slab was

dried under vacuum at 110°C for four hours, mounted on a cardboard form, and placed in firm contact with Kodak N-S 2T Medical X-ray film. Duration of exposure was up to twenty-one days, depending on the radioactivity of the samples initially applied. Comparison of the auto-radiograms was made from a contact print of the exposed film.

Estimations of the molecular weights of membrane proteins were made by running a series of protein standards (Weber and Osborn, 1969). The standards and their respective molecular weights included cytochrome c (12k), lysozyme (14k), trypsin (23k), ovalbumin (43k), bovine serum albumin (63k), and β-galactosidase (130k).

J. Labelling of Membrane Proteins

Two procedures were used to obtain radioactively labelled membrane proteins: *Spirillum serpens* was grown at 30°C for twenty generation times in medium supplemented with 0.8 μCi/ml L-aspartic acid (UL), specific activity 160 mCi/mmole (ICN Isotope and Nuclear Division, Irvine, Calif.), and 20 μg/ml cold carrier aspartic acid. For cells grown at 42°C, the medium contained 4.0 μCi/ml L-aspartic acid (G-^{3}H), specific activity 820 mCi/mmole (ICN), and cold carrier. When cells had reached mid-logarithmic phase of growth, they were pooled, and the procedures of spheroplast formation and separation of inner and outer membranes were followed, as presented

earlier. The membranes pooled from the sucrose density gradient were thus differentially labelled according to their growth temperature.

For comparison of membrane proteins by autoradiography, cells were grown either at 30°C or at 42°C in yeast extract-tryptone medium containing 2.5 μCi/ml ^{35}S-methionine, specific activity 265 mCi/mmole (ICN) and 20 μg/ml unlabelled carrier methionine. Each batch of cells was then separately harvested and converted to spheroplasts; membranes were isolated as previously described. The resulting preparations therefore were ^{35}S-methionine labelled membranes from cells grown at 30°C, and ^{35}S-methionine labelled membranes from cells grown at 42°C. Before loading these samples onto the slab polyacrylamide gel, the radioactivity was determined for each sample to ensure that the same number of counts was placed into adjacent wells.

K. Preparation of Antisera and Isolation of Gamma Globulin Fractions

Four samples were prepared for injection into rabbits (2.5 kg, New Zealand white, female) in order to raise specific antisera: (i) a preparation of flagellar filaments isolated by shearing of whole cells and differential centrifugation; the material was confirmed by electron microscopy to be free of contaminating membrane vesicles; (ii) a preparation of outer membrane

vesicles of Sp. serpens grown at 30°C; (iii) outer membrane vesicles from cells grown at 42°C; (iv) formalinized whole cells of Sp. serpens. The material containing up to 100 μg flagellar protein or 290 μg of membranes protein was mixed with an equal volume of Freund's complete adjuvant (Difco) and injected into the foot pad at six day intervals for four injections. After a test bleeding demonstrated the presence of precipitating antibodies, a booster was given after one month, and the animals exsanguinated. The globulins were fractionated as described below.

In lieu of using membrane vesicles as a battery of antigens, an attempt was made to be highly selective of the protein to which antisera might be raised. Hence, a preparative polyacrylamide slab gel was considerably overloaded (approximately 1600 μg outer membrane protein), and electrophoresis carried out as usual. The fixed, stained, and destained slab was then dried, and horizontal slices excised, corresponding to the desired proteins. These protein-containing gel slices were then reswollen in saline, and aliquots were injected into rabbits at weekly intervals for a total of five injections (Weintraub and Raymond, 1963). Blood was withdrawn by the marginal ear vein, allowed to clot overnight in the cold, and gamma globulin isolated as follows.

A saturated solution of ammonium sulphate was added at room temperature to whole serum to effect a final

saturation of 0.33. After stirring for two hours to avoid trapping of serum components other than gamma globulins, the suspension was centrifuged for thirty minutes at 1400 xg. The precipitate was dissolved in 0.85% saline solution to restore the original volume, and the ammonium sulphate precipitation repeated three times. The final precipitate was taken up in a minimum volume of borate-buffered saline, and exhaustively dialyzed against this solution. Additional details of the procedure are described by Campbell et al. (1964).

L. Coupling of Antibodies to Fluorescein and to Ferritin

Gamma globulin raised against isolated flagellar filaments was coupled to fluorescein by the method of Walker et al. (1971). The reaction was carried out by mixing fluorescein and protein in a ratio of 1:20 (mg:mg) at pH 9.0, and stirring overnight in the cold. The conjugate was separated from free fluorescein by gel filtration chromatography on Sephadex G-50 Medium equilibrated with 0.05 M Tris-HCl, pH 7.5, 0.5 mM EDTA. Storage of the conjugate was at 4°C.

Gamma globulin preparations raised against four outer membrane proteins eluted from a preparative polyacrylamide slab gel were separately coupled to ferritin, yielding conjugates for electron microscopy. Since ferritin of high quality is essential for optimum results, cadmium-free crystallized horse spleen ferritin

(lot 71793, Calbiochem) was further purified, according to Hsu (1967). The ferritin was then conjugated to anti-membrane protein antibodies as described by Schick and Singer (1961), using the bifunctional reagent toluene 2,4-diisothiocyanate. The conjugate was always centrifuged (10,000 xg, fifteen minutes) before use to precipitate aggregated protein.

M. Crossed Immunoelectrophoresis

The method adopted for identification of outer membrane proteins required casting a 1% (w/v) agarose gel (Sea Kem HGT(P) Agarose, Marine Colloid, Inc., Rockland, Maine) containing 0.1% Triton X-100 (Research Products Inc.) in barbital-HCl buffer, I=0.02, pH 8.6. Plates of 50 mm x 50 mm (Kodak Slide Glass Cover) which had been thoroughly washed were overlaid with 3.2 ml molten gel and allowed to harden. A well was cut and a 50 μl sample of solubilized membrane proteins was introduced. The first dimension electrophoresis was carried out at room temperature at 6 V/cm for seventy-five minutes, until a tracker dye had migrated 40 mm. The antigen-containing strip was then left intact on the slide, and the remaining agarose discarded. A second gel (2.7 ml) of the same composition as above was then cast, containing aliquots of concentrated gamma globulin raised against membrane vesicles. The second dimension electrophoresis was at right angles to the

first, and continued for fourteen to sixteen hours at 2.5 V/cm, room temperature. Gels were pressed, washed repeatedly with saline and then distilled water to remove unbound proteins, stained with Coomassie brilliant blue, and destained. Procedures for crossed immunoelectrophoresis have been extensively reviewed by Axelsen et al. (1973) and specific examples of identification of bacterial membrane proteins are found in papers by Owen and Salton (1975) and by Smyth et al. (1976).

N. Electron Microscopy

(i) Negative Staining. 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 M ammonium acetate, pH 7.5, or 1% potassium phosphotungstate, pH 7.5.

(ii) Fixation and Embedding. Samples were fixed in 2% glutaraldehyde buffered in 0.10 M sodium cacodylate, pH 7.5, for one hour at room temperature. After enrobing the material in agar blocks, the samples were postfixed with 1% osmium tetroxide and then with 1% uranyl acetate. Embedding was in Epon 812. Sections were cut with glass knives on a Reichert ultramicrotome, picked up on 200-

mesh copper grids covered with Formvar/carbon, and then stained with 1% uranyl acetate and with lead citrate prior to electron microscopic observation.

(iii) Freeze Etching. Vesicle preparations or whole cell preparations were suspended in 20% glycerol as cryoprotectant and centrifuged (27,000 xg, twenty minutes, ambient temperature) to concentrate the sample, and a portion was deposited on copper discs. The material was immediately frozen in Freon 22 and stored in liquid nitrogen. Freeze etching was carried out according to the method of Moore (1966) using a Balzers apparatus (model BA510 M, Balzers AG, Lichtenstein). After the sample was fractured, it was etched for one minute and shadowed with platinum and carbon. The replicas were washed with distilled water, concentrated H_2SO_4, and Javex, and then transferred to 200-mesh copper grids.

(iv) Microscopy. Samples prepared for electron microscopy were examined with a Phillips EM 300, operating at 60 kV, with an aperture of 50 μm. Micrographs were taken on 35 mm Kodak Fine Grain positive film.

CHAPTER III

RESULTS AND INTERPRETATION

A. Growth of *Spirillum serpens* VHL

Spirillum serpens VHL was grown in complex liquid medium at 30°C, 37°C, or 42°C. Since flagellation and motility reflect active processes, it was considered important to harvest cells which were of known physiological age. Hence, growth curves were determined for cells grown at each of the above temperatures, and a correlation was established between optical density readings and cell number. Figure 6 shows typical growth curves for cells which were grown at 37°C and which were fully motile, and for cells grown at 42°C and which were determined by phase contrast microscopy to be non-motile. Klett readings were taken at either fifteen or thirty minute intervals, and in the case of 37°C grown cells, a small sample was fixed with 0.5% formalin in saline and the cell number was determined using a Coulter counter. The figure indicates that the generation time for both cultures was sixty to sixty-five minutes, and that stionary phase was reached at a Klett reading of 150.

The cell number per ml also reached a plateau in a manner similar to the optical density measurements.

The extent of flagellation of Sp. serpens was determined by electron microscopic observation. Samples were withdrawn periodically from the culture during logarithmic phase or early stationary phase and negatively stained. The number of flagella per pole was highly variable, and could be up to ten; this number was independent of the phase of growth. Attempts were made to enrich for flagellation by selecting rapidly swimming cells from the periphery of semi-solid agar plates which were inoculated at the centre. The progeny from repeated selections initially appeared to have more flagella per cell as judged by electron microscopy, but after several weeks of sub-culturing, this enrichment was no longer evident. Variations in growth conditions were also introduced in order to obtain more highly flagellated cells; tryptose was substituted for tryptone at the same concentration; cells were aerated more or less rapidly; and growth was maintained at various temperatures. None of these conditions was successful in consistently observing, for example, ten flagella per pole in Sp. serpens. The more usual case was to find by electron microscopy a value between zero and five. Figure 7 shows a negatively stained cell with two tufts of flagella splayed from the poles. Judging by its length of 6 μm, it is probably a new-born cell, since it appears vibrioid rather than

having a sinusoidal curvature.

B. Properties of flagella from Spirillum serpens

Flagellar filaments were readily isolated from Spirillum serpens by shearing cells in a blender, followed by successive differential centrifugations at 2,500 xg, 12,100 xg, and 95,000 xg. The final pelleted material was subjected to a second cycle of differential centrifugations in order to minimize contamination by membrane fragments. Negatively stained preparations of flagella filaments were relatively free of membranous vesicles (Figure 8a); the constituent subunits are close packed and reveal no subunit structure; the polarity of assembly is evident, exemplified by arrow heads proximal to the cell surface and V-shaped wedges at the distal end of the flagellum. The diameter of the filament is 16 nm. Attached at the proximal end of some filaments is an enlarged and slightly curved hook region, but in sheared preparations there were very small numbers of hooks and no basal complex structures were observed.

Polyacrylamide slab gel electrophoresis was carried out on a protein solution of flagellar filaments which had been heat-dissociated at 60°C for twenty minutes. By electron microscopy, no intact filaments were observed after heat treatment. Flagellin migrated as a single protein band in 7.5% (w/v) acrylamide, and was shown to have a slower mobility than ovalbumin (43k) employed as a

standard in an adjacent well (Figure 8b). This would confer a molecular weight on flagellin from Sp. serpens of 45k, slightly higher than the value of 40k reported by Martinez et al. (1967).

Antisera were raised in rabbits to flagellar filaments such as those shown in Figure 8a, and the gamma globulin fraction was obtained as described in Materials and Methods. To demonstrate its specificity, the protein was coupled to fluorescein and the conjugate separated from the free fluorescein by gel filtration chromatography. The conjugate was first adsorbed with washed cells of Sp. serpens grown at 42°C, a temperature at which no components of the flagellar apparatus could be observed by electron microscopy. Antibodies specific for cell surface antigens were therefore removed by this step. After reacting the conjugate with formalin-fixed Sp. serpens, threads of fluorescence were observed at the polar regions of flagellated cells, indicating the specificity of the gamma globulin-fluorescein conjugate (Figures 9a, b). No flagella were labelled when cells were pre-treated with unlabelled gamma globulin and then reacted with conjugate (Figure 9c), or when cells were treated with fluorescein alone (Figure 9[illegible]). It is also to be noted that staining of the walls was minimal, again indicating the high specificity of the antiserum preparation for native flagella.

These experiments confirmed current concepts of the

flagellar filament of *Sp. serpens*: that it is a homopolymer of the monomeric protein flagellin, and can act as an effective immunogen to produce highly specific antisera.

Although it was possible to observe the flagellum connected to the hook region in sheared preparations, it was desirable to maintain the native association of the basal complex with the flagellum. Hence, preparative conditions were selected which would minimize the shearing proximal to the hook region, with the intention of isolating "intact flagella". Spheroplasting provided the circumstances for lysis by a non-ionic detergent (2% Triton X-100); fractionation by ammonium sulphate precipitation, and dialysis against Tris-Mg^{++} (see Materials and Methods) were sufficiently gentle to obtain intact flagella. The basal organelle (Figure 10) consisted of two pairs of discs: the upper pair (L and P; nomenclature of DePamphilis and Adler, 1971b) appeared very closely apposed, and were approximately 18 and 21 nm in diameter, respectively; the lower pair (S and M) was probably not entirely free of adhering membranes, and these remnants therefore made their width appear slightly expanded at 28 and 31 nm, respectively. The connecting rod was 10 nm in diameter, a value which will become critical in the revisions to the model of insertion of flagella into the Gram-negative cell envelope. Attention should also be directed to the regular fibrillar projections which

appeared at the base of the lowermost disc. The regularity of these four elements may provide some evidence for an attachment into cytoplasmic membrane.

C. Recognition of a Structure Auxillary to the Flagellum

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). Cells which had been twice washed in distilled water were sonicated for two x two second bursts, and the lysate was transferred to a copper grid. The grid was extensively washed with distilled water prior to negative staining because of release of cytoplasmic contents during preparation. For the enzymatic digestion of the outer membrane, approximately 10^7 cells in 1.0 ml distilled water were treated with 100 μg of AL-1 enzyme (1.0 mg/ml in 0.05 M Tris-HCl, pH 7.5) for five minutes at room temperature. This treatment with protease loosened some of the envelope structures in the immediate vicinity of the basal complex. With either sonication (Figures 11, 12) or enzymatic digestion (Figure 13), the outline of a halo lateral to the basal organelle of the flagellum was observed. If the specimen lay in the plane of wall or membrane, a collar-like disc was recognizable. It is important to note that conditions for the observation of these struc-

tures did not require the addition of compounds to the incubation medium or to the buffers which would result in their preservation, assembly, or aggregation. They were subsequently found to be very stable when subjected to mechanical handling and to the wide variety of fractionation procedures used for membranes separation. This structure, made up of concentric, membrane-associated rings (CMR's) (Figures 14, 15) was the object of further study to determine its anatomical position in the Gram-negative cell envelope.

D. Separation of Inner and Outer Membranes of *Spirillum serpens*

Confirmation that CMR's were associated with one of the two membranes was obtained by preparing spheroplasts of *Spirillum*. The method finally adopted for spheroplasting has been presented in the chapter on Methods: 0.5 mM EDTA and 50 μg/ml lysozyme were added to cells suspended at a selected optical density. Higher densities of cells ($A_{660\ nm} \doteq 12$) did not allow effective spheroplasting, nor was it advisable to plasmolyze cells in 0.75 M sucrose, or to subject cells to washings, to cold shock (4°C), or to low temperature (4°C) incubation with lysozyme. All such ideas have been mentioned in the literature (Birdsell and Cota-Robles, 1967; Miura and Mizushima, 1968; Osborn *et al.*, 1971; Witholt *et al.*, 1976) as being desirable for good spheroplasting, but

these conditions were not applicable to *Sp. serpens*. Preparations of thin sections of spheroplasts (Figure 16) showed that the cell had completely lost its helical shape and had become rounded. The rigid dense layer identified as mucopeptide was no longer evident; outer membrane had formed small vesicles on its lower surface and had peeled away from the underlying cytoplasmic membranes; discontinuities indicated lesions in the outer membrane, whereas the inner membrane remained intact and enclosing the cytoplasm. Effective spheroplasting was the critical preliminary step to separation of inner and outer membranes, and hence to structural localization of CMR's. If this process were incomplete, then hybrizdized membranes of intermediate buoyant density were obtained following sucrose density gradient centrifugation. It was also mandatory to maintain the low ionic strength of the spheroplast buffer, 3.3 mM Tris-HCl, pH 7.6.

Lysis of spheroplasts by osmotic shock gave large fragments of membrane vesicles within vesicles (Figures 17, 18). It was preferable to reduce the vesicle size prior to loading on the gradients, and so lysis was carried out by extended sonication (four minutes) which reduced the optical density of the solution to 15% of its initial value. Comparison of total membranes following spheroplasting but before repeated washings was often conducive to recognizing differences in surface characteristics, as shown in Figure 19. Here two distinctly

different membranes are shown, but the identification of inner or outer membrane was ultimately dependent upon their separation.

The composition of the sucrose density gradient was varied considerably in attempts to obtain membrane fractions as free as possible of contaminating membranes. Inclusion of mono or divalent cations (1.0 M NaCl, 1 to 10 mM Mg^{++}, 1 to 10 mM Ca^{++}) did not separate the membranes, but instead caused aggregation and co-sedimentation of the material. Even the buffers (3.3 mM Tris-HCl, pH 7.6 or 5.0 mM sodium phosphate, pH 7.5) had a deleterious effect on separation, and altered the banding profile. The procedure finally adopted was to construct a linear gradient of 30% (w/w) to 55% (w/w) sucrose in unbuffered 5.0 mM EDTA, pH adjusted to 7.5.

Analysis of the fractions recovered after isopycnic ultracentrifugation is presented in Figure 20. A broad band of material adsorbing at 280 nm eluted near the middle of the gradient, with the peak having a buoyant density (ρ) of 1.17 g/ml as determined by an Abbe refractometer. Towards the bottom of the tube was a second band of heavier material (ρ = 1.25) close to the interface of the 55% and 60% sucrose cushion. The trough between fractions 23 and 32 in the $A_{280\ nm}$ scan 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 (Osborn et al., 1971). Each fraction of the gradient was assayed for succinate dehydrogenase and d-lactate dehydrogenase, and the results are also given in Figure 20. In both instances the markers were predominantly localized to the material in the lighter band, which was therefore presumed to be inner membrane. By comparison, very little enzyme activity was observed in the heavier band, the presumptive outer membrane.

Membranes harvested from the sucrose density gradient by ultracentrifugation of pooled fractions (14 to 22, and 33 to 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 1. The protein content of the outer membrane was always higher than that of inner membrane fractions, perhaps indicating a more tightly bound arrangement of these proteins than those of the cytoplasmic membrane which may be more readily removed by washings. The carbohydrate content was significantly higher in the outer membrane which is known to contain the bulk of the lipopolysaccharide. 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

Table I. Analysis of membrane vesicles recovered from sucrose density gradient.

	Outer Membrane	Inner Membrane
succinate dehydrogenase (Units/mg protein)	0.08	2.58
d-lactate dehydrogenase (Units/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/protein (mg/mg)	0.138	0.066
total heptose	317 μg/ml	80 μg/ml
ratio heptose/protein (μg/mg)	32.4	13.6
organic phosphorous	815 μg/ml	543 μg/ml
ratio phosphorous/protein (μg/mg)	83	93

These values are representative of analysis of any given preparation of membranes, and show the average of at least three separate assays for each membrane macromolecule in this preparation.

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 SDH and d-LDH associated with the outer membrane would reflect the efficacy of separation achieved free of inner membrane. As indicated in Table 1, only 3 to 4% of the total recoverable activity of each of these markers was found in outer membrane preparations. The procedure employed for the separation of membranes is therefore satisfactory.

Examination of negatively stained preparations of the pooled membranes revealed differences in each of the two fractions. Outer membranes were composed of closed vesicles with some evidence of pebbling where the negative stain had penetrated the surface of the vesicle (Figure 21). The vesicles were devoid of cytoplasmic contents as confirmed by both thin section (Figure 22) and freeze etching (Figure 23). Such samples revealed double track unit membranes which had sealed upon themselves and showed no evidence of material within the vesicle. Freeze etching in the presence of cryoprotectant also demonstrated a cleavage plane of outer membrane vesicles. The convex surface (Figures 24a, b) showed granular particles which were more densely packed than the concave fracture which revealed a sparse particle distribution (Figures 24c, d, e).

In the absence of cryoprotectant, the vesicles did not fracture, but instead showed the highly pebbled outer surface of the outer membrane (Figure 24f).

Cytoplasmic membranes on the other hand were not formed into as uniform spheres as was the case for the outer membrane preparations, and often appeared as sheets of membranes with irregular features of the surface, perhaps indicative of some state of disaggregation (Figures 25, 26).

E. Location and Characterization of Concentric Membrane Rings

Extensive scanning of the two membrane fractions was required to localize the concentric membrane rings. Outer membrane preparations included these structures, but the cytoplasmic membrane showed none.

