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A Study Of Cell Wall And Division Of Gram-negative Bacteria

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A STUDY OF CELL WALL AND DIVISION
OF
GRAM-NEGATIVE BACTERIA

by

Pamela D. Steed-Glaister

Department of Bacteriology and Immunology

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

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ABSTRACT

The test objects selected for this study of the wall and division in Gram-negative bacteria were an enteric rod-shaped bacterium (Escherichia coli), a rod-shaped bacterium with a spiral axis (Spirillum serpens), and thin, flat, ribbonlike filamentous gliding bacteria (Simonsiella crassa and Alysiella filiformis). Electron microscopic examination of sectioned specimens revealed a multilayered wall profile similar to that observed in other Gram-negative bacteria. Behaviour of the different layers within this common wall structure was investigated by exposing cells to the action of agents such as lysozyme (a bacteriolytic enzyme) and sodium lauryl sulphate (an anionic detergent known to disrupt the lipoprotein constituent of the wall). Observations from these studies allowed a distinction to be made between outer, non-rigid layers and an inner rigid element. The sites of lipoprotein, lipopolysaccharide and mucopeptide components known to occur in the wall were depicted in schematic models and compared with models presented by other investigators.

Variations in the methods of growth and fixation revealed that the appearance of division by constriction or by septation is affected by the conditions used. The role played by the wall in cell division was

analysed and the labile nature of septa was demonstrated. Mesosomes, the complex membranous bodies associated with division in Gram-positive bacteria, were found to occur in Gram-negative species previously thought to be lacking in these structures. An overall similarity in the division mechanisms of Gram-positive and Gram-negative bacteria was found.

An additional layer was observed at the surface of the common wall profile in Spirillum serpens VHA. This component was identified as the hexagonal pattern elaborated by this bacterium. Isolation and chemical analysis of this surface pattern material was carried out. The pattern was found to contain protein with all normal amino acids except cystine and histidine, and was associated with a "backing" substance which was mainly phospholipid (phosphatidylethanolamine) in nature. A pattern-less mutant, Spirillum serpens VHL, appeared to produce the "backing" without the hexagonal pattern.

A comparative study of S. serpens VHA and the pattern-less mutant S. serpens VHL showed that pattern-producing cells were lysed under certain conditions of growth. The lytic response was inhibited by calcium and strontium but not by other cations tested.

A brief look at the ultrastructure of Lineola longa revealed that this Gram-negative bacterium possesses a distinctive wall profile. Discussion of this species was presented with a generalized view of structure, composition and behaviour of bacterial walls.

INTRODUCTION

Gram-positive and Gram-negative bacteria are distinguished by their reaction to a staining procedure originally devised by the Danish pathologist, Christian Gram (1884) to detect bacteria in tissue sections. Differentiation by the Gram stain is based upon the ability of certain organisms (Gram-positive) to retain a dye-mordant complex after controlled extraction with ethanol; bacteria which lose the complex during extraction are termed Gram-negative. Subsequently, it was realized that Gram-positive bacteria differ from Gram-negative species in aspects other than the Gram stain reaction. The nature of the cell wall, in particular, was subject to investigation and, with development of improved techniques, the analytical study of isolated bacterial walls became a possibility.

Results of an investigation by Salton (1953) demonstrated a marked difference in the wall composition of Gram-positive and Gram-negative bacteria. Electron microscopic studies of sectioned bacteria revealed a corresponding degree of variation in wall structure. Differences in behaviour of bacterial walls, such as their susceptibility to lysis and their role in cell division, were also recognized.

In recent years, an overwhelming amount of information about composition, structure and behaviour of bacterial walls has

flooded the literature. Numerous reviews such as those by Perkins (1963), Salton (1964) and Rogers (1965) have also been published as valiant attempts to relate the diversified data bearing on the subject. At the present time, a major problem is the satisfactory correlation of ultrastructure with composition and behaviour of walls in terms of the many complicated details now known. This problem is of major concern in the studies on cell wall and division of Gram-negative bacteria which constitute this thesis.

HISTORICAL REVIEW

(A) Evidence for a Cell Wall

(1) Light Microscopy

The early cytologists attributed the characteristic shape of a bacterial cell to the presence of some kind of envelope at the cell surface. A cell wall was suspected by Cohn (1875) when he noted bacterial resistance to dissolution by acid and alkali. In 1887, Vincenzi attempted isolation of bacterial walls for chemical analysis and succeeded in obtaining empty cells or hulls. Direct demonstration of a wall in living bacteria was achieved by Fischer (1891) when he plasmolysed bacteria, causing them to undergo reversible retraction while the "delicate membrane of the cell wall" remained unaltered. In 1903, Fischer defined three layers at the surface of bacteria: the plasma membrane, the cell wall, and the capsule or slime layer. However, the distinction between the cell wall and cytoplasmic membrane as discrete morphological entities remained obscure for many years. To confuse the issue still further, a dismaying amount of ill-defined terminology crept into the literature until Knaysi (1938) suggested that terms used for plant cells be applied; thus "cell wall," "cytoplasmic membrane," and "cytoplasm" were used to describe in bacteria the

equivalents of these morphological and functional components of plant cells.