Confirmation that this structure was associated with one of the two membranes of this organism was obtained by lysing spheroplasts in the presence of Triton X-100 at a final concentration of 1% (v/v). When partial dialysis of the non-ionic detergent allowed electron microscopic examination, negative stain revealed that the CMR's had not been lost or solubilized (Figure 27a, b). The Triton X-100 resistant material, demonstrated to be outer membrane in other Gram-negative bacteria (Schnaitman, 1971) was found with CMR's overlying the vesicles.

Indirect methods were used to probe the nature of these structures and to 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 CMR's 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 CMR's 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 Figure 28. The vesicle itself remained morphologically intact, but the rings of the CMR's became progressively less distinct. The inner collar which was released showed unusual lateral mobility over the surface of the vesicle. Where formerly the dimension of the CMR's 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 CMR's in any given preparation were sensitive to protease digestion, however, and this was considered to

be due to the presence of CMR's on the inside surface of the vesicle. If the vesicle was everted during the preparative procedures, such a closed sphere would show a different distribution of macromolecular components as compared to the native orientation. It was therefore of interest to locate the CMR's anatomically 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 CMR's demonstrated that the structures in the intact cell were resistant to such treatments. This suggested that the membrane was interposed, preventing the access of enzyme.

Phospholipase-C was employed in a similar fashion to determine whether the underlying membranes could be loosened sufficiently to allow selective release of the rings. Short digestions of vesicles accentuated the morphological appearance of the CMR's, whereas prolonged treatment caused a loss of the discrete aspect of the structures without the complete digestion observed for proteases (Figure 29).

Attempts were made to isolate the CMR's because direct biochemical analysis of the structures would only be possible if they could be isolated in their native form. The first approach was to use agents disruptive to intrinsic and to extrinsic membrane proteins (Singer, 1974) in anticipation of being able to slip the structures

off the underlying membrane. Incubation of outer membranes in 1% $NaDodSO_4$ severely disrupted the shape or form of the vesicles (Figure 30) and the CMR's were not observed 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 (Figure 31), and the CMR's were not recovered. Partitioning of plasma membrane of ox erythrocytes in a two phase system of n-butanol/water has yielded effective separation of protein into the aqueous phase and lipids into the organic phase (Maddy, 1966), 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 CMR's were not observed by electron microscopy. Both chloroform-methanol extraction and treatment of vesicles with saturated aqueous phenol gave a result similar to n-butanol extraction.

The most effective procedure for removal of CMR's from membrane was provided by 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 (Figure 32a), some CMR's were found to be detached from the underlying layer (Figure 32b, c). Vesicular morphology was maintained and some substructure material was also observed as a result of dissociation of macromolecular components of the membrane. Complete

dissociation was not achieved under such mild conditions either by EDTA alone, or by the concerted action of EDTA and Triton X-100. A decrease in the optical density ($A_{400\ nm}$) 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 76%.

F. Double Labelling of Outer Membrane Proteins

A method that has often been exploited to detect differences in the presence or absence of biological macromolecules has been to utilize a double labelling technique. Samples are radioactively labelled under different physiological conditions with two different isotopes, the preparations pooled, the samples into which label has been incorporated are fractionated or isolated, and two channel counting is carried out to assess possible differences in the incorporation of precursors into product.

Such a technique was adapted with the intention of detecting CMR protein in the outer membrane of *Sp. serpens* as follows: Cells were grown for twenty generations in ^{3}H-aspartic acid (42°C) and ^{14}C-aspartic acid (30°C). The cultures were mixed, and doubly labelled outer membranes were isolated. At the higher of these two temperatures, no components of the flagellar apparatus (filament, hook, basal complex, or CMR's) were observed by electron

microscopy. One might anticipate that the electrophoresis pattern of radioactivity on $NaDodSO_4$-polyacrylamide gels would indicate one or more membrane-associated polypeptides whose synthesis was restricted by growth at the higher temperature. This observation could then be related to absence of the structural array of CMR's at 42°C.

The spectrum of radioactively labelled outer membrane proteins (Figures 33 and 34) was determined by slicing the gel into 140 x 1 mm sections and counting for ^3H and ^{14}C; the difference in the figures is in the temperature of solubilization with 1% $NaDodSO_4$ + 1% β-mercaptoethanol: 70°C, thirty minutes, or 100°C, three minutes. In both cases, there is an apparent coincidence of the two radioisotopes. The same result was found if membranes were differentially labelled with ^3H-leucine and ^{14}C-leucine. In all cases, peaks of radioactivity from proteins of 30°C grown cells and of 42°C grown cells were detected as overlapping.

For purposes of comparison, a second pair of polyacrylamide gels was run, using the same membranes preparation as above, but stained with Coomassie brilliant blue (Figure 35). A qualitative comparison may be made between the series of radioactively labelled proteins and the homologous proteins which are stained. However, it would seem that the width of the protein bands might have precluded detection of such a minor component as CMR protein. Despite the success of this polyacrylamide gel system and

radioactive labelling technique as the source of considerable information on the outer membrane of Escherichia coli (Inouye and Guthrie, 1969; Inouye and Lee, 1972; Hirashima et al., 1973; Halegoua et al., 1976), it would not appear to have demonstrated the differences in labelling pattern which were anticipated for a minor component of the outer membrane of Sp. serpens.

G. Crossed Immunoelectrophoresis

Recent technical developments in crossed immunoelectrophoresis (CIE) were exploited in pursuit of identification of the protein-containing components which contribute to the structural array of CMR's. The potential of this technique lies in the ability to detect in a sensitive manner a multiplicity of membrane antigens (Axelsen, 1973). Such multiplicity is a feature of outer membrane of Sp. serpens, to judge by the large number of polypeptides revealed on $NaDodSO_4$-polyacrylamide gel electrophoresis.

The presence of CMR's on outer membrane vesicles from cells grown at 30°C and by the absence of these structures on outer membrane vesicles from cells grown at 42°C define two populations of membranes. Antisera raised against 30°C vesicles would be expected to be directed towards a battery of membrane components including the CMR protein(s), whereas antisera raised against 42°C vesicles would be directed against a similar battery of antigens but lacking

the CMR protein(s). From theoretical considerations, therefore, one might detect a difference in reactivity of the two antisera.

The antisera made for the CIE experiments were first tested for the presence of precipitating antibodies (bacterial agglutination test) and found to be positive. Gamma globulins were then partially purified by three successive ammonium sulphate precipitations, as indicated in Materials and Methods.

The antigen preparations against which the above antisera were directed were purified outer membrane vesicles from cells grown either at the temperature permissive for CMR's, or at the temperature restrictive for CMR's. These vesicles were derived from the heavy banding region of the sucrose density gradient (ρ = 1.25), and were free of cytoplasmic membranes, as previously demonstrated. A wide variety of procedures for solubilizing the outer membrane proteins was carried out. To an aliquot of vesicles was added a concentrated solution of detergent, yielding the final concentrations listed below; samples were incubated as follows:

(i) 1% $NaDodSO_4$ + 1% β-mercaptoethanol; 100°C, three minutes;

(ii) 1% $NaDodSO_4$ + 1% β-mercaptoethanol; 70°C, thirty minutes;

(iii) 1% $NaDodSO_4$ + 1% β-mercaptoethanol; 37°C, sixteen hours;

(iv) 0.1%, 1.0% or 4% Triton X-100; 70°C; thirty minutes;

(v) 0.1%, 1.0% or 4% Triton X-100; 37°C, up to sixteen hours;

(vi) dodecyltrimethylammonium bromide (DTAB), 250 mM; 37°C, sixty minutes.

In all of the above solubilizing systems, the membrane sample containing detergent was either applied directly to the antigen well on the CIE slide, or the sample was centrifuged (28,000 xg, fifteen minutes, 22°C) to give a vesicle-free, protein-containing supernatant. This supernatant was then either used as the antigen sample, or dialyzed against 1000 ml barbital-HCl buffer, I=0.02, pH 8.6, containing 0.1% Triton X-100, in an attempt to decrease the concentration of the solubilizing detergent. Such a procedure would therefore exchange the original detergent in favour of Triton X-100, and avoid anomalous effects due to detergent interfaces. Protein concentrations were determined for all samples applied to the wells.

Figure 36a shows a CIE pattern of outer membrane vesicles (30°C grown cells) solubilized with 1% $NaDodSO_4$ + 1% β-mercaptoethanol, 70°C, thirty minutes, and then dialyzed against barbital-HCl buffer containing Triton X-100; the gamma globulin preparation was directed against 30°C vesicles. It is evident that only six immunoprecipitins have formed on the slide, and that this pattern does not reflect the anticipated heterogeneity of outer membranes. No "rockets" formed in reacting antisera

against vesicles solubilized with $NaDodSO_4$ at 100°C for three minutes, probably because severe denaturation of the polypeptides would not allow for immunological recognition. It was also necessary to dialyze the solubilized proteins to decrease the concentration of the ionic detergent; failure to do so resulted in a CIE pattern with only one diffuse "rocket" (Figure 36b); $NaDodSO_4$ apparently interfered with immunoprecipitation.

Triton X-100 was almost ineffective in solubilizing outer membrane proteins for detection in the CIE system. Three components of weakly anodic mobility were detected by CIE when outer membrane vesicles were treated with Triton X-100 to a final concentration of 4% (Figure 36c). Incubation of outer membranes in the presence of 250 mM DTAB, followed by dialysis of the sample against barbital-HCl buffer containing Triton X-100, yielded four very lightly staining immunoprecipitates (Figure 36d).

In lieu of using membrane vesicles as the preparation from which proteins were solubilized, a "total membranes" sample for use in CIE was prepared as follows: Whole cells of Sp. serpens were harvested and twice washed in 0.85% NaCl. The cells were then sonicated in 2 x 30 second bursts in an ice bath. Unlysed cells were precipitated at low speed (1085 xg, ten minutes, 22°C) and membranes were recovered by centrifugation (12,100 xg, twenty minutes, 22°C). Following two washes of this preparation in distilled water, the "total membranes"

were resuspended in distilled water and Triton X-100 was added to a final concentration of 4%. The sample was incubated (eight hours, 37°C), centrifuged to precipitate the membranes, and the supernatant containing solubilized material was decanted and used for CIE against gamma globulins to 30°C outer membrane vesicles. The pattern (Figure 36e) is biphasic and polydisperse, possibly reflecting heterogeneity in charge and size of this component. It is interesting to note that two forms of lipopolysaccharide have been detected in Sp. serpens (Chester and Murray, 1975; and unpublished data) and these forms may well be indicated by this slide.

The necessity of using an antigen sample of high protein concentration has been indicated by other workers (Smyth et al., 1976). In using $NaDodSO_4$ to extract membranes, the range of protein subsequently applied to the CIE plate well was from 5 µg to 170 µg. These antigen samples after electrophoresis in the first dimension were then reacted with gamma globulin preparation containing from 100 µg to 10.3 mg protein. Despite this wide range, it became evident that the antigenic complexity of the outer membrane of Sp. serpens was not readily established by CIE in terms of multiplicity of immunoprecipitates.

H. Solubilization of Membrane Proteins

These results prompted a search for a better method for solubilization of outer membrane proteins, and an

investigation into the stoichiometry of this phenomenon. A small aliquot of radioactively labelled outer membranes was incubated in a large excess volume of detergent or chaotrope, at a selected concentration for a given time interval and temperature. The extracted membranes were then subjected to ultracentrifugation and the supernatant was decanted and counted for radioactivity. The percentage of total dpm found in the supernatant would therefore indicate the degree to which membrane proteins were released by detergent. As indicated in Table II, $NaDodSO_4$ was most effective in solubilizing outer membrane protein from Sp. serpens, but there was a dramatic decline in the ease of solubilization for almost all other reagents. Only DTAB at 500 mM appeared moderately effective (64% release of counts). The non-ionic detergent Triton X-100 at 4% final concentration showed differences in the extent of solubilization according to the source of the reagent; 16% and 29% of total dpm were released by the reagent from Sigma Chemical Co. and from Research Products Inc. (R.P.I.), respectively.

I. Polyacrylamide Slab Gel Electrophoresis

Parallel information on the identity of proteins which might be solubilized by $NaDodSO_4$ was obtained by electrophoresis on a slab of polyacrylamide gel, and a photograph of the gel is shown in Figure 37. At 37°C, neither 0.1% nor 0.5% detergent resolved outer membrane

Table II. Effect of various reagents in solubilizing outer membrane proteins of Sp. serpens.

solubilizing reagent	concentration	dpm released into supernatant	% of total dpm released
N.C.S. tissue solubilizer		20,200	100
guanidine hydrochloride	6 M	5,060	25.1
DTAB	500 mM	12,900	64.0
sodium deoxycholate	4%	6,970	34.6
urea	6 M	1,890	9.4
Triton X-100 (Sigma)	4%	3,260	16.2
Triton X-100 (R.P.I.)	4%	5,900	29.3
$NaDodSO_4$ + β-mercaptoethanol	1% + 1%	20,400	101
$NaDodSO_4$ + β-mercaptoethanol (70°C, thirty minutes)	1% + 1%	20,200	100
distilled water		1,670	8.3

A five microlitre sample of ^{14}C-leucine labelled membranes was added to 250 microlitres of each of the above reagents at the indicated concentration; the effect of dilution by this addition was considered negligible. The mixtures were incubated for sixty minutes at 37°C, or in one instance at 70°C for thirty minutes. The samples were then centrifuged by R_{av} = 105,000 xg for sixty minutes at 15°C, and the vesicle-free supernatant was decanted into a scintillation cocktail vial. Insta-Gel (4.75 ml) was added to each sample and the radioactivity was determined. Total radioactivity in non-extracted membranes was determined by addition of 0.5 ml of N.C.S. tissue solubilizer (Amersham-Searle), incubated at 37°C for sixty minutes, and similarly counted for radioactivity.

proteins on the PAGE slab; at 70°C, 1.0% $NaDodSO_4$ solubilized a "major" protein of molecular weight 38,000 daltons, whereas 37°C incubation with 1.0% detergent was incapable of doing so.

These results should be compared with the electrophoresis pattern of outer membrane proteins incubated with 1.0% $NaDodSO_4$ and 1.0% β-mercaptoethanol at 100°C for three minutes (Figure 38); total disruption of the membrane gave the maximum number of proteins (twenty three) detectable by staining. It is important to mention, however, that the resolution of protein bands by staining with Coomassie brilliant blue is inferior by comparison with ^{35}S-methionine autoradiography of electrophoresed proteins, as presented elsewhere in this Chapter. Hence, only those proteins which comprise a relatively large fraction of the total membrane proteins would be detected, as is shown in Figure 38.

Autoradiography of radioactively labelled proteins afforded the opportunity of direct comparison between the spectrum of components inserted into the membranes of two separate preparations. Neither inspection of stained slabs nor densitometry of the stained protein bands was able to identify differences between proteins associated with outer membranes from cells grown at 30°C ("permissive" for CMR's) and the proteins associated with outer membranes from cells grown at 42°C ("restrictive" for CMR's). Because of the absence of the structural array of CMR's

at the higher temperature, it is reasonable to suggest that one or more proteins which contribute to such an assembly would be absent from the electrophoresis pattern of 42°C outer membranes.

Therefore, a culture of Sp. serpens was labelled at 30°C with ^{35}S-methionine, and a second culture was labelled identically at 42°C, as presented in Materials and Methods. This provided for two preparations: one of outer membrane vesicles from 30°C grown cells which, by electron microscopy, displayed CMR's; and the other of outer membrane vesicles from 42°C grown cells, which were confirmed by microscopy not to possess the assembled aggregate of CMR's. Each sample was solubilized in 1% $NaDodSO_4$ + 1% β-mercaptoethanol, 100°C for three minutes, a procedure known to ultrastructurally disrupt CMR's. To two wells on a $NaDodSO_4$ slab polyacrylamide gel were added the same number of radioactive counts from 30°C vesicles as 42°C vesicles, in order that the intensity of the bands on the exposed film might be compared.

The autoradiogram of the proteins labelled at the two different temperatures is shown in Figure 39. It is readily apparent that the membrane proteins from 30°C vesicles differ in two respects from those of 42°C vesicles: (i) four bands, with relative mobility (M) in 7.5% acrylamide gel of 0.80, 0.77, 0.72, and 0.36, and having estimated molecular weights of 46k, 54k, 72k, and greater than 200k, respectively, are present among the

30°C proteins but distinctly absent from the 42°C proteins; (ii) the intensity of selected pairs of proteins other than those listed above appears to differ, although the significance of this finding is uncertain.

The correlation between CMR protein and one or more of the four proteins found only in 30°C membranes may therefore be tentatively established. It was somewhat unexpected that as many as four proteins did not appear in the autoradiogram of 42°C outer membrane vesicles. Nevertheless, the identification of CMR protein was considerably narrowed from the spectrum of twenty nine proteins in *Sp. serpens*, as derived by this technique.

J. Ferritin-Gamma Globulin Reactions

Localization of macromolecules in biological specimens by the use of ferritin-conjugated antibodies was first investigated by Schick and Singer (1961). A purified antigen was selected for an immunization series in rabbits, and following several injections the antisera were confirmed for specificity towards the given antigen. Gamma globulin was then fractionated and coupled to ferritin by means of a bifunctional reagent, toluene 2,4-diisothiocyanate. The conjugate was allowed to react with tissues which had been fixed and embedded in a water soluble matrix. Localization of antigen was possible using the electron microscope, since the electron dense core of ferritin could be readily recognized.

The principle of such specific staining was adapted for possible detection of membrane antigens in Sp. serpens as follows: ^{35}S-methionine autoradiography showed that four proteins were present in outer membrane at 30°C but absent at 42°C. A preparative polyacrylamide slab gel was heavily overloaded with 1600 μg outer membrane proteins (30°C) which had been solubilized at 100°C for three minutes in 1% $NaDodSO_4$ + 1% β-mercaptoethanol. The fixed, stained, and destained gel was then dried to a thin film, and compared with the autoradiogram. Those four proteins (46k, 54k, 72k, and >200k) shown on the X-ray film were then located as very faint blue bands on the dried slab gel. Thin horizontal strips of the stained material were cut out, resuspended in saline, and used as purified membrane antigens to immunize rabbits. Gamma globulin was isolated from the antisera, and was covalently coupled to ferritin to produce four reactive conjugates. An aliquot (5 μl) of each conjugate was suspended in 500 μl of 0.05 M Tris-HCl, pH 7.6, 0.1 M NaCl, 10 mM $MgCl_2$, and reacted with a small sample (5 μl = 92 μg protein) of outer membrane vesicles of Sp. serpens which displayed CMR's. Reaction conditions were at 37°C for four hours and then overnight at 4°C. Samples were withdrawn and examined by negative staining with ammonium molybdate.

Comparison of the electron micrographs of samples withdrawn after reaction of ferritin-gamma globulin with CMR's yielded an important result. In the case of the

conjugate coupled to anti-72k protein, a considerable density of ferritin molecules was superimposed upon the membrane-associated array of CMR's (Figure 40). This specificity was not displayed by the anti-46k, anti-54k, and anti->200k, prepared in an identical manner, and allowed to react with outer membrane vesicles under the conditions elaborated above (Figures 41, 42, 43). In these situations, a random distribution of ferritin particles was observed. If the vesicle preparations were initially reacted with gamma globulin to 72k protein, and then with conjugate, no labelling was observed. Blocking of the available sites on the CMR's had occurred. Furthermore, vesicles from outer membranes of 42°C grown cells which showed no CMR's did not reveal any such clustering of ferritin as was the case for anti-72k protein.

The interpretation of this information is that one protein of estimated molecular weight 72,000 daltons is responsible either totally or in part for the structural assembly of concentric membrane rings. This conclusion is based upon finding by ^{35}S-methionine autoradiography four differences in the protein composition of outer membranes at 30°C and 42°C. Testing the immunological specificity of these four proteins by use of ferritin-conjugated antibodies showed failure of three of the conjugates to bind to CMR's. Immunological specificity of one component was retained, and by electron microscopy this 72k protein was correlated with CMR's.

K. Perforation of the Murein Sacculus

One of the reasons for selecting a bipolarly flagellated organism in a search for membrane-associated components of the flagellar apparatus was that ultrastructural observations would be directed to a specific area of the cell surface. Since the basal organelle is known to penetrate the layers of the cell envelope, it seemed logical to suggest that perforations should be observed in the peptidoglycan layer. Hence, preparations of the murein sacculus were examined in the electron microscope by negative staining and by casting a carbon/platinum replica. _Sp. serpens_ possesses a variable and low number of flagella at each cell end, as discussed earlier in this Chapter. It was difficult to assess with confidence which regionof the polar cap to search for holes through which flagella might once have penetrated. For this reason, the more richly flagellated _Sp. volutans_ (Wells strain) was used as a source for sacculi, and examined in the same manner as described above. This organism also possesses CMR's on the lower face of the outer membrane, and has a basal organelle similar to that of _Sp. serpens_. Ammonium molybdate and uranyl magnesium acetate as negative stains were not effective in demonstrating surface irregularities at the poles of _Sp. volutans_. Shadow cast replicas of murein sacculi gave the same result. However, 4% phosphotungstate clearly penetrated the flagellar holes, and furthermore showed an electron transparent

"halo" surrounding each perforation (Figure 44). A number of holes equivalent to the number of flagella appeared at both ends of the cell, and no holes were observed along the lateral walls.

The diameter of these stain-containing pits in Sp. volutans was estimated by measuring over 200 holes, and yielded an average value of 15 nm. A comparable value was obtained for the size of the perforation in Sp. serpens, wherever such could be identified. The diameter of the "halo" was determined as 35 nm. These numerical values will later become significant in positioning of the P ring of the basal organelle relative to the peptidoglycan layer of the cell envelope.

L. Insertion of the Basal Organelle into Cytoplasmic Membrane

Three different sets of information proved valuable in assessing the attachments or insertions of the flagellar basal organelle into the cell envelope, with particular attention to the cytoplasmic membrane.

Autolyzed preparations of Sp. serpens were useful for electron microscopic examination only if cells were devoid of cytoplasm. A culture of the bacterium maintained at room temperature for several days was harvested by centrifugation, twice washed with distilled water, and negatively stained with ammonium molybdate. Some cells, free of cytosol, still retained the orientation of the flagellar

hook and basal complex firmly attached to the cell envelope (Figure 45) despite considerable retraction of the cytoplasmic membrane at regions other than the polar cap. Presumably the flagellar apparatus caused the adherance of wall and membrane at the flagellar insertions. These preparations demonstrated the following:

(i) the orientation of an outer membrane and an inner membrane;

(ii) that CMR's were clearly associated with the external membrane and not with the internal membrane;

(iii) that CMR's could be discerned "on edge" by the regular spacing of subunits on either side of flagellar hooks;

(iv) the contour of outer membrane was not continuous and smooth at the insertion points, but was slightly infolded by the presence of CMR's; these structures therefore appeared to confer a rigidity to the area at which the flagellum emerged;

(v) the S ring (third in register) and the lowermost M ring appeared in contact with the plasma membrane, but their specific attachments were unclear.

Electron microscopic study of these autolyzed preparations yielded maximum information on the most probable "native" orientation of cell envelope and basal organelle. Actively growing cultures were not amenable to these observations, because of the necessity to disrupt the cells, generally by sonic oscillation. This procedure

sheared off both filament and hook, and usually also led to such mutilation of the basal complex that it could not be recognized.

Fractionation of the inner and the outer membranes made it possible to assign CMR's to outer membrane. Extensive examination of cytoplasmic membrane vesicles by negative staining, however, at no time displayed any structural features which might have been associated components of the flagellar apparatus. No "hole" through which stain might have penetrated (as through outer membrane) nor regular surface elements could be detected in isolated membranes.

The second set of information on the orientation of basal organelle and membranes was derived from freeze-etchings of *Sp. serpens*. Because of (a) the number of flagella only at the poles and (b) the embedding of the lower rings into cytoplasmic membrane as shown in autolyzed preparations, one might expect that some irregularities in the freeze etch fracture face of the cytoplasmic membrane could give a clue as to the level of insertion of either the S or the M ring. One can readily appreciate that this hypothesis necessitated fortunate fractures at the polar caps of cells well endowed with flagella. Suspensions of whole cells of *Sp. serpens* were prepared for freeze etching in 15% glycerol as cryoprotectant, and processed as described in Materials and Methods. Such samples are known to cleave preferentially through the

hydrophobic interior of the cytoplasmic membrane (Snellen and Starr, 1976) so that the convex face is highly studded with particles (Figure 46). Attention must be directed to a parallel series of micrographs (Figure 47) where several large depressions appear on this fracture face. In the centre of each of these depressions is a circular "plug", and in a strikingly regular fashion, the periphery of each depression is lined with a ring of fifteen studs.

The interpretation of these micrographs is as follows: The cleavage has fractured through the polar cap of cytoplasmic membrane into which flagella were once inserted. The depressions (diameter = 23 nm) have been caused by the S ring which has been torn out during cleavage. The only remnant of the basal organelle is the central rod which appears in the centre of the depression as a "plug" with a diameter of 10 nm. The circumferential particles are distinguished by their uniformity of size and spacing, and are thus the unique feature of the cytoplasmic membranes' association with the basal organelle.

Thin sections of fixed and stained whole cells of _Spirillum_ provided the third kind of information on the relationship of basal organelle and cell envelope. As with the identification of the holes in the murein sacculus, it became advantageous to use _Spirillum volutans_ because of its considerable enrichment for polar flagella. Again fortunate sections were necessary, and where obtained (Figure 48) showed the following:

(i) a tuft of flagella in thin section, appearing as strands at the cell poles;

(ii) additional electron density where the flagellum penetrated the cell envelope; this was presumably attributable to the four rings of the basal organelle, although they could not be individually differentiated;

(iii) additional electron density beneath the double track cytoplasmic membrane; this was manifest as a bulbous component, not recognizable by any of the other preparative techniques for electron microscopy. This protuberance could not be attributable to deformation of cytoplasmic membrane because of its regularity beneath those regions from which flagella were splayed from the pole.

CHAPTER IV

DISCUSSION

The study of bacterial membranes has advanced considerably in recent years, chiefly due to the multiplicity of approaches in studying these membranes. Genetic manipulation of membrane components, biochemical fractionation of cell envelopes, physiological response to the milieu, and structural integration of macromolecules into each layer of the cell envelope has provided insight into the nature and complexity of these processes in prokaryotic cells. This complexity may be higher in eukaryotes, but since bacteria are readily subject to experimental manipulation, more definitive answers concerning observed biochemical defect and altered physiology and structure can be obtained by their study (Leive, 1973).

The membrane is now regarded as the site of most functional differentiation in the bacterial cell. A variety of internal membrane systems has been studied, and most are extensions of the plasma membrane where a concentration of function occurs. These include meso-

somes, specialized vesicles, lamellated membranes, photosynthetic, and spore membranes. Isolated fractions from bacterial membranes are also known to have multifunctional properties: electron transport, active transport carrier systems, phospholipid biosynthesis, and synthesis and assembly of cell wall polymers. The diversity of structural and functional interplay is also reflected in studies of bacterial flagella. In this system, synthesis of specialized components of basal organelle, hook, and filament, ordered assembly into a highly differentiated organelle at a specified time often coordinate with cell division, response to signals, and activation into cell movement all provide for study of a comparatively simple sub-cellular organelle. Hence, conditions which govern the genetic circumstances for flagella synthesis have been determined; production of flagella is known to favour certain physiological conditions; flagella have been readily isolated for study of their chemical properties and biophysical polymerization; and experiments have been conducted on living cells to gain information about their behavioural responses.

One aspect of bacterial flagella that has received considerable attention is the elucidation of their morphology and ultrastructure. As presented in the Introduction, one can appreciate that advances in electron microscopy and in biochemistry of cell envelopes contributed to correlation of structure with function.

Examination of autolyzed preparations provided limited insight as to the existence and orientation of specialized structures which anchored the flagellum to the cell surfaces. By isolating intact flagella, it became possible to appreciate the complexity of fine structure of the basal organelle with its four stacked discs and central rod, attached firmly to the hook, and ultimately to the filament.

But the dissolution of the layers of the Gram-negative cell envelope included the possibility, usually overlooked, that some component of the apparatus might have been lost or degraded. Furthermore, the evidence for the intercalation of the basal organelle with each layer of the envelope has thus far been indirect. The anatomical arrangements put forward in the literature have not specifically considered those regions of wall and membrane which were once the site of flagellar insertions.

This study is the result of a search for additional components of the flagellar apparatus, with particular attention directed towards the cell envelope layers. It includes the isolation and characterization of specialized regions of wall and membrane into which fits the basal organelle. Recognition of a structural arrangement which has now been termed concentric membrane rings (CMR's) was first made by examination of whole cell preparations of *Sp. serpens* under the electron microscope. Negatively stained material led to an appreciation of more

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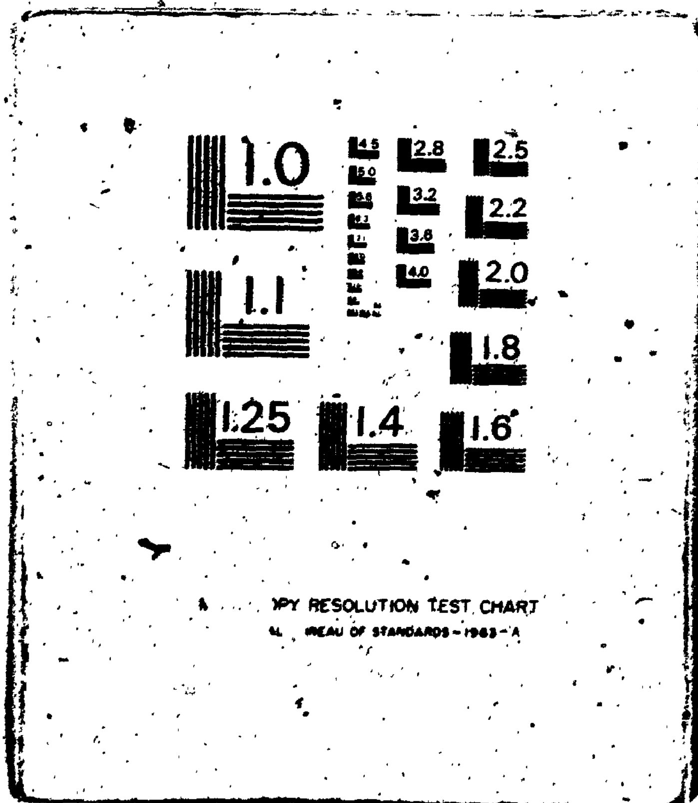

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than just a set of "basal organelles" at the polar cap of this Gram-negative bacterium. Preparations of lysates by sonication or incubation with non-ionic detergent or cell wall proteases enhanced the clarity of microscopy, and assisted in localizing this structure to one of the cell envelope layers.

Isolation of inner membrane and outer membrane of Sp. serpens was the approach of choice to conclusively demonstrate the association of CMR's on one or the other membrane. This task necessitated development of a rigorous procedure and set of conditions allowing reproducible preparations. Many procedures have been reported in the literature to be suitable for such separation. Miura and Mizushima (1968, 1969) separated membranes of E. coli; Osborn et al. (1972) obtained good fractionation of membranes from Salmonella typhimurium; Proteus mirabilis (Oltmann and Stouthamer, 1973; Hasin et al., 1975), Acinetobacter sp. (Scott et al., 1976), Rhodospirillum rubrum (Collins and Niederman, 1976) have all been the object of study. None of these methods, however, proved entirely effective for membrane isolation in Spirillum. Various combinations of ideas presented in other work were incorporated into the final procedure, as outlined in Materials and Methods.

The anatomical positioning of CMR's on the outer membrane was based upon observation using the electron microscope. Furthermore, by taking advantage of the

structure's sensitivity to protease, it was determined that this auxillary component is harboured on the underside of the outer membrane, "facing" the mucopeptide layer. Sensitivity to various detergents, organic solvents, and enzymatic digestions demonstrated the peculiar resistance of CMR's to a wide spectrum of agents frequently employed in membrane research. Such resistance to solubilization appears characteristic of outer membranes of Gram-negative bacteria, and is probably attributable to the presence of lipopolysaccharide on the exterior surface of the bilayer. Our experiments confirmed this property for outer membrane of Sp. serpens as demonstrated by the low levels of ^{14}C-leucine labelled proteins released upon treatment of vesicles with ionic, non-ionic, and chaotropic agents (Table II). The "minimum" requirements for maximum liberation of proteins in the membrane were determined to be incubation with 1% $NaDodSO_4$ + 1% β-mercaptoethanol, 70°C, thirty minutes.

Identification of a constituent protein responsible for CMR's was achieved by a two-fold process. The electrophoresis pattern of radioactively labelled membrane proteins indicated the possibility that one or more of four polypeptides (46k, 54k, 72k, and >200k) were incorporated into the structural array under "permissive" conditions. These four candidates were then purified on a preparative polyacrylamide slab gel, and monospecific antisera were raised against each one. Coupling

of these immune sera to the electron-dense ferritin produced conjugated markers suitable for electron microscopy. Labelling of CMR's by anti-72k protein-ferritin complex indicated its unique specificity for the membrane-associated structural array, and eliminated the possibility that the other three proteins might be constituents.

It was initially desirable to search for mutants of *Sp. serpens* which were in some respect structurally or functionally altered with respect to their flagellar apparatus. The selection of such mutants proved elusive, however, for reasons which are not immediately apparent. Mutagenesis with nitrosoguanidine (Adelberg *et al.*, 1965) or ultraviolet light was followed by screening on semi-solid agar plates or agar into which anti-flagellar anti-serum had been incorporated. Differences in colony morphology (Iino and Enomoto, 1971) were used as a criterion of altered flagellation. Slow swimming *Spirilla* were selected from the centre of swimming cells on semi-solid agar plates (Armstrong *et al.*, 1967); cells which settled in long columns were eluted in anticipation of enriching for non-flagellated or paralyzed mutants. None of these experimental approaches resulted in selection of *fla*⁻ phenotypes.

In the absence of 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 the two poles. Similarly in Bacillus subtilis, Ryter (1971) has demonstrated that there is no redistribution of these organelles once they have appeared through the cell envelope. It is suggested that CMR's act as a stabilizing plate about the insertion of the flagellum such that it remains rigidly inserted into the cell envelope. The structure might act to exclude the fluid phase in which other proteins are floating (Singer and Nicholson, 1972), thereby minimizing lipid interactions in the vicinity lateral to the flagellar insertions.

Recognition of CMR's on outer membrane of a Gram-negative bacterium appears to be the first report of specialized structural differentiation attributable to this membrane. It furthermore enhances the understanding of the functional roles which this layer contributes to the cell. The notion of outer membrane being considered solely a permeability barrier is gradually being superseded by ascribing to it selected functions (Costerton et al., 1974). It has been firmly established as an exclusion layer to compounds of molecular weights greater than 700 daltons (Payne and Gilvarg, 1968) and therefore accounts for resistance to many antibiotics, detergents, and dyes (Sanderson et al., 1974). The lipopolysaccharide which extends its carbohydrate strands into the external milieu (Shands, 1965) contributes most to the bacterium's

antigenicity, and has been the key to serological identification and genetic studies of assembly of a biological polymer (Nikaido, 1973). Bacteriophages may be differentiated into rough-specific and smooth-specific according to their LPS receptor requirements on the host enteric bacteria. But for a long time, no specific role could be assigned to any of the proteins in this membrane. With the discovery of a most abundant lipoprotein (Braun and Rehn, 1969) which is covalently linked to the underlying mucopeptide layer, a structural function was suggested for one such protein, in that it might be responsible for maintaining an intact cell wall. To what extent analogous or similar lipoproteins are involved in Spirillum is a matter of conjecture, but it is attractive in view of the "pitted" appearance and substructure of its outer membrane. More recently, Inouye (1974) proposed that these lipoprotein molecules form an assembly which spans the entire thickness of the membrane, providing a hydrophilic channel and allowing passage of low molecular weight substances.

The multifunctional aspects of outer membrane have now been extended to include a wide variety of receptor proteins. Colicins (Luria, 1973) are a group of bacteria-specific "killer" molecules which attach to membrane proteins in their primary stage of infection. These receptor functions may be shared, in some instances, with other physiological roles such as iron transport

(Hantke and Braun, 1975). Many bacteriophages also utilize a receptor common to components of iron transport systems (Braun *et al.*, 1973, 1976). It would appear that some classes of proteins have bifunctional roles. They may act as scavengers or as a means of retrieval of cations, some of which are rare but of exquisite importance to the cell. Alternatively, they may provide a target for a molecule or a virus that will eventually destroy the cell. Genetic data first demonstrated the coincidence of loci for maltose transport and for the phage lambda receptor (Schwartz, 1967; Szmelcman and Hofnung, 1975) since the two mapped together on the *E. coli* chromosome. Purification of the maltose-binding protein in the outer membrane has been achieved (Randall-Hazelbauer and Schwartz, 1973) and this protein is also known to interact reversibly with the phage (Schwartz, 1975). A final example of shared function is to be found in the case of vitamin B12 transport and the E colicin receptor (DiMasi *et al.*, 1973) where again the same macromolecule has evolved dual attributes.

Based upon selected information presented in the chapter of Results, revisions to the currently accepted model for the insertion of flagella into the cell envelope of Gram-negative bacteria are now proposed. These revisions are established by identification of specific locations on the outer membrane, mucopeptide layer, and cytoplasmic membrane which supported a

flagellum, and which may be recognized ultrastructurally in fractionated preparations of the layers of the envelope.

DePamphilis and Adler (1971b, c) isolated the flagellum and basal complex free of adhering membranes, and accurately measured the diameter and thickness of the four rings, L, P, S, and M. Their intercalation of flagellum into the envelope was made by superimposing measurements derived from negatively stained preparations onto the thin section profile from fixed and stained whole cells. The numerical values are at best an approximation, since measurements from these two different preparations are not necessarily comparable. Disparity in measurements is also complicated by variations in the composition, pH, and uptake of the stains employed. The register of discs and envelope layers is, however, inaccurate and mismatched.

Table III will assist in evaluation and revision. The following information should be incorporated into a revised model for bacterial flagellar insertions, and is based upon evidence from the Gram-negative bacterium *Spirillum serpens*:

1. That the specialized structural differentiation termed concentric membrane rings (CMR's) be anatomically located on the under surface of the outer membrane.

2. That the 12 nm "hole" in the outer membrane is sufficient to allow for the diameter of the central

Table III. Dimensions of cell envelope layers and dimensions of the flagellar basal organelle: a comparison.

	Escherichia coli	Spirillum serpens
	DePamphilis and Adler (1971b, c)	this study
diameter of L ring (n.s.)	22.5 nm	18 nm
P		21 nm
S		28 nm
M		31 nm; best approximation, assuming identical diameter for each ring = 22 nm
diameter of connecting rod (n.s.)	7 nm	10 nm
diameter of "hole" in outer membrane (n.s.)	n.d.	12 nm
diameter (maximum) of CMR's (n.s.)	n.d.	90 nm
diameter of "hole" in mucopeptide (n.s.)	n.d.	15 nm
diameter of "halo" in mucopeptide (n.s.)	n.d.	35 nm
diameter of depression on convex cytoplasmic membrane (f.e.)	n.d.	23 nm
diameter of rod within the depression on convex cytoplasmic membrane (f.e.)	n.d.	10 nm
thickness of basal organelle, "top" to "bottom" (n.s.)	27 nm	31 nm

Table III (continued)

	Escherichia coli	*Spirillum serpens*
	dePetris (1965, 1967); t.s.	Steed-Glaister (1967); t.s.
thickness of outer membrane	6.0 nm	7.0 - 8.5 nm
thickness of mucopeptide	2.5 nm	4.0 - 4.5 nm
thickness of cell wall layers	13.0 nm	13.5 - 16.5 nm
thickness of cytoplasmic membrane	6.0 nm	7.5 - 8.5 nm
thickness of cell envelope	23.0 nm	25.0 - 29.0 nm
thickness of cell envelope:		
(Inouye, 1975); t.s.	25.0 nm	
(Bayer, 1974); t.s.	20.0 nm	

Legend:

n.s. - negative staining
f.e. - freeze etching
t.s. - thin section
n.d. - not determined

rod (10 nm); the L ring is therefore embedded in this membrane.

3. That the 15 nm perforation in the murein sacculus is sufficient to accommodate the central rod, but is clearly too small for the P ring's diameter (21 nm); the P ring cannot be inserted into the mucopeptide layer, but should be positioned exterior to this layer.

4. That the depression (23 nm) on the convex fracture face of the cytoplasmic membrane is the position of the "S" ring; it is therefore situated on the lower half of the bimolecular leaflet and not "supramembrane".

5. That the fifteen circumferential protein studs of the convex cytoplasmic membrane fracture face are the only identifiable membrane-associated components of this layer of the envelope.

6. That account be made for the blebs which are found beneath the cytoplasmic membrane in thin sections; these blebs should now be considered to include the lowermost "M" ring.

7. That the regular splayed fibrils emanating from the lowermost ring, as seen in negative stain, may provide additional anchoring onto the plasma membrane.

Diagrammatic representation of all of these features is shown in Figure 49. Caution must be exercised in superimposing measurements from electron microscopic data using different preparative techniques, and the hazards of such a course have been referred to above. Nevertheless,

Figure 49 is a "best fit" of rings to envelope layers. The coincidence of measurements of the basal organelle with the minimum and maximum dimensions of the cell envelope layers is excellent in the case of Spirillum serpens, and should clearly be revised in the case of Escherichia coli to be consistent with the ideas presented above.

One of the most attractive features of the structural arrangements proposed here is the correlation which may be made with information on morphogenesis of bacterial flagella. Many pieces of evidence have demonstrated that the monomeric protein flagellin is synthesized on ribosomes within the cytoplasm, and is subsequently directed towards the cell envelope for "export" (Martinez and Gordee, 1966). Since flagella are also known to extend in length by addition of sub-units at their distal tip and not in the region proximal to the cell surface (Iino, 1969) a mechanism for transport of flagellin must be invoked. The lowermost M ring situated along the hydrophilic side of the lipid bilayer could act as a nucleating centre for flagellin. The protein would be directed towards this acceptor region of the basal organelle, enter the hollow rod, and proceed past the hook, to add on to the growing tip of the flagellar filament.