Evidence for the existence of a membrane limiting the bacterial cytoplasm accumulated from studies of permeability characteristics (reviewed by Knaysi, 1946; Mitchell and Moyle, 1956) but the actual location of such a membrane, in relation to the cell wall, proved difficult to ascertain by ordinary staining methods. Staining procedures producing good contrast between the cell wall and cytoplasm of Gram-positive bacilli were described and illustrated by Robinow (1945). A more sophisticated method, combining the advantages of protoplast retraction and a dye with membrane-staining affinity, was developed by Robinow and Murray (1953), thus allowing direct microscopic differentiation between the cytoplasm, cytoplasmic membrane, and cell wall of stained bacteria. Prior to this demonstration, the numerous cytological techniques described in the literature (reviewed by Bisset, 1953) could not reliably make this distinction. However, a Gram-dependent response to staining procedures experienced by earlier cytologists (Gutstein, 1925; Maneval, 1929) was also observed by Robinow and Murray (1953) who obtained successful results only with Gram-positive bacteria, the Gram-negative species failing to react satisfactorily. Trials of other cell wall staining methods have confirmed this observation (Cassel, 1951; Hale and Bisset, 1956).

(2) Electron Microscopy

The electron microscope became "a new tool for bacteriological

research" (Marton, 1941) and many special techniques were developed for its use in studying bacterial anatomy. Although the early studies with unstained preparations of intact, sonicated, and lysed cells yielded little new information, the electron micrographs obtained reaffirmed the relative disposition of cell wall, cytoplasmic membrane, and cytoplasm that had been determined by light microscopy and showed that the wall provided the rigidity and shape of the cell. The need to enhance the natural electron density of cellular components was soon recognized and, in 1942, Mudd and Anderson employed heavy metal salts as electron stains. Additional staining techniques for electron microscopy were described by Williams and Wyckoff (1944); Lamb, Stuart-Webb, Bell, Bovey and Danielli (1953), and were recently reviewed by Zobel and Beer (1965). Methods for sectioning bacteria prior to their examination in the electron microscope were developed by several groups of workers, the major developments being the use of plastic-embedded specimens by Newman, Borysko and Swerdlow (1949), and introduction of the glass knife by Latta and Hartmann (1950).

The difficulty of interpreting the complex bacterial structure revealed by electron microscopy contributed to a state of confusion and disagreement among investigators. Cell walls and cytoplasmic membranes in sectioned bacteria were not clearly defined structures and were subject to misinterpretation until Murray (1957) and Chapman and Kroll (1957) demonstrated their true relationship at the cell surface. The multilayered nature of the cell wall in Escherichia coli

was revealed by Kellenberger and Ryter (1958), and a similarly layered cytoplasmic membrane was described in several bacterial species (Tokuyasu and Yamada, 1959; Glauert and Hopwood, 1959, 1960; and Fitz-James, 1960). These important observations established the existence of the wall and cytoplasmic membrane as two distinctive highly organized structures at the surface of bacterial cells.

(B) Cell Wall Composition

(1) General

Bacterial walls were tested originally for the presence of materials resembling chitin and cellulose, which were known to be important structural components of other cell walls. Vincenzi (1887) has been credited with the first attempt to analyze isolated bacterial walls; he concluded that the wall was not composed of cellulose. Routine chemical analysis of bacterial walls awaited the development of satisfactory isolation procedures, which began with Dawson's demonstration of staphylococcal walls in 1949. Cell disruption methods used in conjunction with differential centrifugation led to the production of homogeneous wall suspensions (Mitchell and Moyle, 1951; Salton and Horne, 1951). The purity of their preparations was estimated by examination in the electron microscope, this instrument being, in fact, an indispensable tool for the analytical control of subcellular components. The absence of cytoplasmic contamination of wall suspensions as determined by ultra-violet (UV) absorption for nucleic acids, was introduced

as an additional criterion for pure wall isolations (Salton, 1956).

In 1953, Salton published the results of an investigation of wall composition in Gram-positive and Gram-negative bacteria. The outstanding differences were a higher lipid content and a wider range of amino acids in Gram-negative bacterial walls. He was amazed by the "diversity of complex substances" which apparently made up the structure responsible for mechanical rigidity. However, the existence of wall components not involved in rigidity was indicated by behaviour of the type-specific M-protein antigen possessed by Streptococcus pyogenes and studied earlier by Lancefield (1943). It appeared to be merely an accessory component and not an essential unit in the cell wall complex. Inability to detect a morphological change in the wall upon removal of the M-protein led Salton to conclude that morphological homogeneity of wall preparations belied a pronounced degree of molecular and antigenic heterogeneity. Subsequent cell wall studies allowed a distinction to be made between the component responsible for the rigidity of the bacterial wall and a variety of "non-rigid" components.