The ideas hereby presented constitute a challenge to currently accepted information on the cell envelope associations of bacterial flagella, and it is hoped that

study of other organisms will continue to expand the understanding of such relationships.

Figure 1

Diagrams to summarize the relationship of flagella to envelope structure in the region of the polar tip of *Spirillum serpens*; the inset shows details of the envelope layers. Legend: a. cell wall: outer membrane plus underlying mucopeptide; b. inner cytoplasmic membrane; c. polar membrane with linkers to inner membrane; f. flagella emerging through the cell envelope, with an internal expansion or bulb.

From Murray and Birch-Andersen (1963). By permission, National Research Council of Canada, Ottawa.

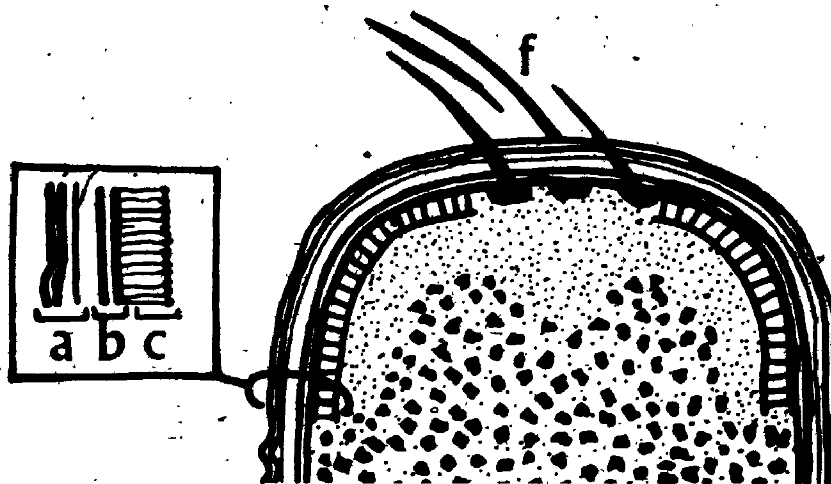
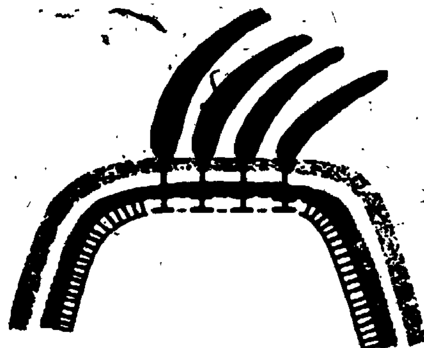


FIGURE 1

Figure 2

a. Schematic representation of the structural relationship between the cortical layers of the polar region of Rhodospirillum and the basal region of the flagella.

b. Schematic representation of the various components of the basal organelle of a flagellum and their position relative to the cell membrane and cell wall. A "flagellar membrane" is represented connecting the paired discs of the basal organelles to each other and to the polar membrane.
From Cohen-Bazire and London (1967). By permission, American Society for Microbiology, Washington.



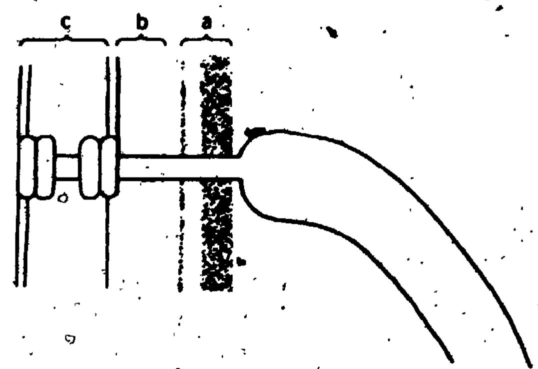


FIGURE 2

Figure 3

a. Diagram illustrating the fine structure of the basal portion of the flagellum of *Ectothiorhodospira mobilis*; basal discs (BD) and basal organelle (BO).

b. Insertion of the flagellum into the polar disc (PD); basal discs (BD) appear to "clamp" flagella in to polar disc.

c. Relationship of polar disc (PD) to polar plate (PP); flagella enter the polar disc through a central pore (P).

From Remsen *et al.* (1968). By permission, American Society for Microbiology, Washington.

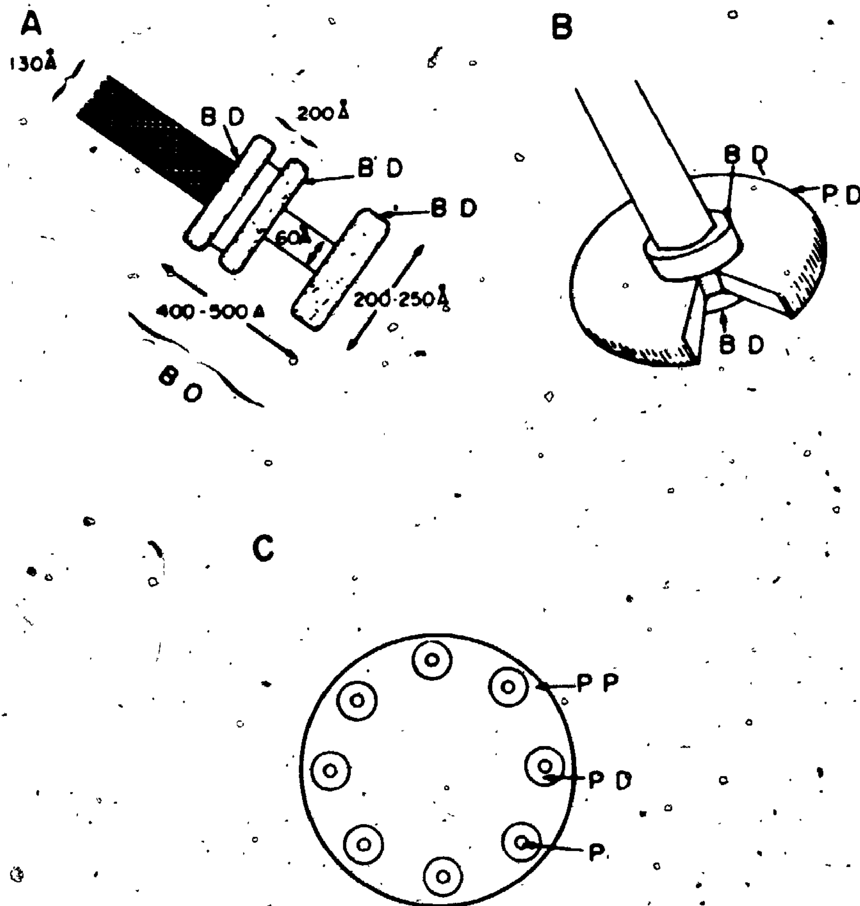


FIGURE 3

Figure 4

Model of the basal end of a flagellum from <u>Escherichia coli</u>. Dimensions are given in nanometers.
From DePamphilis and Adler (1971b). By permission, American Society for Microbiology, Washington.

Figure 5

Model of the attachment of the flagellar basal body of <u>Escherichia coli</u> to the cell envelope. Dimensions are expressed in nanometers. The basal body rings are shown being thinner than the cell envelope layers. The dimensions of the flagellar basal end come from negatively stained preparations, whereas the dimensions of the cell envelope layers come from thin-sectioned material.
From DePamphilis and Adler (1971c). By permission, American Society for Microbiology, Washington.

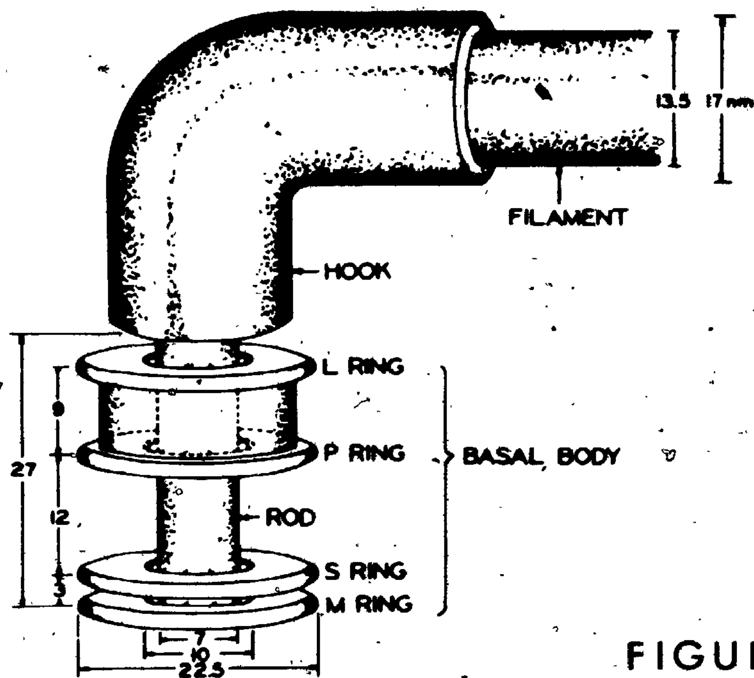


FIGURE 4

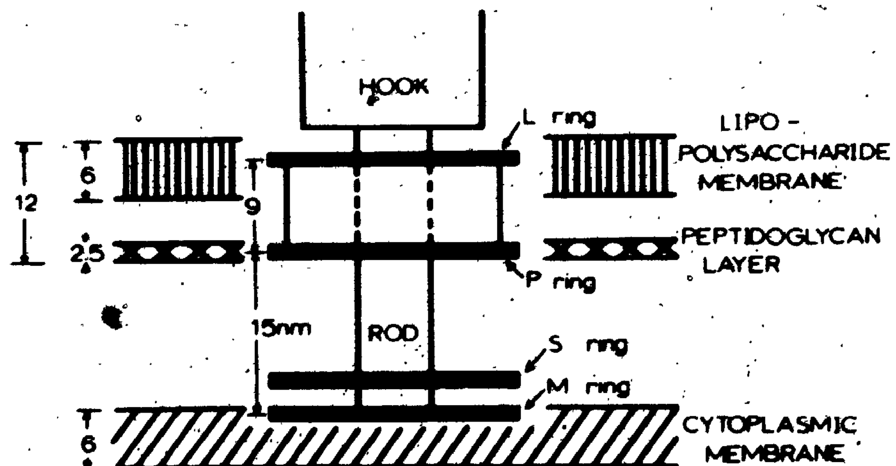


FIGURE 5

Figure 6

Growth curves of Spirillum serpens. Cells in complex liquid medium were monitored in a Klett-Summerson photoelectric colorimeter (filter 54) at either 37°C (x - x) or 42°C (Δ - Δ). At both temperatures, the generation time was estimated to be sixty to sixty-five minutes. Cell numbers (⊙ - ⊙) were determined using a Coulter counter for the culture growing at 37°C to provide a correspondance between turbidity measurements and number of bacteria.

FIGURE 6.

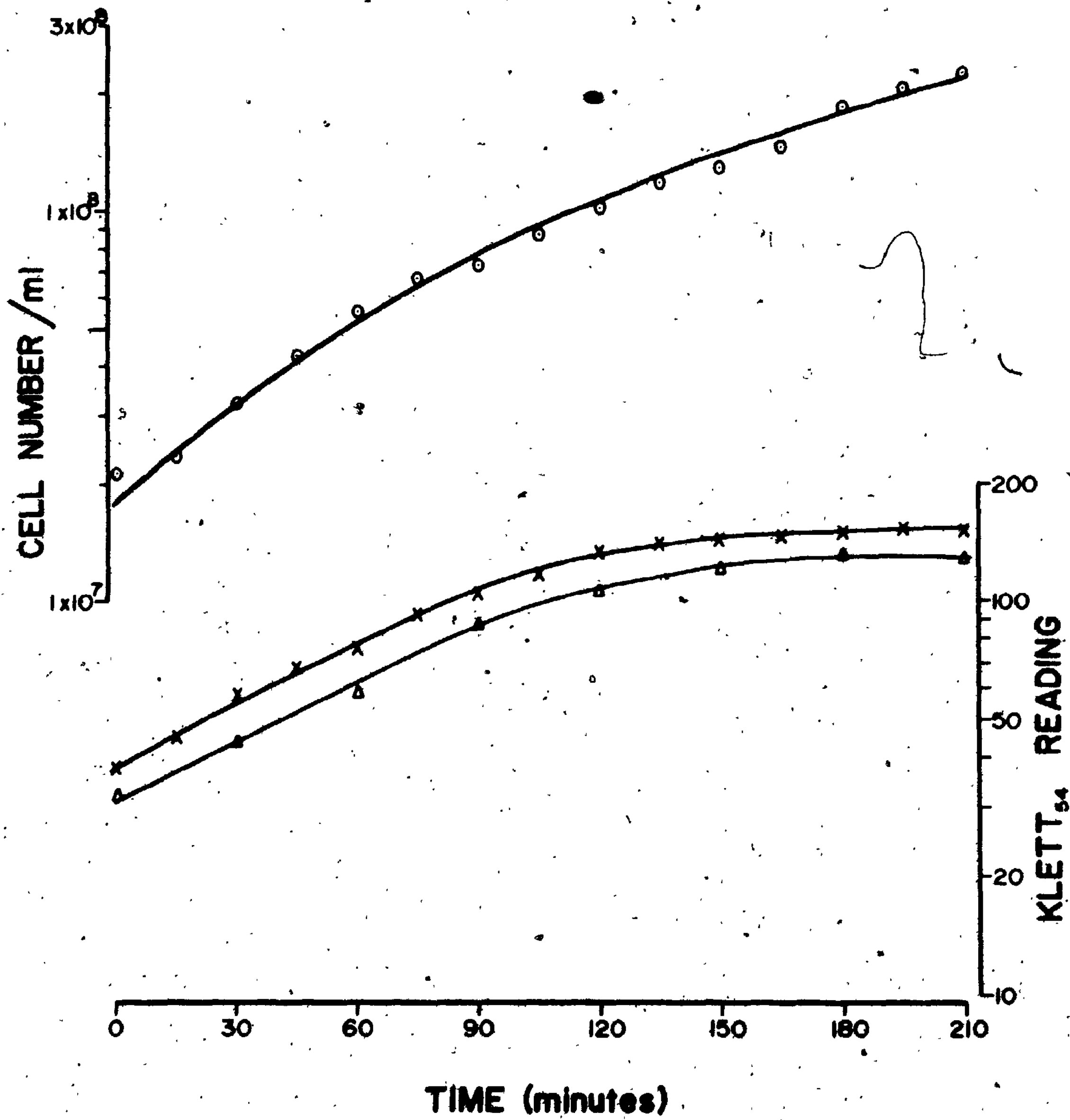


Figure 7

A new born cell of Spirillum serpens, showing tufts of flagella splayed from both polar regions of the cell. Negatively stained with 1% phosphotungstic acid. Bar equals 1 μm.

Figure 8

a. Flagellar filaments (F) isolated by shearing cells of Spirillum serpens in a blender, followed by differential centrifugations. The preparation is free of membrane contamination. Slightly curved hooks (H) are found attached to long or to short flagella, depending upon the point of shearing. Negatively stained with 1% phosphotungstic acid. Bar equals 0.5 μm.

b. Flagella were dissociated into monomers of flagellin by heating (60°C, twenty minutes). $NaDodSO_4$ electrophoresis demonstrates that this protein is a single molecular weight species of 45,000 daltons (well 1), migrating slightly behind ovalbumin of 43,000 daltons (well 2).

Figure 9

Fluorescence microscopy of flagella from <u>Spirillum serpens</u>.

a, b. Gamma globulin to flagella was coupled to fluorescein, and reacted with whole cells. Fluorescence of the conjugate indicates specificity of the antibody preparation for flagella.

c. Cells were pre-treated with gamma globulin and then reacted with the fluorescein conjugate. No labelling of the flagella is observed.

d. <u>Sp</u>. <u>serpens</u> reacted with fluorescein alone demonstrated non-specific partitioning of dye into the cell envelope; again, no flagella are labelled.

9a
9b
9d
9c

Figure 10

Intact basal organelle, hook (H), and filament (F) from _Spirillum serpens_. The basal organelle is composed of four rings: L, P, S, and M (nomenclature of DePamphilis and Adler, 1971b). A rod (R) connects the upper pair of rings to the lower pair of rings, and also emerges from the basal organelle to join the hook. Negatively stained with 1% phosphotungstic acid. Bar equals 100 nm.

10

Figure 11

The hook region (H) of the flagellum inserting into ring-like structures which form a plate (CMR's) lateral to the basal organelle. Lysed fragments of the cell envelope were negatively stained with 1% ammonium molybdate. Bar equals 200 nm.

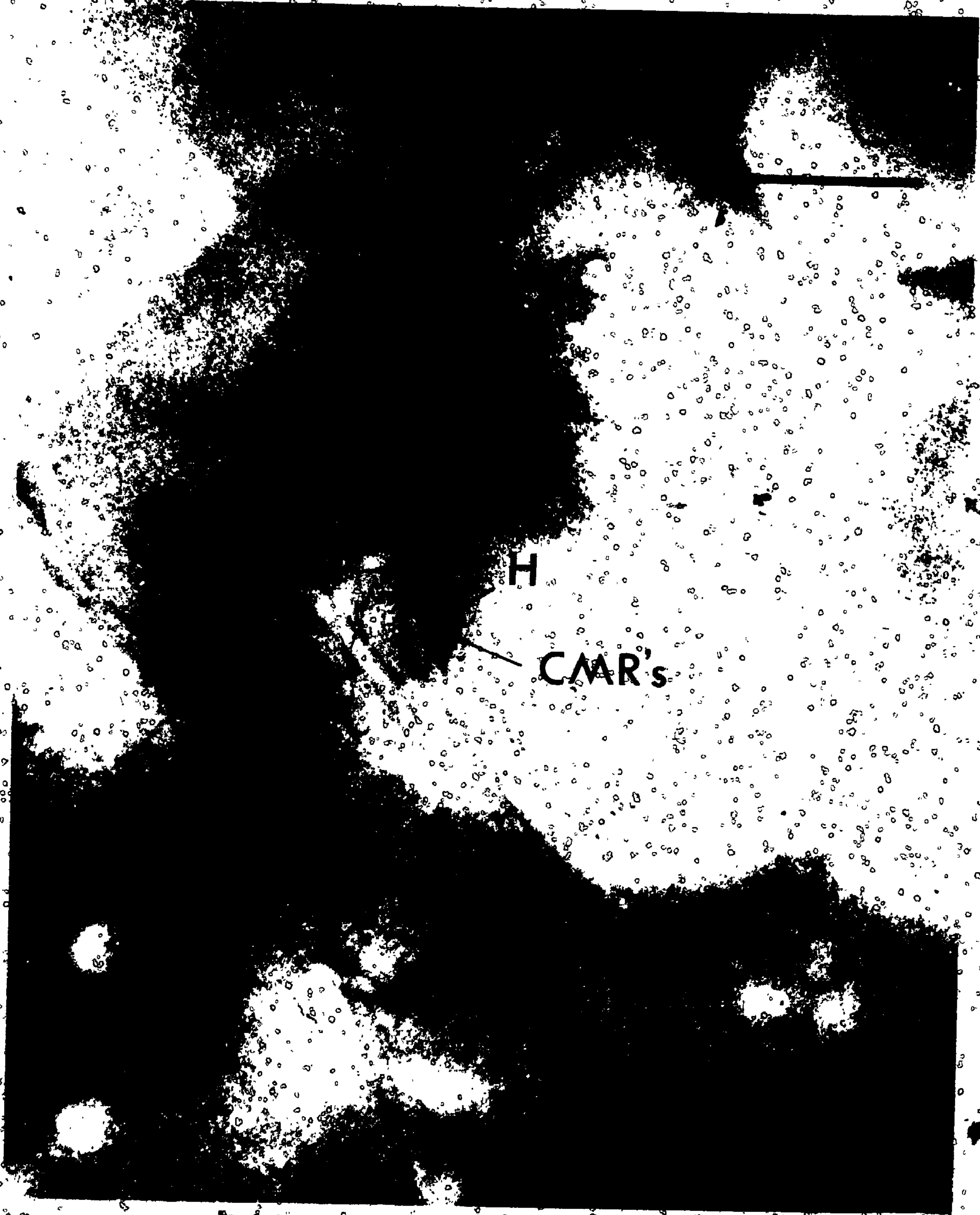
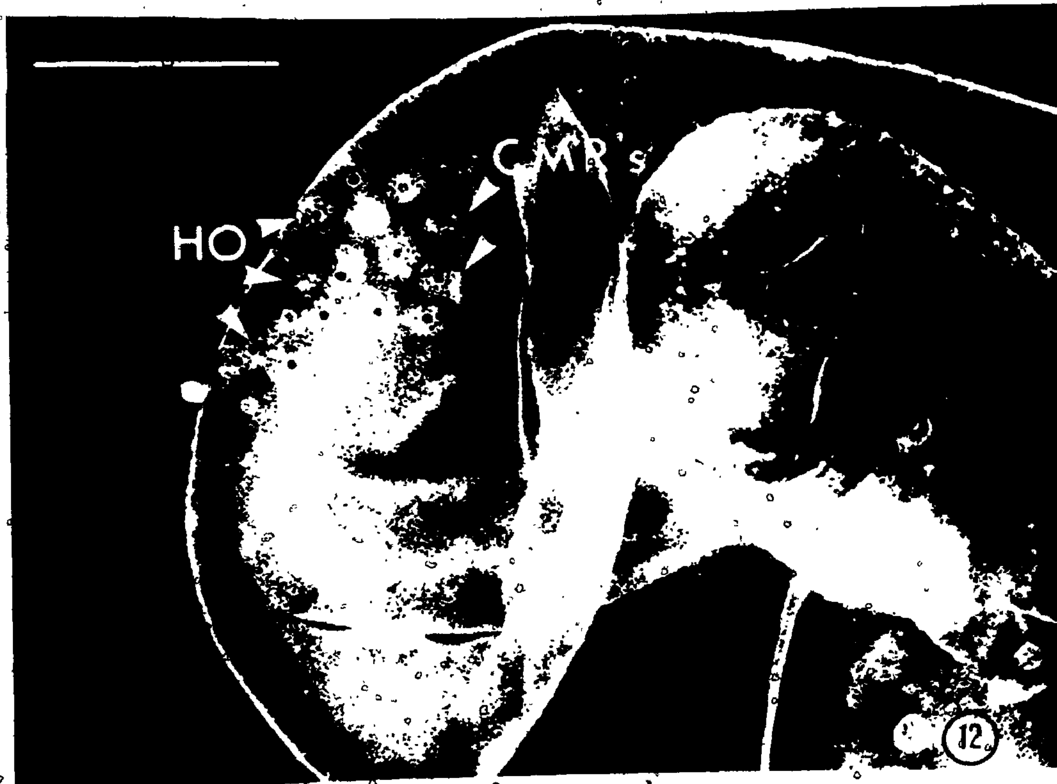

H
CMR's

Figure 12

The polar tip of Spirillum displays an array of holes (HO) into which flagella were once anchored. Sonication of the preparation sheared away the filaments, voided the cells of cytoplasm, and allowed for penetration of negative stain (1% phosphotungstic acid). In the immediate vicinity of the holes is the outline of an additional membrane-associated component of the flagellar apparatus (CMR's). Bar equals 0.5 µm.

Figure 13

Loosening of the cell envelope with Myxobacter AL-1 protease enhances resolution of the association of flagellum inserted into concentric membrane rings (CMR's). The underlying membrane (M) fragment persists. Negatively stained with 1% phosphotungstic acid. Bar equals 200 nm.


CMR's
HO
12

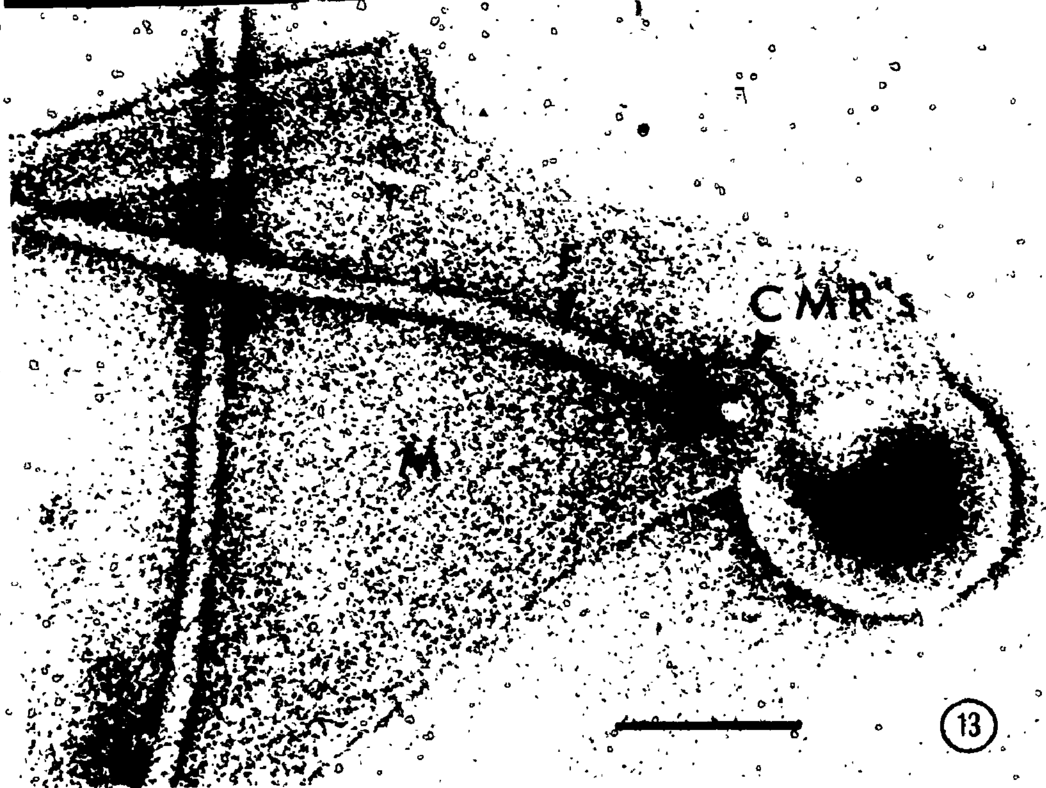
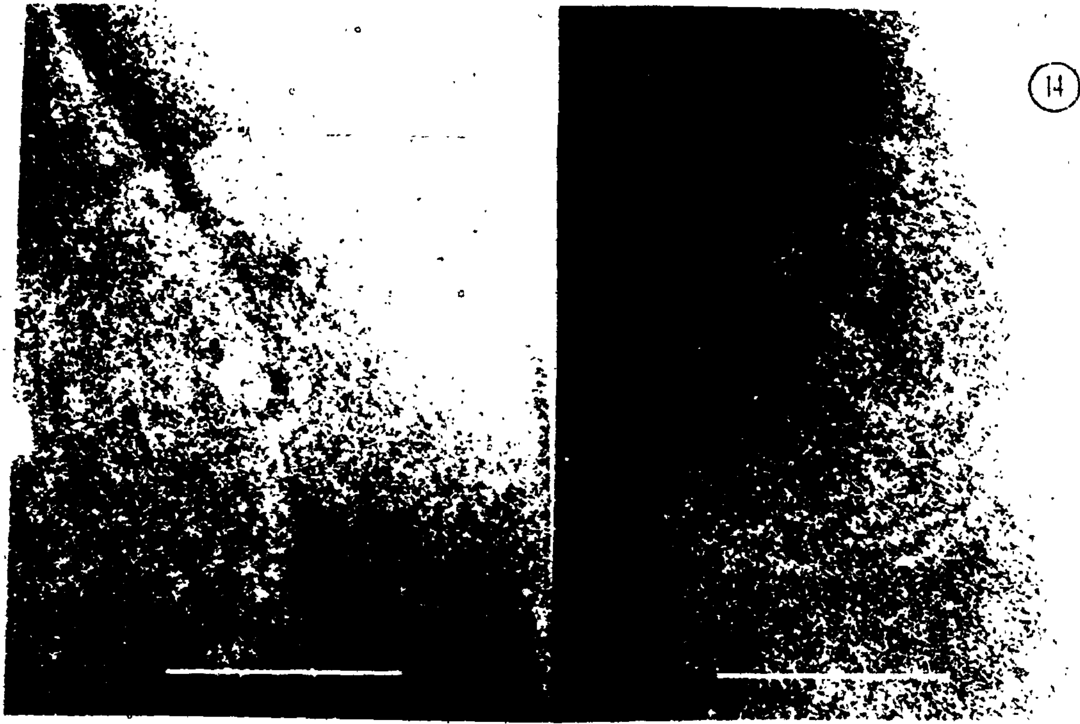
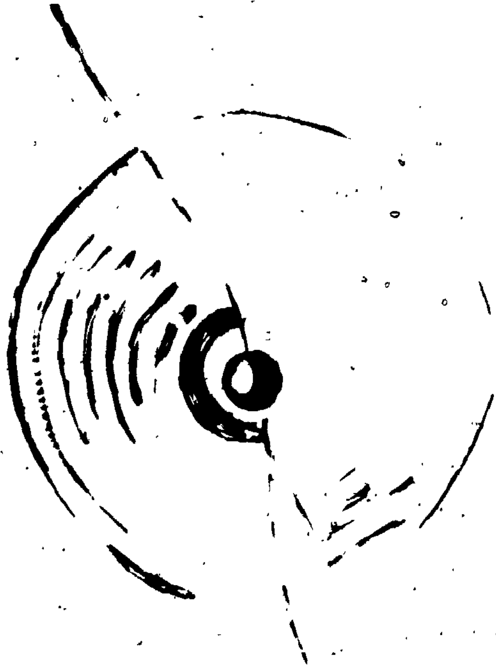

CMR's
M
13

Figure 14

High magnification of membrane rings (CMR's) obtained as Triton X-100 resistant material following spheroplasting. Freehand drawings serve to interpret the micrographs, showing sequestering of negative stain (1% ammonium molybdate) by these structures. A maximum of seven rings concentric to a central hole (12 nm) forms a plate of diameter 90 nm. An electron-translucent collar is located lateral to the central hole. Bar equals 50 nm.





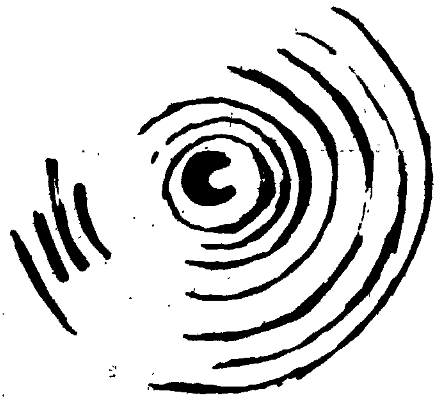


Figure 15

A composite plate of concentric membrane rings from four different preparations. Constancy and uniformity of structure are demonstrated: each ring of the array is discretely spaced from its adjacent ring; the central hole into which stain collects is 12 nm in diameter and is surrounded by a collar in all instances. Negatively stained with 1% phosphotungstic acid (a) or 1% ammonium molybdate (b, c, d). Bar indicates 50 nm.

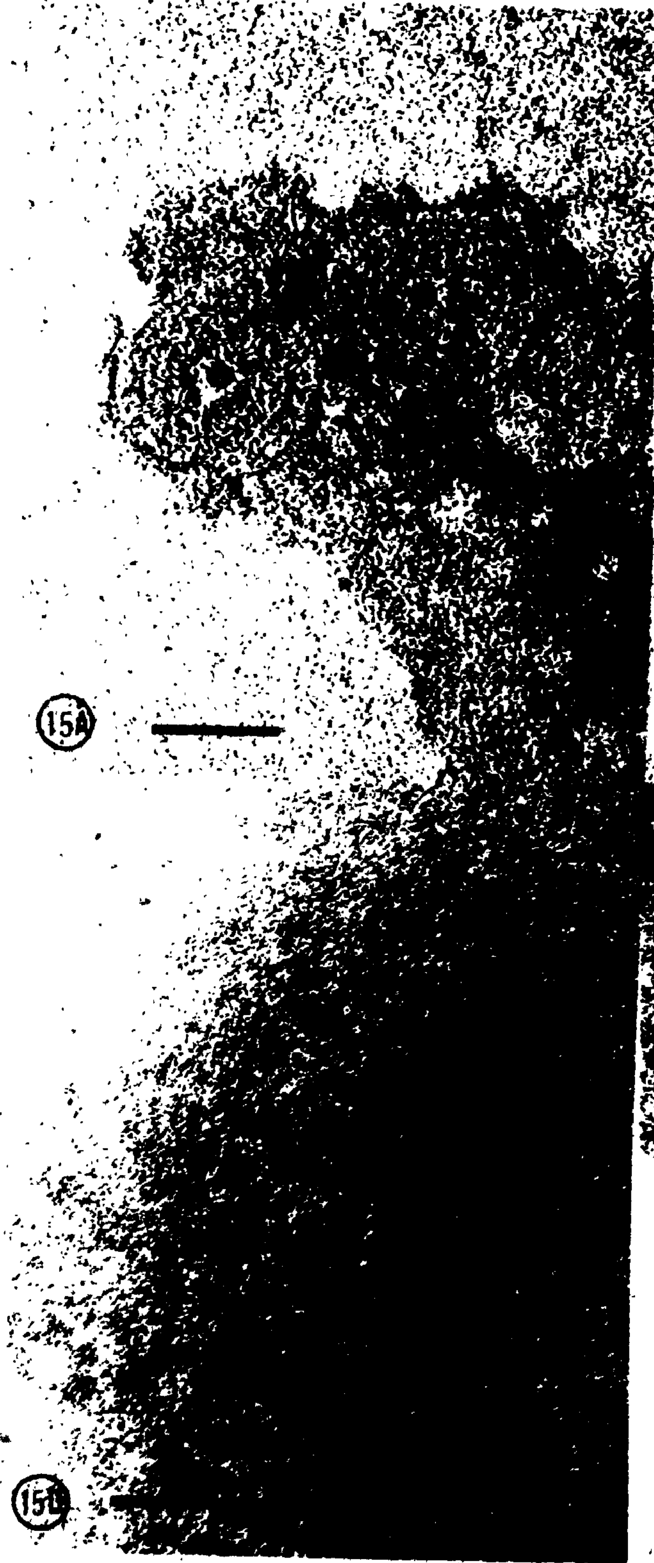
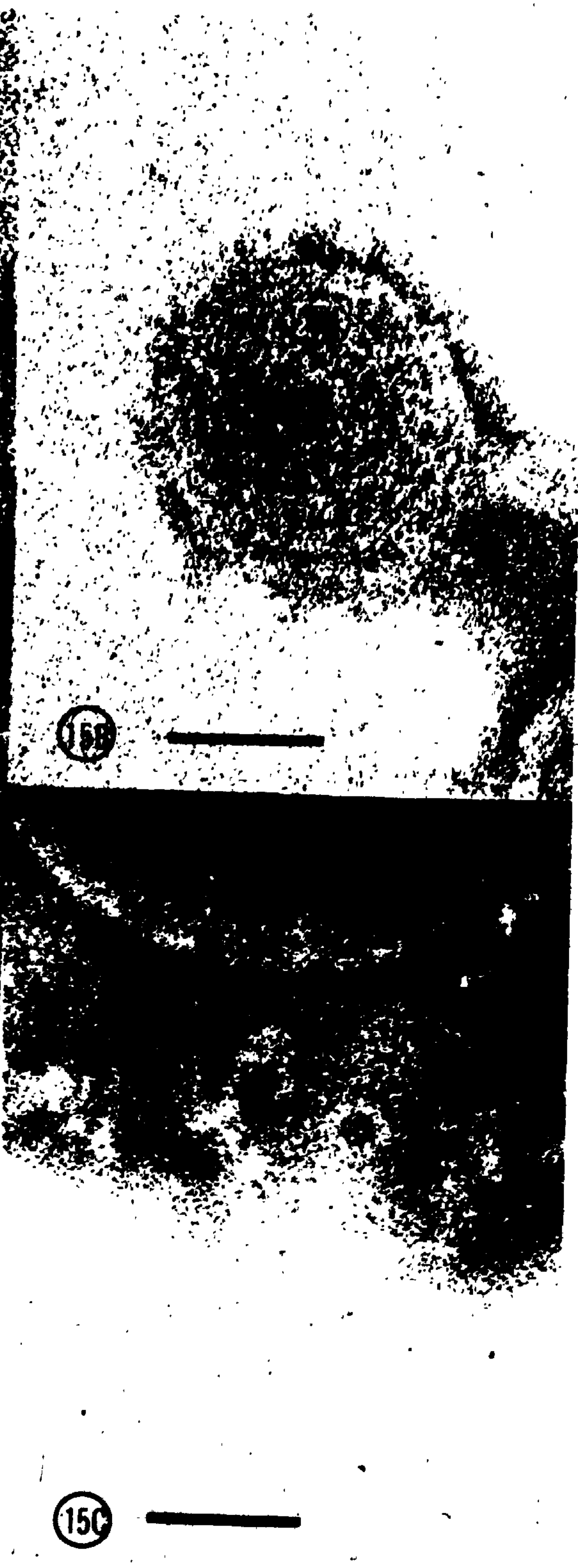

15A
15D

15B
15C

Figure 16

Thin section of a spheroplast of *Sp. serpens*. The outer membrane (OM) has peeled away from underlying cytoplasmic membrane (CM), leaving vesicles (V) on its lower surface. Lesions (L) in outer membrane are indicated by discontinuity. The cytoplasm is still densely packed with ribosomes (R) and condensed nucleoid (N). Fixation: 2% glutaraldehyde, followed by post-fixation with 1% OsO_4 and 1% uranyl acetate. Embedding was in Epon 812. Bar equals 0.5 μm.

CM
V
L

Figures 17 and 18

Lysis of spheroplasts of Sp. serpens by osmotic shock maintains the orientation of outer membrane (OM) surrounding inner membrane, and does not produce small size vesicles. Cytoplasmic membrane re-seals itself into smaller vesicles (CMV) as shown both by thin sections (Figure 17) and by negative staining with 1% ammonium molybdate (Figure 18). Bar equals 0.5 μm.

OM
CMV
17

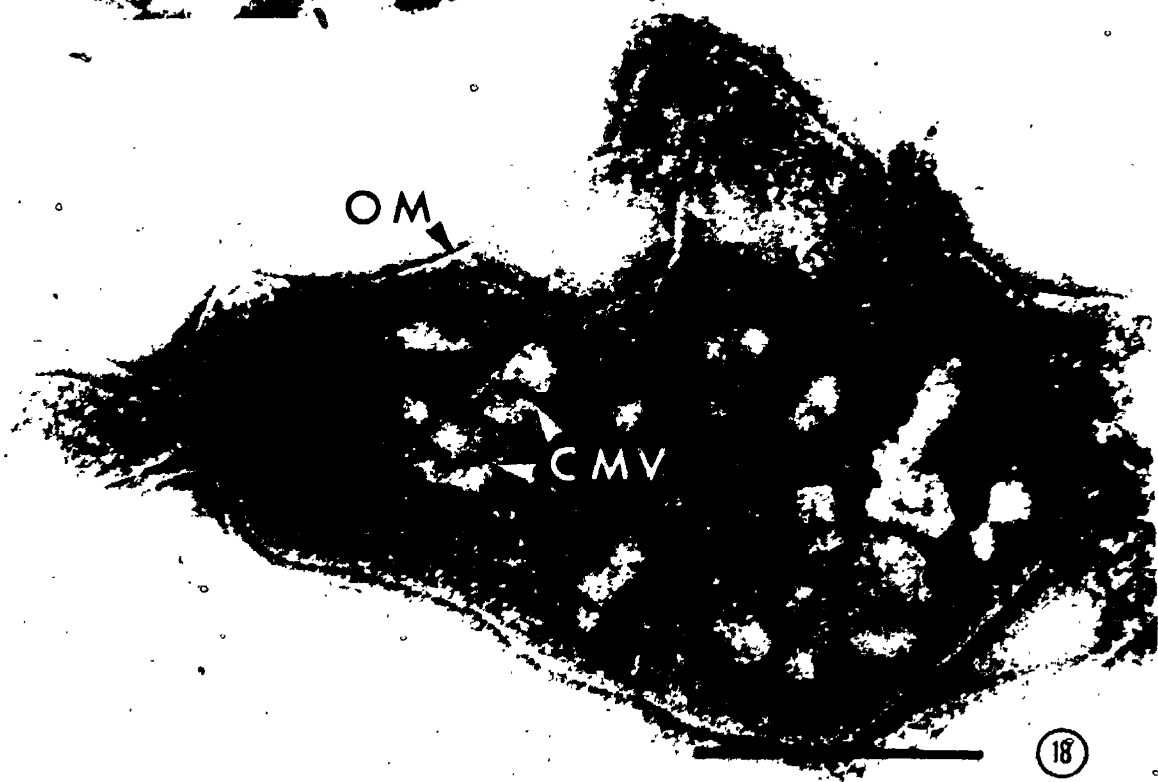

OM
CMV
18

Figure 19

Spheroplasts lysed by sonic oscillation, yielding a "total membranes" preparation. Outer membrane (OM) displays a "pitted" appearance by negative staining (1% ammonium molybdate) whereas cytoplasmic membrane (CM) is densely packed with granular material on its surface. Bar indicates 0.5 µm.

19

Figure 20

Elution profile following sucrose density gradient centrifugation of total membranes preparation. The buoyant density of membranes in sucrose is shown in gm/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: d-LDH (△-△) and SDH (●-●). Two peaks of membranes were identified: cytoplasmic membrane (ρ = 1.17) and outer membrane (ρ = 1.25).