(2) The Rigid Component

The bacteriolytic enzyme, lysozyme, discovered by Fleming in 1922, was shown to attack the bacterial surface (Kern, Kingade, Kern and Behrens, 1951). In 1952, Salton demonstrated the dissolution of isolated bacterial walls by this enzyme. The antibacterial

action of penicillin, another of Fleming's discoveries (Fleming, 1929) was also found to involve wall disruption. However, this agent differed from lysozyme in that it affected only growing cells and was thought to interfere with a rigid wall component common to all bacteria (Duguid, 1946). Chemical analysis of the breakdown products resulting from lysozyme dissolution, and of the products that accumulated during penicillin treatment, played a major role in the elucidation of wall composition and architecture. Since these two agents acted on the rigid component of the wall, the various constituents identified as a result of their action were considered to be units of the wall complex responsible for cellular integrity.

In 1959, Perkins and Rogers, working with Gram-positive bacteria, proposed the term, mucopeptide, for the rigid wall component whose acid hydrolysate yielded a characteristic but limited range of amino acids, some in the D-configuration, one or two hexoses, and the hexosamines glucosamine and muramic acid. In a companion paper by Mandelstam and Rogers (1959) the biosynthesis of mucopeptide in Gram-positive cocci was studied by following the incorporation of labelled amino acids. They found that glycine, glutamate, alanine or lysine, were incorporated almost exclusively into the cell wall mucopeptide, and that this synthesis was inhibited by penicillin and bacitracin, but not by chloramphenicol. The role of uridine and cytidine nucleotides as intermediates in the biosynthesis of bacterial walls, and their relation to the action of certain antibacterial agents were reviewed by

Strominger (1960). He proposed schemes for biosynthesis of mucopeptide in Staphylococcus aureus and Escherichia coli, and summarized the evidence for a "basal structure" common to both Gram-positive and Gram-negative bacterial walls.

The structure and metabolism of some specific constituents of mucopeptides were reviewed by Work (1961) together with a discussion of the cross-linkage involved in the architecture of this mucopolymer. A study of mucopeptides of Gram-negative bacterial walls by Mandelstam (1962) led to his conclusion that, although the chemical make-up was similar in mucopeptides of both Gram-positive and Gram-negative bacteria, the relative amounts of the constituents varied considerably among Gram-positive bacteria, but not so in the Gram-negative species studied. He also pointed out the fact, already well substantiated (Weidel and Primosigh, 1958; Salton, 1958) that the mucopeptide occurred as a minor wall element in Gram-negative bacteria whereas it represented 10 to 20 percent of the Gram-positive bacterial mass and constituted almost the entire cell wall. Salton (1963) suggested that the total amount and the physico-chemical state of the mucopeptide in bacterial walls could have some bearing on the response to the Gram stain; the quantitative rather than qualitative difference between Gram-positive and Gram-negative bacterial walls was emphasized. A picture of the wall in Escherichia coli was described by Milner, Anacker, Fukushi, Haskins, Landy, Malmgren and Ribi (1963) in which the rigid mucopeptide, substrate for lysozyme and phage enzyme, was

depicted as polypeptide balls held in a rigid network by comblike structures whose teeth of peptide chains were fixed to a backbone of amino sugars.

A most important feature of bacterial cells was brought to the attention of wall isolators and analyzers by Weidel, Frank and Leutgeb (1963), namely, the presence of autolytic enzymes and the consequences of their action during wall preparation. When taken into account, the autolytic destruction of mucopeptide components explained some odd results of cell wall studies that had failed to fit other observations. The labile, rather than static, nature of bacterial walls was emphasized by the authors who described a method to "freeze" the mucopeptide structure so as to avoid degradation.

Weidel and Pelzer (1964) described the mucopeptide in a novel manner by regarding it as a "bag-shaped macromolecule" forming a "murein sacculus." The balance and co-ordination between synthetic and degradative enzymes required for incorporating new units in the growing complex were discussed in terms of cell wall growth and the problem of preserving the intact structure for analytical purposes. The extent of cross-linkage in the "murein sacculus" of Escherichia coli cell wall was investigated by Takebe (1965) and found to involve 60% of the whole peptide side chains. Chaloupka, Řihová and Křečková (1964) estimated that approximately 15 to 20 percent of the rigid layer in Bacillus megaterium wall was degraded during one cell

division. These authors also found that resistance of cell walls to lysozyme varied with age of the cells and increased during the stationary phase.

(3) The "Non-Rigid" Components

Both Gram-positive and Gram-negative bacteria were known to possess antigens and bacteriophage receptor sites at their surfaces, and these were thought to be part of the cell wall. Morgan, (1936, 1937) interpreted the smooth-rough variation among Gram-negative bacilli as a loss of somatic antigen. In 1943, Lancefield removed the M-protein antigen from Streptococcus pyogenes with trypsin without decreasing the viability of the cells. Antigens of protein and of polysaccharide nature exhibiting type- and group- specificity respectively, were studied by McCarty (1952) and Cummins (1954) in Gram-positive bacteria. The behaviour of these antigenic substances revealed their lack of function in cell wall rigidity and were regarded as "accessories" to the mucopeptide component by later investigators.

A most useful technique for sorting out cell wall components was made available when Weibull (1953) produced protoplasts of Bacillus megaterium. It appeared that Gram-positive bacteria exposed to the action of lysozyme in a protective osmotic environment were converted to naked protoplasts bounded by the cytoplasmic membrane. Gram-negative bacteria failed to undergo similar conversion unless certain adjuvants, such as ethylenediaminetetraacetate (EDTA), were

used with the enzyme (Repaske, 1956, 1958; Kohn, 1960). Lysozyme action was studied by Salton (1958) who noted the quantitative rather than qualitative difference between Gram-positive and Gram-negative bacteria with respect to lysozyme-sensitivity. The characteristic behaviour of lysozyme-exposed Gram-negative bacteria was attributed to the presence of a lipoprotein moiety in the cell wall which protected the lysozyme substrate. This non-rigid lipoprotein layer was thought to be susceptible to the action of the adjuvants which assisted lysozyme in producing the osmotically-sensitive spheres called spheroplasts (McQuillen, 1960). Penicillin-induced protoplasts and spheroplasts were demonstrated by Lederberg (1956) and in 1958, Salton and Shafa related morphological and chemical data when they revealed the residual lipoprotein layer at the surface of penicillin-induced spheres of Vibrio metchnikovii. Other correlated studies were made by Weidel and Primosigh (1958) who removed the lipoprotein layer from the underlying rigid component of Escherichia coli wall and estimated the location of different bacteriophage receptor sites within the wall.

In 1959, Ribí, Milner and Perrine showed that the endotoxic and antigenic lipopolysaccharides found in smooth enteric Gram-negative bacilli were restricted to the cell wall. The absence of cell wall antigens in protoplast membranes of Bacillus megaterium (Vennes and Gerhardt, 1959) and their presence in washed membranes of Escherichia coli B spheroplasts (Holme, Malmberg and Cota-Robles, 1960) further substantiated the existence of "accessory" wall materials

in Gram-negative bacteria.

Weidel, Frank and Martin (1960) extracted the outermost lipoprotein layer with sodium dodecyl sulphate, and the intermediate lipopolysaccharide layer with phenol-saturated water, to expose the innermost rigid wall component in Escherichia coli. In a subsequent paper, the relative proportions of these three layers were estimated as 60%, 12%, and 21% of the total wall weight respectively (Martin and Frank, 1962 a).

Milner, et al. (1963) described a picture of the non-rigid components of Escherichia coli cell wall in which the lipopolysaccharide layer, containing endotoxic O antigen and phage-receptor sites, was depicted as lying above and bound tightly to the mucopeptide layer; external to both was a thick, amorphous layer of lipoprotein. These workers extracted the endotoxin in the form of fibrils, but believed its natural state in the bacterium was a gel. Wardlaw (1963), estimated that the lipopolysaccharide fraction of walls in a rough strain of Escherichia coli was 1% compared with 9% in a smooth strain. A morphological correlation of the outer wall layer and endotoxic particles was contained in the ultrastructural study of Veillonella by Bladen and Mergenhagen (1964). The somatic antigen was identified in cell wall profiles of Gram-negative bacteria by use of specific antibody conjugated with electron-dense ferritin (Shands, 1965).

A special class of non-rigid wall components identified in some Gram-positive bacteria are the polymers of ribitol and glycerol

phosphates called teichoic acids (Armstrong, Baddiley, Buchanan, Carss and Greenberg, 1958). The presence of intracellular teichoic acids lying between the cell wall and cytoplasmic membrane was revealed by Hay, Wicken and Baddiley (1963) who discussed their possible role as a non-specific permeability barrier to charged molecules. Baddiley (1964) presented a further study of teichoic acids and considered their significance as a source of antigenic properties.