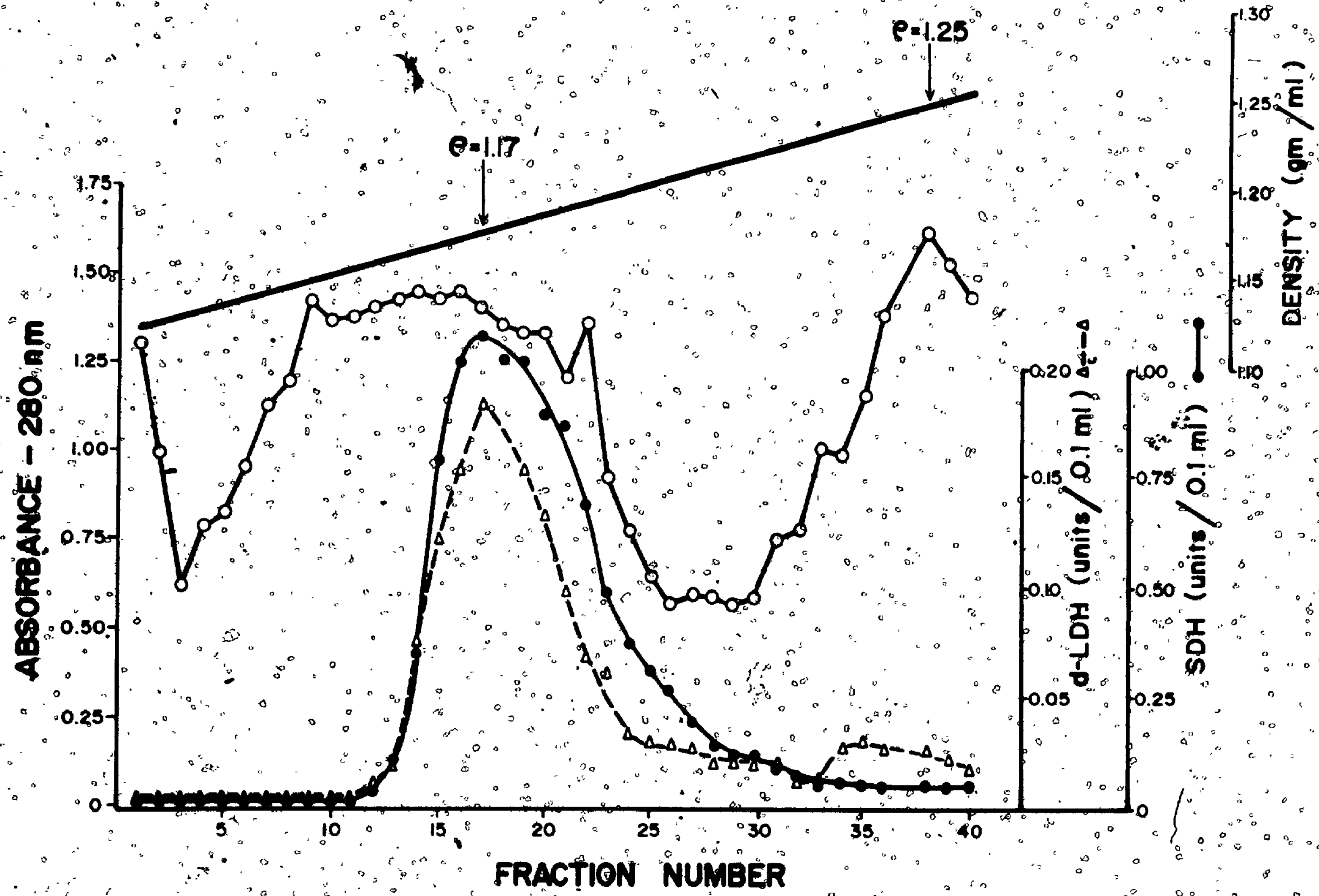


FIGURE 20

Figure 21

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.

21

Figure 22

Thin section of outer membrane vesicles of *Sp. serpens*. The collapsed spheres are devoid of cytoplasmic contents, and exhibit a "double track" membrane profile (arrow). Fixation: 2% glutaraldehyde in 0.10 M sodium cacodylate buffer, pH 7.8; post-fixation in 1% OsO_4 in cacodylate buffer, and 0.5% uranyl acetate. Embedding was in Epon 812. Bar equals 200 nm.

Figure 23

Freeze etching of outer membrane vesicles in the presence of glycerol as cryoprotectant. The membranes appear as closed spheres which have been crossed cleaved to reveal unit membrane structure (arrow). Bar equals 200 nm.

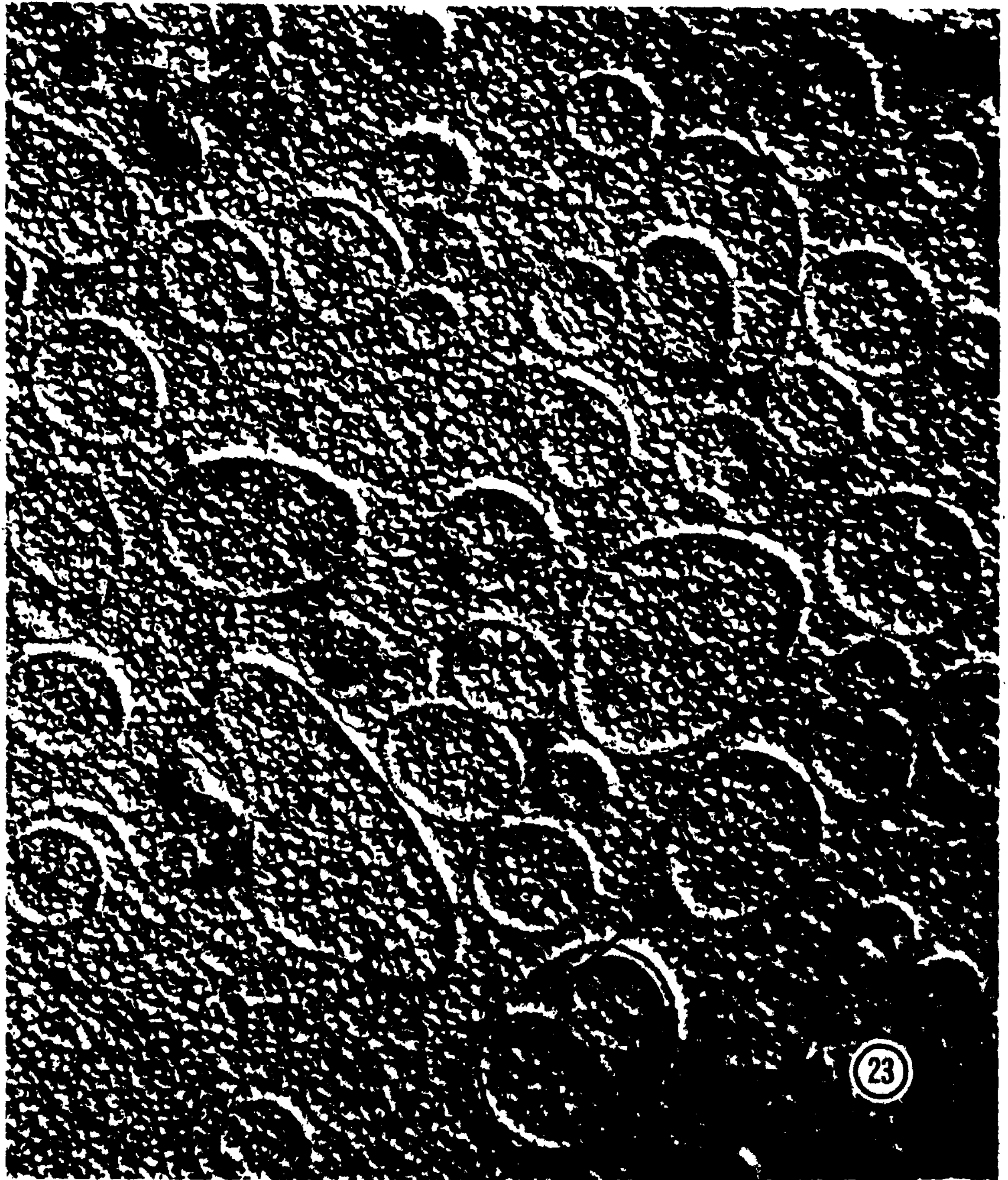

23

Figure 24

Freeze etching of outer membrane vesicles with glycerol for cryoprotectant. Fracture faces of membrane vesicles demonstrate granular particles on the convex surface (a, b) but sparse particle distribution on the concave cell wall layer (c, d, e). In the absence of glycerol, the outer pitted surface of outer membrane is revealed (f). Direction of shadowing with platinum and carbon is indicated by the arrow. Bar indicates 200 nm for all micrographs.

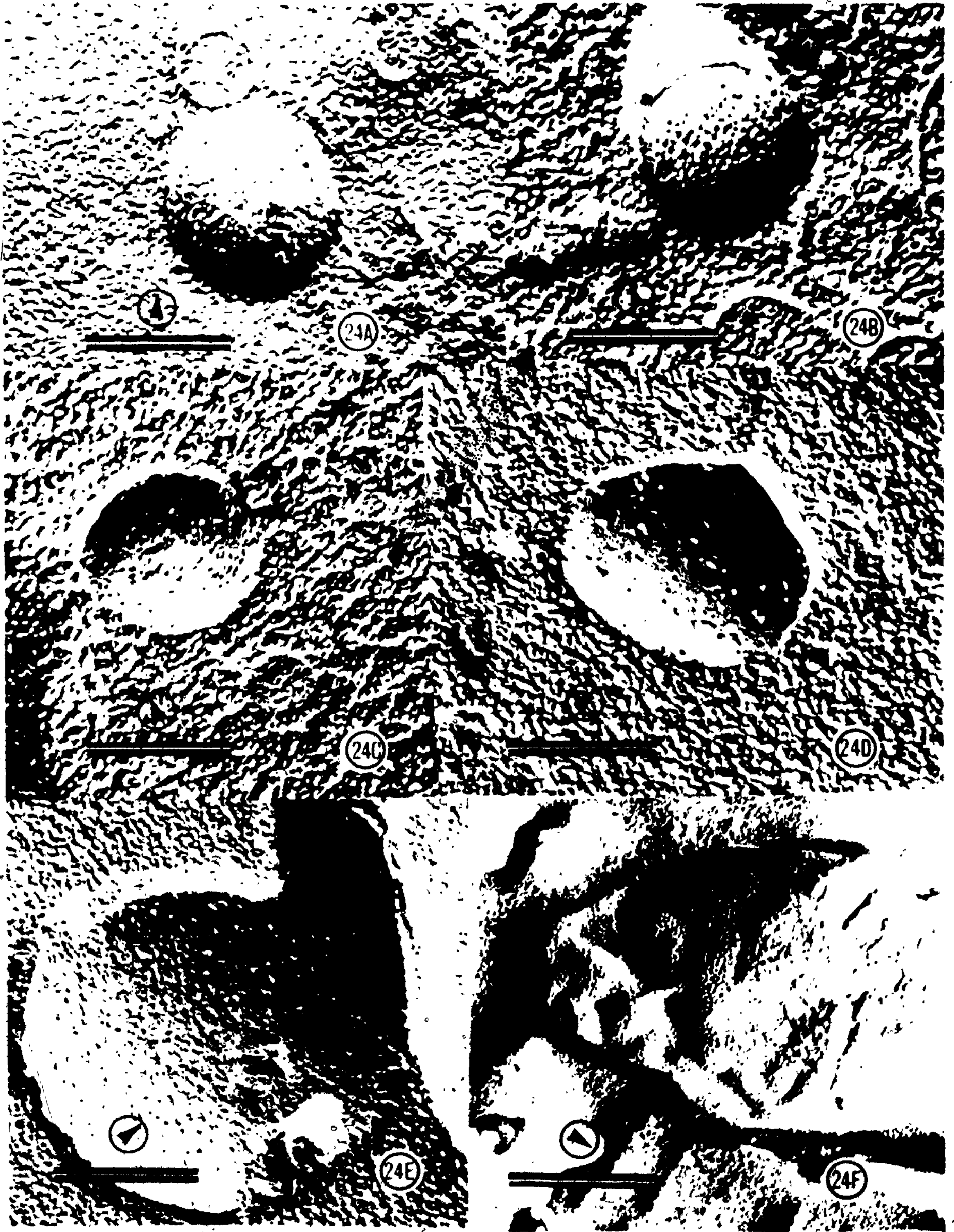

24A
24B
24C
24D
24E
24F

Figure 25

Negatively stained preparation (1% phosphotungstic acid) of cytoplasmic membranes harvested from the light region of the sucrose gradient (ρ = 1.17). Membranes are irregular flat sheets, not discretely collapsed spheres as shown in Figure 21. Bar equals 200 nm.

Figure 26

Thin section of cytoplasmic membranes, showing vesicles within vesicles. Fixation: 2% glutaraldehyde in 0.10 M sodium cacodylate, pH 7.8; post-fixation in 1% OsO_4 in cacodylate buffer, and 0.5% uranyl acetate. Embedding was Epon 812. Bar equals 200 nm.

25

26

Figure 27

Spheroplasts of Sp. serpens lysed by 1% Triton X-100. The outer membrane (OM) is resistant to morphological dissolution by this non-ionic detergent, and is shown as lamellae of vesicles. Cytoplasmic membrane (CM) has aggregated into clumps. Concentric membrane rings (CMR's) are observed superimposed on outer membrane. Negatively stained with 1% ammonium molybdate. Bar equals 200 nm.

27

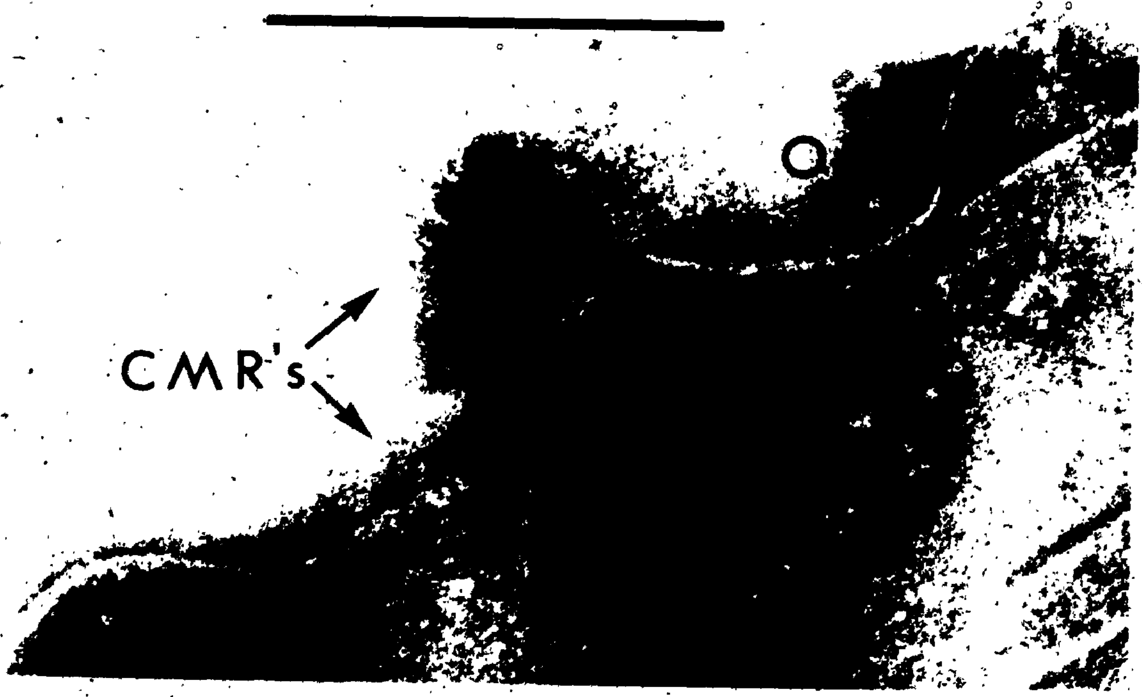


Figure 28

A series of electron micrographs showing outer membrane vesicles with concentric membrane rings (CMR's) after digestion with Myxobacter AL-1 protease.

a. Some CMR's appear accentuated on the vesicle after fifteen minute digestion.

b. Prolonged incubation of vesicles for seventy-five minutes completely digests the concentric rings, but the electron translucent collar (C) is not susceptible to proteolysis.

c. Aggregation of the collar remnants indicates possible redistribution of these structures over the surface of the vesicle.

Negatively stained with 1% ammonium molybdate. Bar equals 200 nm.

CMR's
28

C
28B

28C

Figure 29

Treatment of outer membrane vesicles of *Sp. serpens* with phospholipase-C. CMR's are not digested by such treatment, but lose the integrity of the rings, and seem to be in relief on the vesicle surface. Stained with ammonium molybdate (1%). Bar equals 200 nm.

29
CMR's

CMR's

Figure 30

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

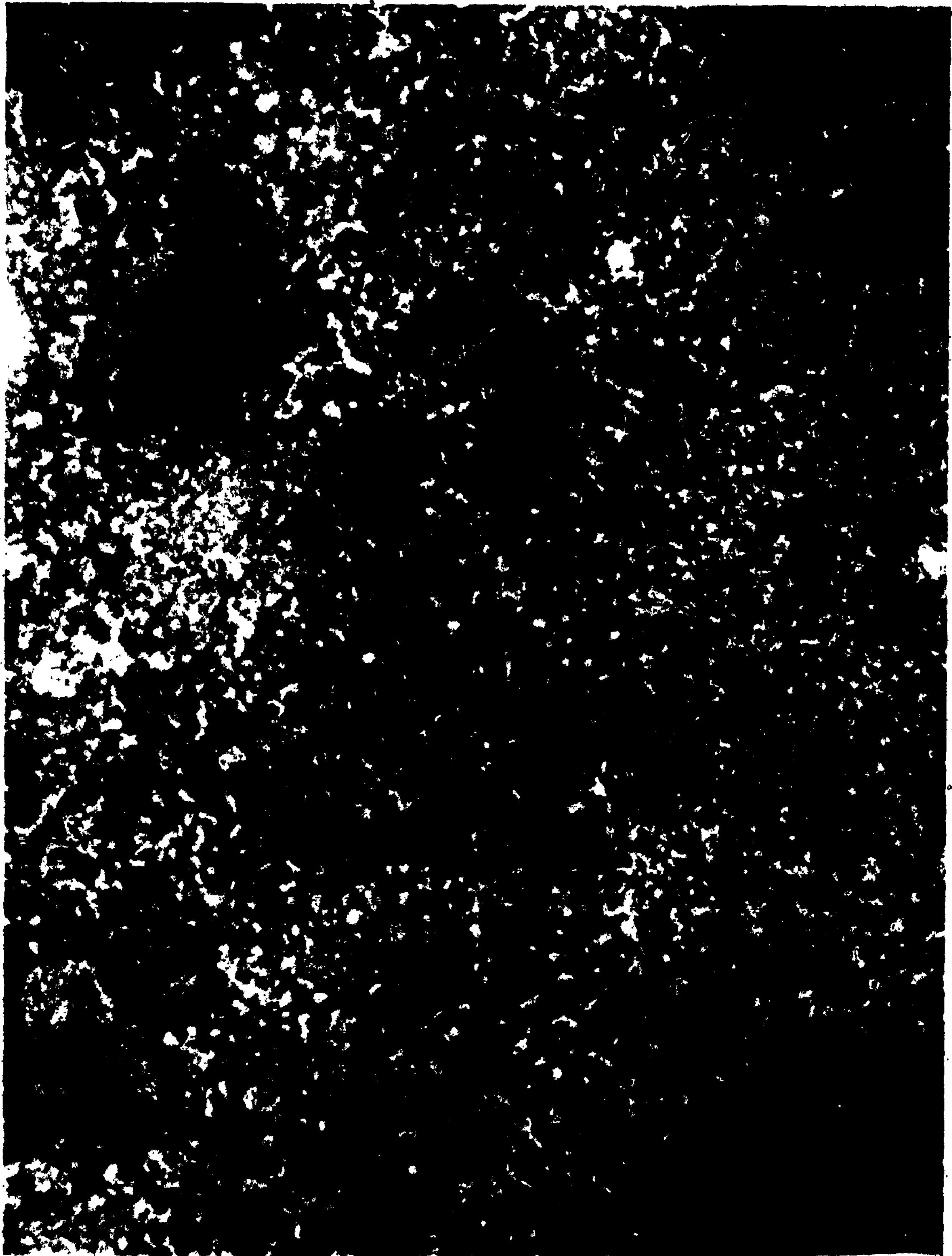


Figure 31

Non-ionic detergent (Brij-58), when added to membranes at a concentration of 2%, caused the vesicles to reseal into smaller diameter spheres, and no CMR's were recovered. Negatively stained with 1% ammonium molybdate. Bar equals 200 nm.

31

Figure 32

Dialysis of outer membranes against 5.0 mM EDTA, pH 7.5, usually left CMR's overlying the vesicles (Figure a). In some instances, this treatment was effective in removing the structures from the membrane (Figures b, c), allowing them to float free in suspension. Negatively stained with 1% ammonium molybdate. Bar equals 200 nm.