(C) Ultrastructure

(1) General

The demonstration of a multilayered cell wall in sections of Escherichia coli (Kellenberger and Ryter, 1958), and the revelation of the residual lipoprotein layer in sections of penicillin-induced spheres of Vibrio metchnikovii (Salton and Shafa, 1958), initiated the quest for correlation of biochemical, functional and morphological properties of bacterial walls at an ultrastructural level. The dimensions of cell wall profiles had been estimated by earlier workers, such as Chapman and Hillier (1953), Birch-Andersen, Maaløe and Sjöstrand (1953), and Chapman and Kroll (1957), but identification of components within the wall profile was not made. Kellenberger and Ryter (1958) interpreted the composition of the triple-layered Escherichia coli wall on the basis of chemical data available at that time, suggesting that it represented a polysaccharide coated on either side by protein and/or lipid. Salton and Shafa (1958) identified the residual surface

layer after penicillin treatment as the lipoprotein wall component because it was thought to be the non-rigid element insusceptible to penicillin action. An electron-dense layer of lipoprotein beneath a polysaccharide region was proposed for the surface structure of Escherichia coli (Ogura, 1963).

A different line of approach was taken by a group of investigators who stripped the multiple wall layers from Gram-negative bacteria by means of various chemical treatments and examined the remaining wall at each stage of the process (Weidel, Frank and Martin, 1960; Martin and Frank, 1962 a, b). However, their observations of shadowed preparations, which allowed location and identification of the different layers relative to one another, were difficult to relate, with any degree of certainty, to cell wall profiles of sectioned bacteria. Clarke and Lilly (1962) proposed a general wall structure for Gram-negative bacteria in which they postulated the existence of a rigid layer sandwiched between two "unit" type protein-lipid-lipid-polysaccharide membranes, namely the cytoplasmic membrane and the outer wall unit. In 1963, Martin presented elegant models of Escherichia coli walls before and after attack by lysozyme and penicillin.

During the last few years, improved techniques in the field of electron microscopy have led to the discovery of considerable structural complexity in the walls of many Gram-negative species (Coster-ton, Murray and Robinow, 1961; Cota-Robles, 1963; Morita and Stave,

(1963). Murray (1963a) obtained a great deal of information about the wall structure of Spirillum serpens by using several different techniques to aid interpretation. The loose triplet-layer resembling the Escherichia coli wall profile was found to be external to a thin, taut, electron-dense component. A similar wall profile was demonstrated in other Gram-negative bacteria (Poindexter and Cohen-Bazire, 1964; Reyn, Lautrop and Birch-Andersen, 1964). Claus and Roth (1964) proposed that the taut, innermost layer might represent the mucopeptide element of the cell wall. A wide range of wall complexity among Gram-negative bacteria was indicated by the extremely complicated layered structure in Beggiatoa and related species (Morita and Stave, 1963; Maier and Murray, 1965), the surface envelope in Lampropedia hyalina (Chapman, Murray and Salton, 1963; Murray, 1963b) and the relatively simple wall profile of halophilic species (Brown and Shorey, 1963), while the "typical" Gram-negative wall profile was exemplified by an inner, taut, dense layer and outer, loose, dense-light-dense component. The thin, innermost element was implicated in wall rigidity and lysozyme action by Bladen and Mergenhagen (1964) who also observed removal of the outer triplet-layer after extraction with aqueous phenol.

The existence of a layer containing globular elements, demonstrated in sections of Escherichia coli, was interpreted as lying between the inner rigid and outer soft layers of the wall (de Petris, 1965).

This author described a wall profile in which the rigid component occupied an electron transparent zone outside the cytoplasmic membrane. Bayer and Anderson (1965) used a cryostat procedure to study the Escherichia coli wall and its response to various reagents. They found evidence of surface protrusions and channels and, relating their findings to biochemical data, described a wall profile model which accounted for many aspects of the structure, function, composition and behaviour of Gram-negative bacterial walls.

A most unusual cell wall organelle was reported in Nitrosocystis oceanus (Murray and Watson, 1963), and a later comparative study of Nitrosocystis, Nitrosomonas, and Nitrobacter species revealed distinctive wall profiles in each of these nitrifying bacteria (Murray and Watson, 1965).

(2) Regular Wall-Patterns

The presence of a cell wall component exhibiting a regular pattern was first demonstrated in a species of Spirillum by Houwink (1953). This pattern consisted of an hexagonal array of globular units associated with, and apparently bound to, a surface matrix of unknown composition. A similar arrangement was observed in the walls of Rhodospirillum rubrum (Salton and Williams, 1954), and of Halobacterium halobium (Houwink, 1956). A regular arrangement of structural units in the envelope of Lampropedia hyalina sheets was reported by Murray (1960/1961), whose later study included a discussion of the role played

by such a structure in this microbe (1963b). A detailed description of the dimensions and architecture of the macromolecular pattern in Spirillum serpens accompanied numerous electron micrographs depicting isolated wall fragments of the pattern in shadowed, stained and sectioned preparations (Murray, 1963a). During the examination of pH effect on Halobacterium cutirubrum, the units of the wall pattern were found to disappear at low pH values (Kushner and Bayley, 1963). Martin and Frank (1962b) demonstrated the removal of the lattice-structure from isolated Spirillum walls after sodium lauryl sulphate treatment, thus exposing the underlying smooth mucopeptide basal structure. The action of this anionic detergent was described as dissolution of the amorphous surface matrix resulting in disintegration of the lattice into isolated structural elements. Moiré patterns formed by the hexagonal array of repeating units at the surface of Micrococcus radiodurans were discussed by Glauert (1966). The most recent demonstration of hexagonal wall pattern was that described by Fischman and Weinbaum (1967) in Escherichia coli B; only heated walls always possessed the structure. A different type of wall pattern was detected by Labaw and Mosley (1954) in which the subunits formed a rectangular array. A similar description was published by Baddiley (1964) for a pattern in what was thought to be the mucopeptide layer of Bacillus polymyxa cell wall. Replicas of intact cells prepared by de Boer and Spit (1964) revealed a mosaic of striated structure on the surface of a Gram-negative bacterium.

(D) Growth of Bacteria

(1) Cell Wall Growth

Like other living cells, bacteria grow when placed in a suitable environment. In addition to an increase in the bulk of cytoplasmic contents, replication of surface layers is a necessary outcome of cell growth. Several investigators undertook the task of determining how the cell wall became extended, and two principal modes of cell wall manufacture were described.

A diffuse, uniform intercalation of new material into the existing wall was claimed for several Gram-negative bacteria by a number of workers who used vastly different techniques. Quadling and Stocker (1956) arrived at their conclusion by studying the distribution of flagella among progeny; Williams (1959) referred to cell inclusions as markers during the study of cell elongation in Spirillum anulus; May (1963) and Cole (1964) reported uniform dispersion of new wall material in Salmonella by means of fluorescent serological methods. Use of this latter technique indicated diffuse extension of the cell surface in Spirillum volutans (McElroy, Wells, Jr., and Krieg, 1967).

Cell wall extension by addition at specific sites was the other method described for several bacterial species. Bisset (1951) and Bisset and Pease (1957) considered the pole to be the growing point of the cell. The fluorescent antibody technique revealed that new cell wall material was manufactured at a single, or multiple, but specific sites with a definite relation to septation, in the equatorial zone of the

cell (Cole and Hahn, 1962; May, 1963; Chung, Hawirko and Isaac, 1964 a, b; and Wagner, 1964).

Hoffman and Frank (1965) showed that cell elongation was a discontinuous process when they observed the growth of Escherichia coli by time-lapse photomicrography.

(2) Growth Rate

The period of time required for a bacterial cell to duplicate, termed the generation time, was determined by the plate method (Buchner, Longard and Riedlin, 1887), and by direct microscopic examination (Ward, 1895, 1899) and found to be of the order of 20 minutes. Ward (1895) also obtained data for the rate and mode of growth in filaments of bacilli, noting that intercalary growth took place along the length of the filament. These filaments were regularly divided into segments by transverse septa, and complete segmentation gave rise to individual, rod-shaped bacilli. Results of his growth rate measurements showed a period of slowest growth at the time of insertion of septa, when the rate of elongation was reduced because "the cell-contents were too busy with the act of division to be then growing at quickest rates." This opinion was expressed some 60 years later by Hoffman and Frank (1965), who found that, at the time of cell division, the synthesis of cell wall material appeared to be diverted entirely into cross-wall formation. From the numerous experiments he conducted, Ward (1895) also obtained information about the effects

of temperature, light, and nutrients, on the growth curves of bacilli.

The effect of the growth rate on cell wall composition was the subject of an investigation by Collins (1964), in which cells of Salmonella enteritidis were grown in a chemostat at progressively faster dilution rates. Agglutination tests showed that the fastest growing cells underwent a partial smooth to rough antigenic variation which reversed when the growth rate was again reduced.

(3) Division

For many years the act of division in bacteria was described by the vague term, fission; this was in fact, an identifying characteristic of schizomycetes. However, the detailed course of events involved in this process is only now being revealed through concerted efforts of investigators working in several disciplines.

(i) Light microscopy

Fischer (1897) reported that bacterial division followed the same course as division in cells of higher plants. The elongated cell was divided into two by a transverse wall, the protoplasm being simply abstricted into two parts by the ingrowing wall. The actual details could not be discerned in the minute bacterial cell, but were assumed to correspond to the stages observed in plant cells. However, in 1903, Fischer presented a diagram of Beggiatoa in which a dividing cell showed partial ingrowth of a transverse septum.