CMR's

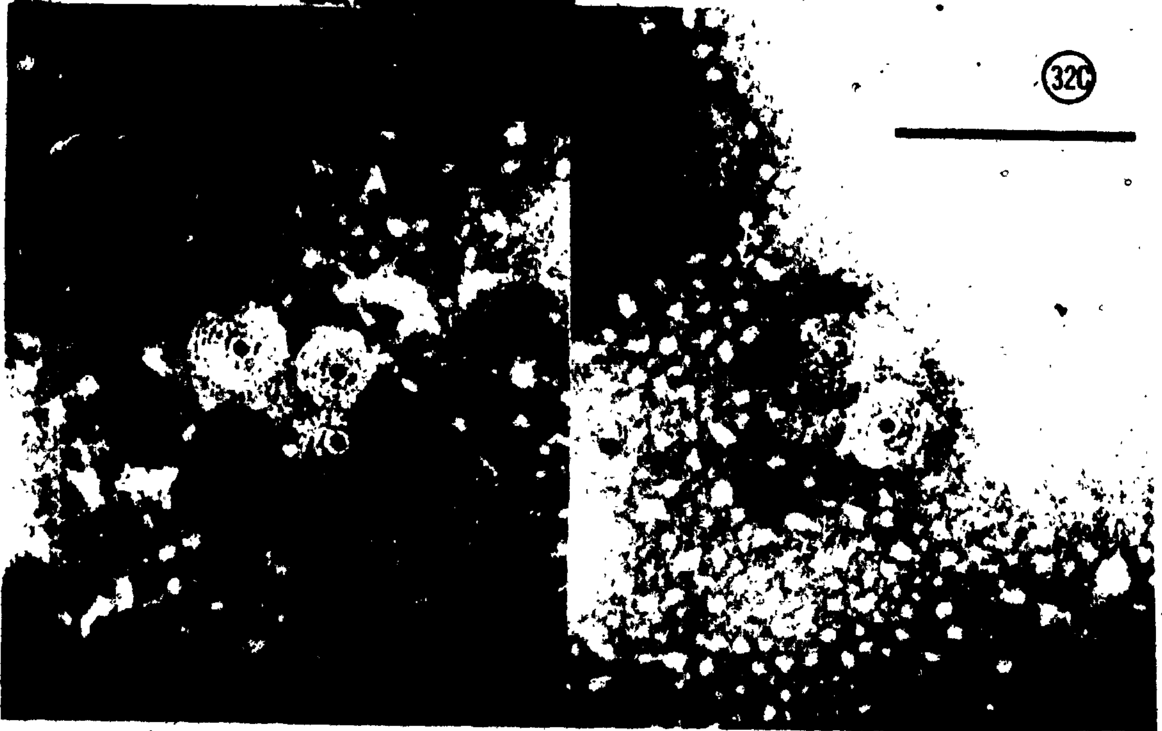


Figure 33

$NaDodSO_4$-polyacrylamide gel electrophoresis of outer membrane proteins. Sp. serpens was labelled with ^3H-aspartic acid at 42°C or with ^{14}C-aspartic acid for 30°C grown cells. Outer membranes were prepared from pooled cell cultures. Solubilization conditions to liberate membrane proteins: 1% $NaDodSO_4$ + 1% β-mercaptoethanol, 70°C, thirty minutes. Direction of electrophoresis is from left to right. The gel was sliced into 140 x 1 mm sections and counted for ^3H and ^{14}C.

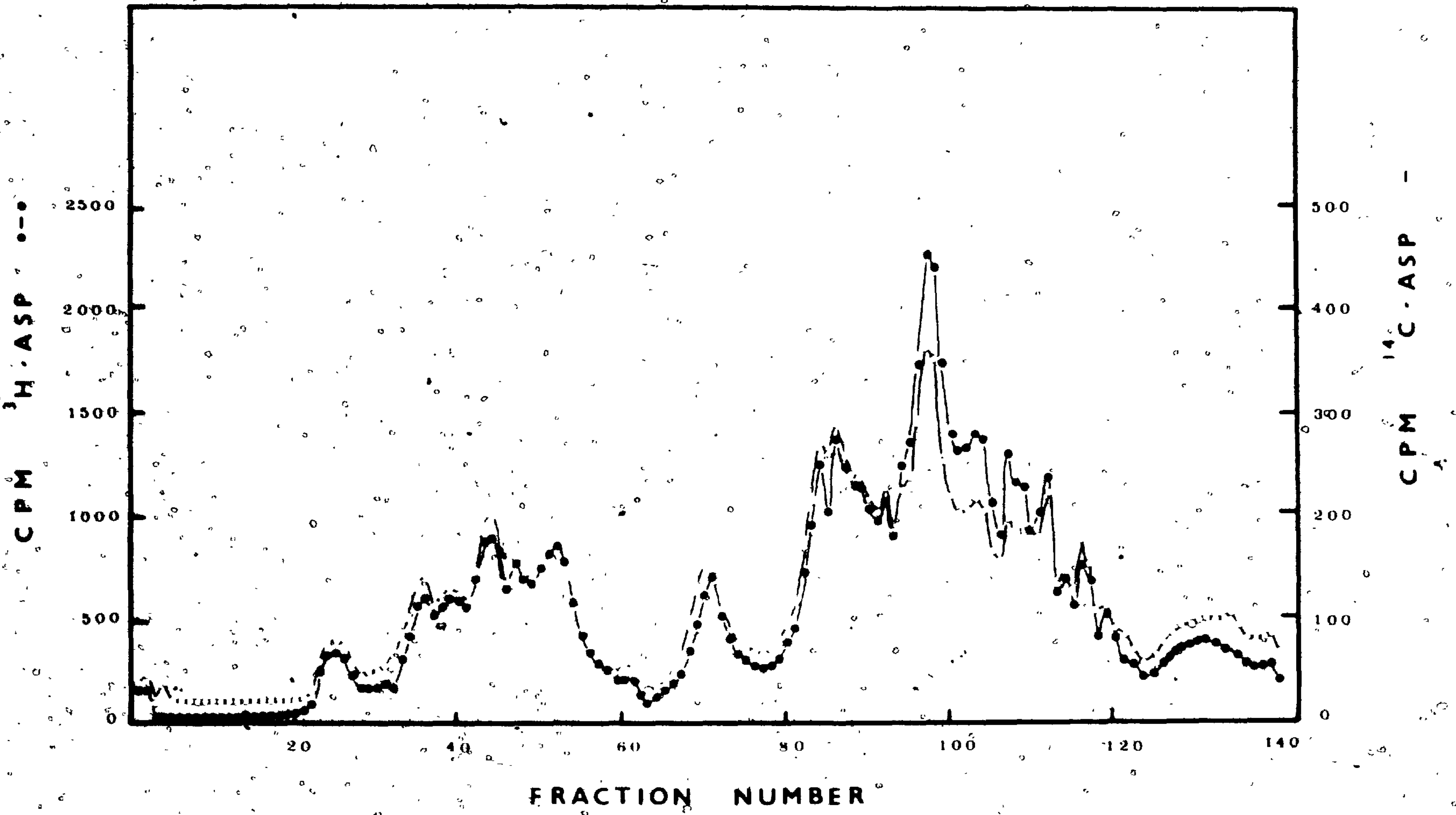


FIGURE 33

Figure 34

$NaDodSO_4$-polyacrylamide gel electrophoresis of outer membrane proteins. Same preparation and electrophoresis conditions as in Figure 33, except that solubilization of membranes employed 1% $NaDodSO_4$ + 1% β-mercaptoethanol, 100°C, three minutes. Proteins between fractions 20 and 60 appear to be heat-labile by comparison with the 70°C, thirty minute treatment (Figure 33).

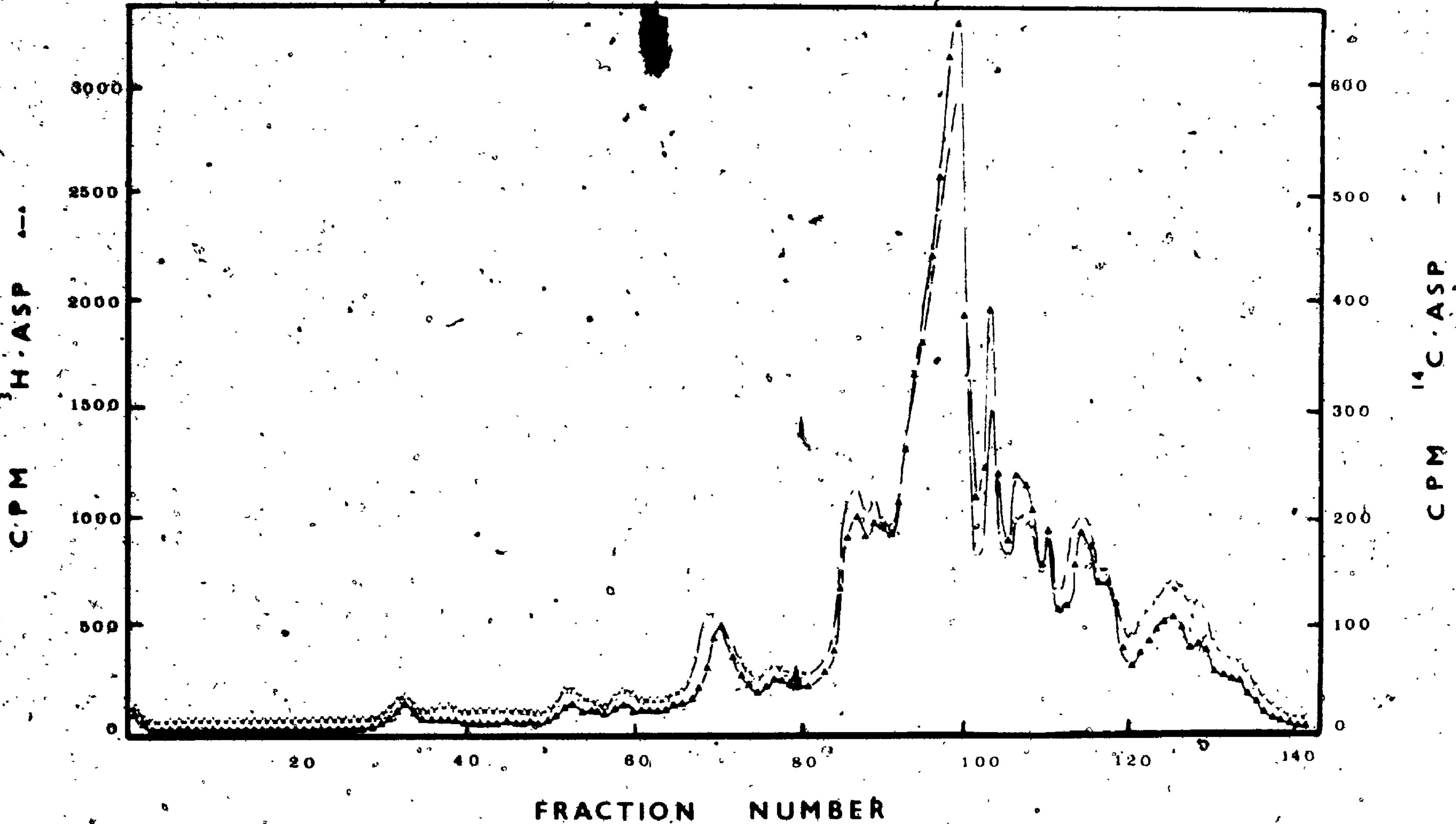


FIGURE 34

Figure 35

$NaDodSO_4$-polyacrylamide gel electrophoresis of outer membrane proteins. In lieu of slicing and counting for radioactivity, the ^{3}H and ^{14}C-aspartate-labelled membrane proteins were stained with Coomassie brilliant blue.

a. Membranes solubilized with 1% $NaDodSO_4$ + 1% β-mercaptoethanol, 70°C, thirty minutes.

b. Membranes solubilized with 1% $NaDodSO_4$ + 1% β-mercaptoethanol, 100°C, three minutes.

a b

35

Figure 36

Schematic representation of the crossed immunoelectrophoresis patterns obtained by using proteins solubilized from outer membrane vesicles of *Sp. serpens* as antigens, and reacting them against gamma globulins raised to membrane vesicles derived from 30°C grown cells.

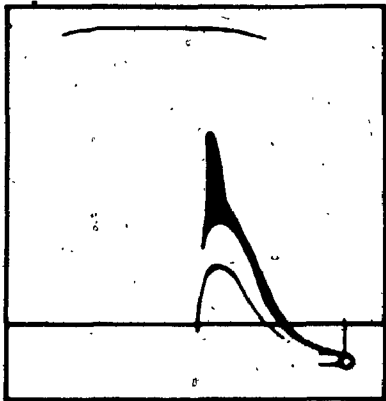
A. An outer membranes suspension was clarified by incubation in 1% $NaDodSO_4$ + 1% β-mercaptoethanol, 70°C, thirty minutes. The preparation was then dialyzed against barbital-HCl buffer, I = 0.02, pH 8.6, containing 0.1% Triton X-100, and applied to the antigen well. The second dimension gel contained 1030 µg gamma globulin protein in 50 µl.

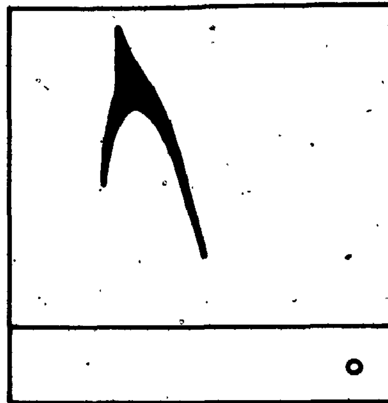
B. Same preparation and solubilization conditions as A, but without dialysis against barbital-Triton X-100 buffer. A decreased amount of immunoglobulins (412 µg in 20 µl) yielded a higher peak height of the major precipitin as compared to its height in A.

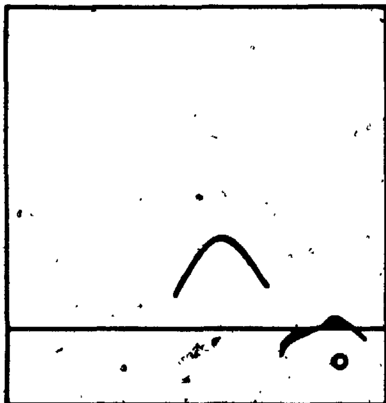
C. Treatment of outer membranes with 4% Triton X-100 (R.P.I.) was followed by centrifugation of the sample to give a white residue and a clear supernatant. The latter was decanted and used directly as antigen sample; the antiserum contained 1030 µg protein. Despite prolonged electrophoresis in the first dimension (more than three hours), it was not possible to significantly displace the antigens from the origin.

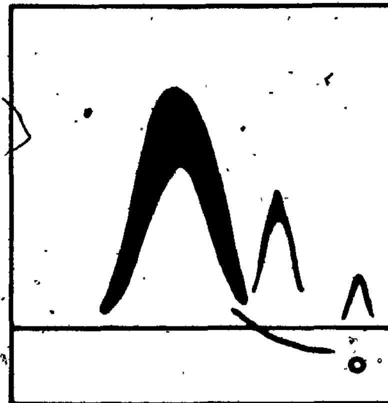
D. Dodecyl trimethyl ammonium bromide (DTAB) was added to outer membranes to a final concentration of 250 mM. After incubation at 37°C for sixty minutes, the preparation was dialyzed against barbital-Triton X-100 buffer, and the recovered sample transferred to the CIE slide. The gamma globulin incorporated into the second dimension contained 1030 µg protein.

E. Total membranes were prepared as described in the text, incubated with 4% Triton X-100 (R.P.I.) for eight hours at 37°C. The sample was centrifuged to yield a vesicle-free supernatant, and this supernatant then constituted the antigen preparation. 412 µg gamma globulins were used for immunoprecipitation.


A


B


C


D

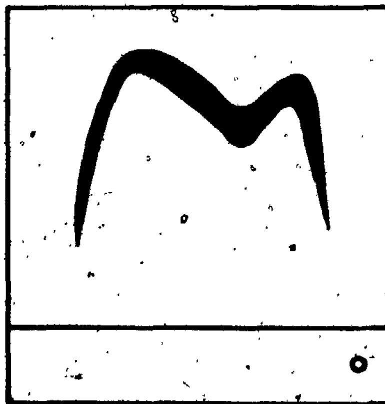

E

FIGURE 36

Figure 37

Polyacrylamide gel electrophoresis of outer membrane proteins from *Sp. serpens*. A small sample of membranes (20 μl containing 112 μg proteins) was incubated with 5 μl of a concentrated solution of $NaDodSO_4$ and β-mercaptoethanol, yielding three different final concentrations; two temperatures were used for the solubilization. After this treatment, 10 μl of glycerol was added, the samples were applied to the wells of the 7.5% acrylamide slab gel containing 0.1% $NaDodSO_4$, and electrophoresis was carried out as described in Materials and Methods. The staining reagent for proteins was Coomassie brilliant blue.

Well 1 0.1% $NaDodSO_4$ + 0.1% β-mercaptoethanol; 37°C, sixteen hours

Well 2 0.1% $NaDodSO_4$ + 0.1% β-mercaptoethanol; 70°C, thirty minutes

Well 3 0.5% $NaDodSO_4$ + 0.5% β-mercaptoethanol; 37°C, sixteen hours

Well 4 0.5% $NaDodSO_4$ + 0.5% β-mercaptoethanol; 70°C, thirty minutes

Well 5 1.0% $NaDodSO_4$ + 1.0% β-mercaptoethanol; 37°C, sixteen hours

Well 6 1.0% $NaDodSO_4$ + 1.0% β-mercaptoethanol; 70°C, thirty minutes

1 2 3 4 5 6

(37)

Figure 38

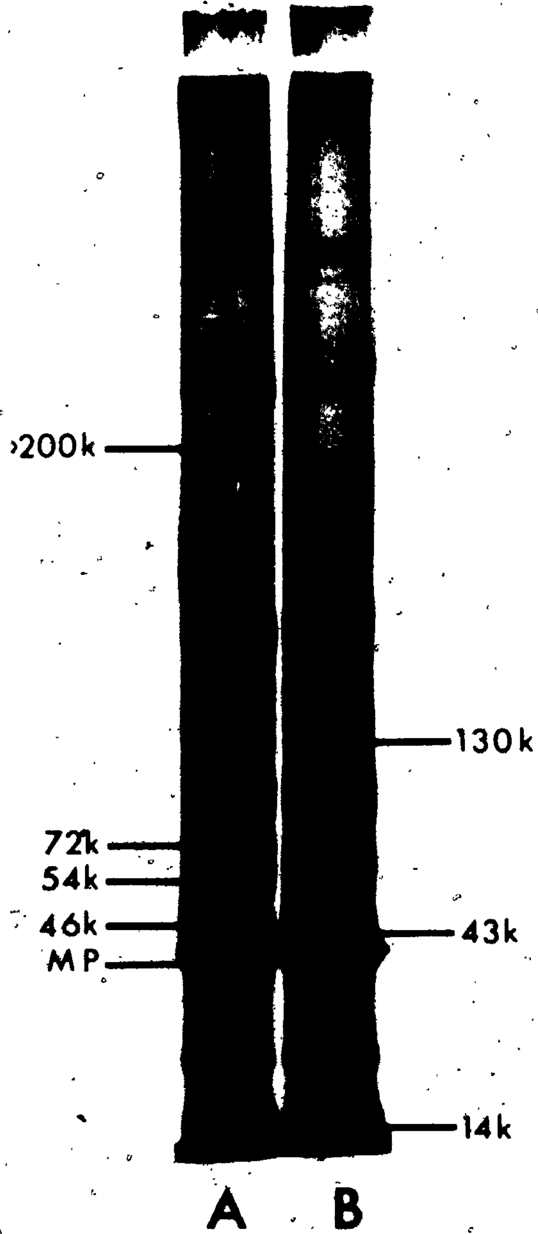
Maximum release of proteins from outer membrane vesicles, employing 1% $NaDodSO_4$ + 1% β-mercaptoethanol at 100°C for three minutes. Outer membranes (20 μl containing 100 μg protein) were incubated with the above detergent, and applied directly to the well of the PAGE slab gel. A total of twenty-three protein staining bands could be counted by visual inspection, although they do not all appear upon photographic reproduction.

Figure 39.

Autoradiogram of ^{35}S-methionine labelled outer membrane proteins of *Sp. serpens*. Two cultures, one of cells grown at 30°C and the other of cells grown at 42°C, were separately labelled with radioactive methionine. Outer membranes from each were prepared, and were solubilized in 1% $NaDodSO_4$ + 1% β-mercaptoethanol, 100°C, three minutes. Electrophoresis was in 7.5% acrylamide. Standards included lysozyme (14k), ovalbumin (43k), β-galactosidase (130k).

Well A. Outer membrane proteins, 30°C grown cells.
Well B. Outer membrane proteins, 42°C grown cells.

Four bands are distinctly absent at the higher temperature but appear in the spectrum of 30°C membrane proteins; they are labelled >200k, 72k, 54k, and 46k. The major outer membrane protein (MP) has an estimated molecular weight of 38,000 daltons.



39

Figure 40

Reaction of ferritin-gamma globulin conjugate with outer membrane vesicles displaying CMR's. The gamma globulin specificity was directed against 72k protein. Ferritin cores are superimposed on CMR's. Labelling of other areas of the vesicle is minimal, as is background labelling. Negatively stained with 1% ammonium molybdate. Bar equals 200 nm.

Figure 41

Reaction of ferritin-gamma globulin *vs* 46k protein with outer membrane vesicles from *Sp. serpens*.

Figure 42

Reaction of ferritin-gamma globulin *vs* 54k protein with outer membrane vesicles from *Sp. serpens*.

Figure 43

Reaction of ferritin-gamma globulin *vs* >200k protein with outer membrane vesicles from *Sp. serpens*.