Some insight into the complexity of division was indicated by the observation that the first distinctly visible septum appeared some time after the moment of actual cell-division (Ward, 1895). The multicellular nature of some bacteria was recognized by Ellis (1906), who considered an individual bacterium to consist of more than one cell when it was divided by internal partitions. Examination of stained fixed cells led to his description of division stages beginning with a transverse division-wall, followed by constriction, with final separation resulting from combined constrictive and wall-partitioning processes. Consecutive stages in division of vitally-stained Bacillus subtilis were reported by Knaysi (1930). Different modes of cell division, claimed by cytologists for a variety of bacterial species, were classed as three main types in Knaysi's review of bacterial cytology (1938). These were plate formation, cytoplasmic retraction, and direct division by constriction.

Schaudinn (1902) undertook an extensive study of Bacillus butschlii stained with iron-hematoxylin, and, among his descriptions and illustrations, was an account of division by a centrifugal plate derived from a centrally located granule. The fully extended plate split to form the new poles of the separated cells. Chance (1953, a, b) and Clark, Webb and Chance (1957) claimed evidence for this mode of division in many Gram-positive bacteria and several Gram-negative species. The technique employed was a staining procedure originally intended for demonstrating bacterial nuclei, and involved a somewhat

unreliable step of decolourization.

The confused state of bacterial cytology, with respect to cyto-kinetic behaviour, was examined again by Knaysi in 1941. He concluded from his comparative studies of yeast and bacteria, that a break in the cytoplasmic membrane presaged cell division in bacteria. Both Gram-positive and Gram-negative species were investigated and, in all cases, he observed deposition of two walls, there being no indication of a single wall later splitting or of simple constrictive division. Later papers published by Knaysi (1944, 1946, 1949) reaffirmed the importance of the cell membrane as the initial agent in division, being responsible for division of the cytoplasm prior to development of cross-walls. However, confusion still reigned in the subject of bacterial cytokinesis as it appeared that no single mechanism of division applied to all bacteria. Robinow (1945) did much to clarify the situation when he analysed differences in modes of growth and multiplication in such a way as to allow their interpretation as differences in time-relationship of distinct processes common to the various species studied. Thus, cytokinesis in Gram-positive and Gram-negative bacteria, though often different in appearance, was described as a common procedure differing primarily in the time sequence of events involved. Other important observations related to the subject were the multicellular nature of many Gram-positive and Gram-negative species, and the development of transverse septa from the inner surface of the cell wall. However, the question of a single-

or double-layered septum was left unanswered for lack of adequate resolution and conclusive evidence.

Supplementing his earlier studies of bacterial colonies, Bisset (1950) ascribed division by constriction to morphologically smooth bacteria, and division by septation to rough forms. In both cases, the cell membrane was thought to form a transverse septum which then proceeded to secrete cell wall material; this began at the junction of cell membrane and membranous septum in constrictive division, and within the thickness of the septum in cross-wall formation. The nature and behaviour of bacterial envelopes were subject to revision by Bisset (1953) who again correlated mode of division with the "smooth" and "rough" morphological types of bacteria. The basophilic membrane septum which later secreted new wall material was designated as the first sign of incipient division in both bacterial types.

(ii) Electron microscopy

The electron micrographs which appeared with the account of cellular division in Bacillus cereus (Chapman and Hillier, 1953) allowed visualization of some structures beyond the resolving power of light microscopes. Successive stages in formation of transverse walls were shown to involve cytoplasmic inclusions called peripheral bodies. Unfortunately, adequate preservation of the structures was not achieved, and several years passed before their nature and function were better understood. Fitz-James (1960) applied the term,

mesosome, to the cytoplasmic membrane-derived organelles which seemed to correspond in site and function to the original peripheral bodies. Chapman and Hillier (1953) had assigned a role in the secretion of cross-wall material to these bodies, but failed to relate them to the cytoplasmic membrane, which could not be resolved in their sections. Fitz-James (1960) suggested that mesosomes played a role in septation during division and in spore-formation, and observed their connection with both the cytoplasmic membrane and chromatin material. Other descriptions of mesosome-like elements in various Gram-positive species were published (Glauert and Hopwood, 1959; Giesbrecht, 1960), and their function in nuclear and cellular division was interpreted from a study of serial sections of Bacillus subtilis by Ryter and Jacob (1963, 1964).

Septa, consisting of ingrowing cross-walls lined with cytoplasmic membrane, associated with prominent and complex mesosomes became a common feature in electron micrographs of sectioned Gram-positive bacteria. A corresponding picture was not found in Gram-negative species although some examples of cross-walls and membranous intrusions were known (Bladen and Mergenhagen, 1964; Fitz-James, 1964a). The absence of septa and mesosomes in most Gram-negative bacteria examined suggested that these cells divided by a simple constrictive process, and this view was held for the "typical" Gram-negative species, Escherichia coli and related organisms.