For all of the above three reactions, ferritin does not appear aggregated in the area of CMR's. The specificity of the coupled antisera is therefore not directed against 46k, 54k, or >200k protein. Negatively stained with 1% ammonium molybdate. Bar equals 200 nm.

41
FE
FE
43
CMR's

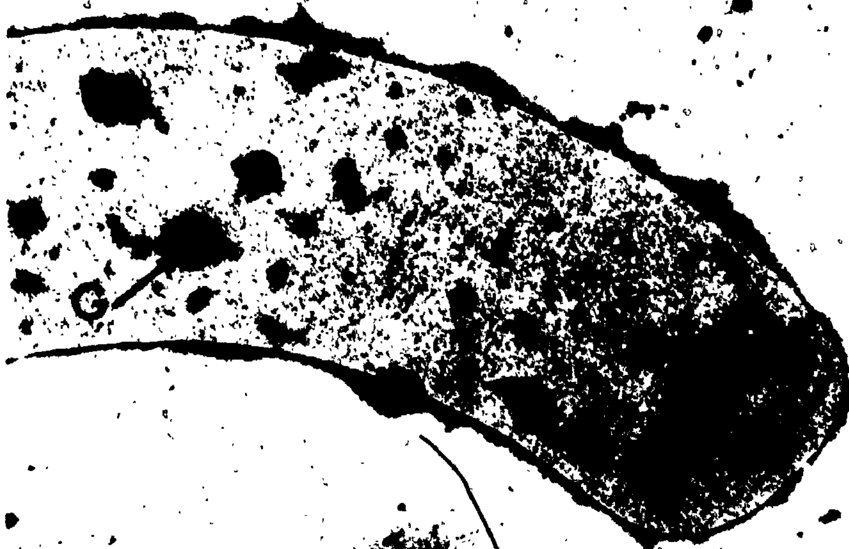
Figure 44

Murein sacculus of Spirillum.

a. The giant bag-shaped macromolecule is devoid of adhering membranes; some poly-ß-hydroxy butyrate granules (G) are trapped within. The sacculus has collapsed and become pleated at the pole. Stain-containing holes are found only at the tip, and are absent from the lateral walls. Stained with 4% phosphotungstic acid. Bar equals 0.5 µm.

b. Higher magnification of the polar cap of Spirillum, displaying perforations (P; diameter = 15 nm) in the murein sacculus, through which flagella once emerged. A "halo" (H; diameter = 35 nm) surrounds each pit. Bar equals 200 nm.

44A



44B

Figure 45

Autolyzed *Spirillum serpens*. Cytoplasmic membrane (CM) has retracted from the cell wall layers (CW) and has rounded up to form an interior vesicle. Flagellar hooks (H) project from the cell surface, each displaying a V-shaped distal tip (V). A rigid zone is conferred by concentric membrane rings (CMR's) in the plane of wall lateral to the insertion of each hook. The upper pair of rings (L, P) are shown inserting into the wall layers, but the orientation of the lower pair of rings (S, M) within the cytoplasmic membrane is unclear. Negatively stained with 1% ammonium molybdate. Bar equals 200 nm.

H
V
L
P
CW
Cm

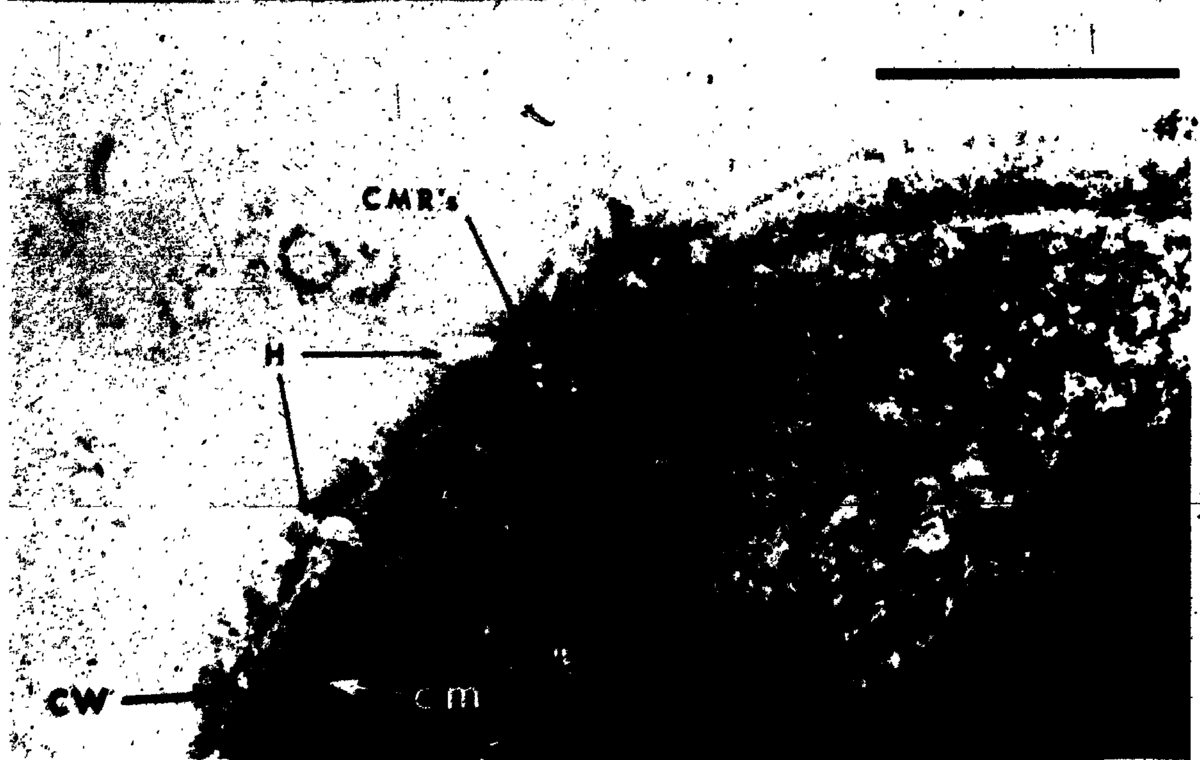

CMR's
H
CW
cm

Figure 46

Freeze etching of spheroplasts of Spirillum serpens. Retraction of the outer membrane (OM) confirms that the level of fracture is through cytoplasmic membrane (CM).

a: Convex cytoplasmic membrane, with densely packed granule distribution.

b. Concave cytoplasmic membrane, showing distribution of particles on this face.

These micrographs assist in the identification of the fractures in Figure 47. Arrows indicate direction of shadowing. Bar equals 0.5 μm.

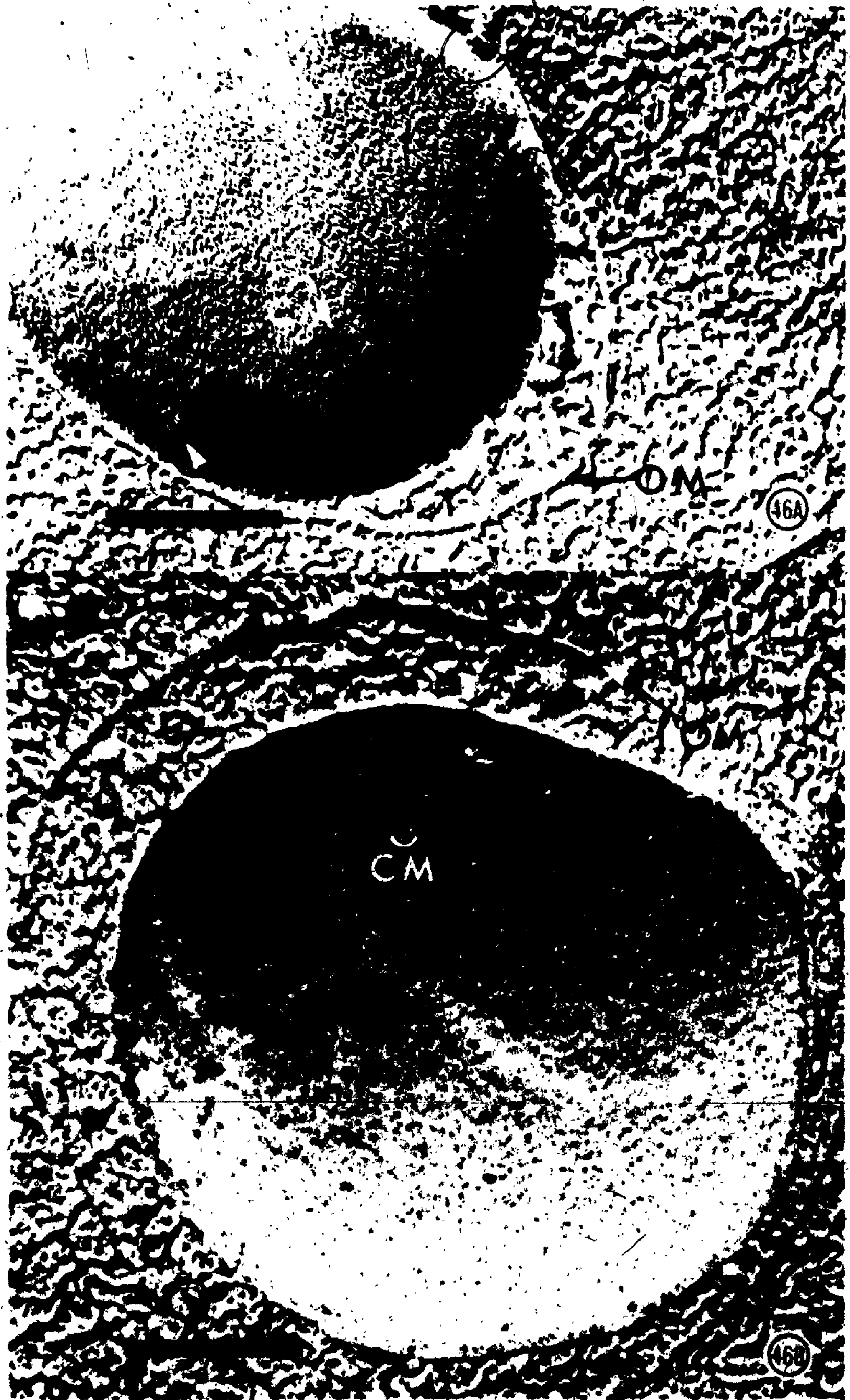

OM
46A
OM
CM
46B

Figure 47

Freeze etching of *Spirillum*, demonstrating convex cytoplasmic membrane fractures at the polar tip of these cells. Numerous depressions are readily apparent on these surfaces, and their diameter (23 nm) is sufficient to accommodate one of the membrane rings of the basal organelle. A "plug" (diameter 10 nm) is found in the centre of each depression; its dimension correlates well with the diameter of the rod of the basal organelle as derived by negative staining. Arrows indicate direction of shadowing. Bar equals 0.5 µm.

47

Figure 48

Thin section through the polar cap of *Spirillum volutans*. Each flagellum (F) emerges individually through the cell envelope layers: outer membrane (OM), mucopeptide (MP), and cytoplasmic membrane (CM). Polar membrane (PM) is also shown. Additional electron density is pronounced in two areas:

(i) in the plane of section through the basal complex (BC); the individual rings cannot be resolved;

(ii) immediately beneath the cytoplasmic membrane, as a bulbous protuberance (B).

Fixation: Ryter-Kellenberger (1958). Bar equals 0.5 μm.

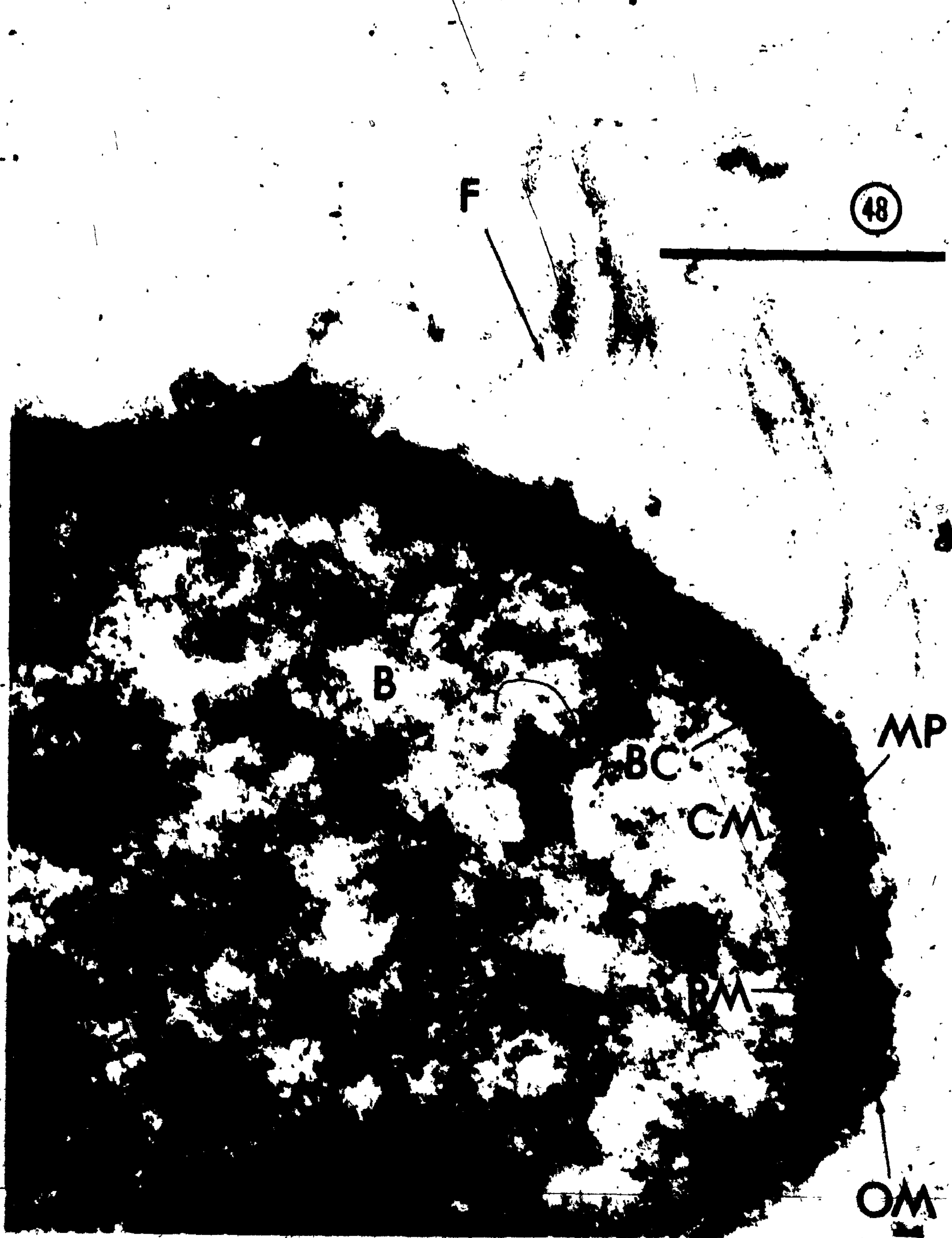

F
48
B
BC
CM
MP
BM
OM

Figure 49

A generalized anatomical model of cell envelope associations of bacterial flagella. Structural features derived from the study of Spirillum serpens have been incorporated into the diagram, and are based upon the information summarized in the Discussion. The nomenclature for each of the four rings (L, P, S, and M) is that of DePamphilis and Adler (1971b). Concentric membrane rings (CMR's) are oriented on the outer membrane (OM). The positions of each ring relative to outer membrane, mucopeptide (MP), and cytoplasmic membrane (CM) of the Gram-negative cell envelope are also displayed.

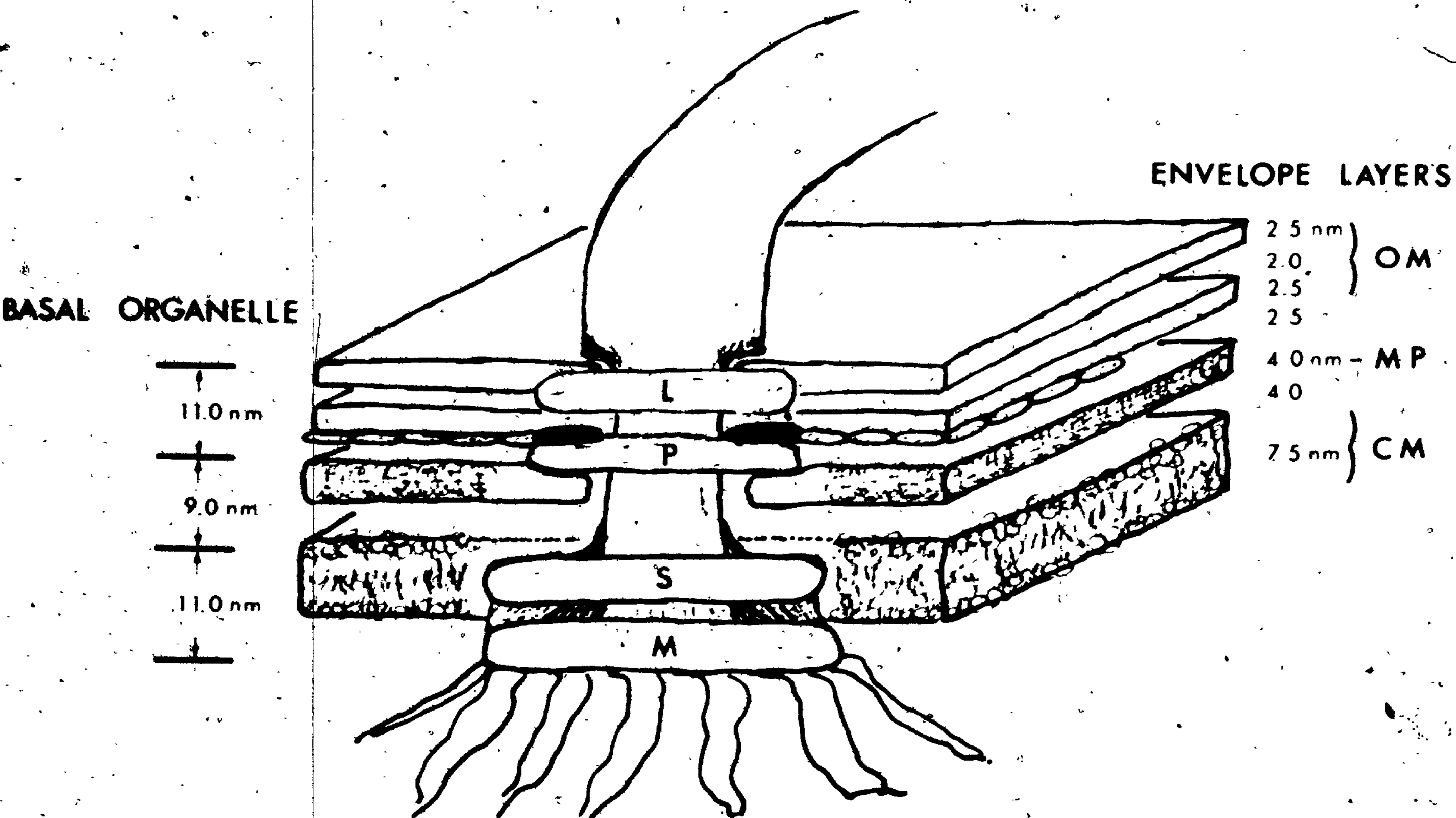


FIGURE 49

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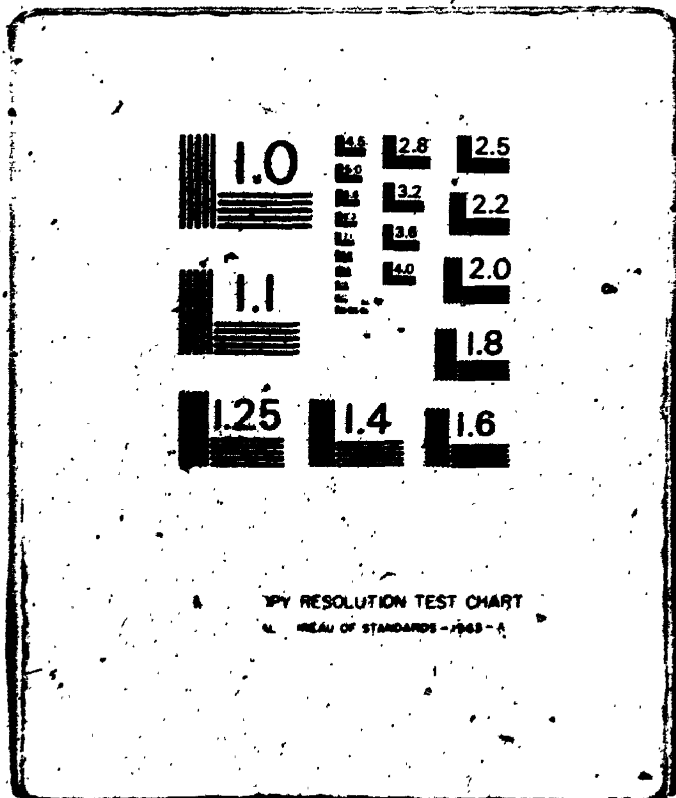

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