The participation of a septum in division of Escherichia coli was indicated in sections examined by Conti and Gettner (1962), but just what elements were involved, was not determined. Electron micrographs of plamolysed Escherichia coli (Cota-Robles, 1963) revealed division of the cytoplasm by the cytoplasmic membrane prior to cell wall involvement; this observation was interpreted as evidence supporting a constrictive mode of division. Fitz-James (1964a) described the division of Neisseria as beginning with a membranous infold and partial constriction followed by slow septation. Mesosomes were present but not at the site of division. Prominent mesosomes were seen to occupy the division site in stalked bacteria, but septa were present only in Asticcacaulis, the closely related Caulobacter seeming to divide by constriction (Poindexter and Cohen-Bazire, 1964). Electron micrographs of sectioned bacteria have revealed that in Gram-negative bacteria dividing by septation, the septum consists of the ingrowing thin, taut, innermost layer of the wall, enclosed within the invaginating cytoplasmic membrane (Morita and Stave, 1963; Maier and Murray, 1965; Bladen and Mergenhagen, 1964). A well preserved septum in Escherichia coli was recently observed by Kohiyama, Cousin, Ryter and Jacob (1966) during their study of temperature-sensitive mutants.

(4) Division Inhibition

One manifestation of division inhibition is the formation of cell filaments in which the cell wall and cytoplasmic constituents continue to grow, without cytokinesis at the end of the generation period. Many different kinds of treatment have been reported to induce filament formation in bacteria. The effects of various division inhibitors which produced filaments in a species of Erwinia, and of other agents able to reverse the inhibitions, were investigated by Grula and Grula (1962 a, b, c, 1964, 1965). Filaments resulting from magnesium deficiency were described by Webb (1949 a, b, 1951, 1953). A role played by iron in cell division or separation was suggested by Ratledge and Winder (1964) to explain filament formation of Escherichia coli in an iron-deficient medium. This effect was observed also by Jordan and Howell (1965), who obtained filaments in an actinomycete as a result of iron-depletion; the magnesium concentration had no effect on morphology, but did influence the rate and amount of growth.

The study of various mutants supplied a large amount of information about certain requirements affecting division processes. Plunkett (1962) described an Escherichia coli mutant which required streptomycin in order to carry out cytokinesis; this requirement did not exist at lower temperatures. A similar temperature-dependent requirement was reported earlier for another Escherichia coli mutant which needed panthothenic acid at high temperatures. Van de Putte, Westenbroek and Rörsch (1963) discussed the relationship

between radiation resistance and filament formation in Escherichia coli on a genetic basis. Unlike the radiation resistant mutant, wild type cells produced filaments after UV-irradiation or incubation with crystal violet. This filament formation was markedly reduced by pantooyl lactone, which also prevented UV - or crystal violet-induced lysis of Bacillus subtilis. An action of UV-irradiation involving synthesis of special structures such as septa, was the suggestion put forward by the authors, and the role of pantooyl lactone was thought to be associated with a "basal structure" common to both Gram-positive and Gram-negative bacterial walls. Selected Escherichia coli mutants showed division inhibitions at elevated growth temperatures which were not reversed by pantooyl lactone (van de Putte, van Dillewijn and Rörsch, 1964). Both types of division inhibition, the radiation-induced and the temperature-induced, were considered to be genetically determined, but by different genes on the chromosome. Ivanovics (1964) studied the unbalanced growth of a Bacillus anthracis mutant which required pyrimidine precursors at higher incubation temperatures. Cytological examination of normal and radiation-resistant Escherichia coli cells revealed concomitant increase in cell length with increased radiation resistance, and a peculiar "budding" was observed in the more radiation-resistant cultures (Pontefract and Thatcher, 1965). Nuclear division was shown to be a prerequisite for ensuing cytokinesis when the reversal of cell division inhibition by pantooyl lactone or high temperature incubation was prevented by exposure to mitomy-

cin C (Adler and Hardigree, 1965).

The other manifestation of interference with the division process is complete inhibition of growth. Such an effect was reported by Hurwitz, Landau and Doppel (1962) when Escherichia coli cells exposed to streptomycin either divided and grew or failed to grow, without any formation of elongated cells or filaments. This behaviour was contrasted with that of streptomycin-dependent cells grown in the absence of the antibiotic.

(5) Lysis

The behaviour of the bacterial cell wall has been studied by examining the consequences of interference with or destruction of specific wall components. Bacterial lysis resulting from the action of physical and chemical agents was discussed by Pethica (1958) and particular mention was made of ionic detergents. McQuillen (1958) presented an account of lysis resulting from metabolic disturbances, and related the data to cell wall composition and behaviour. The chelating agent, ethylene-diaminetetra acetate (EDTA) was found to lyse the cell walls and intact cells of Pseudomonas aeruginosa (Eagon and Carson, 1965) and was considered by Gray and Wilkinson (1965 a, b) as a competitor for a metal essential for cellular integrity. This same agent failed to lyse Escherichia coli cells but did cause a nonspecific increase in permeability (Leive, 1965 a, b). Repaske (1956, 1958) introduced EDTA as an adjuvant for lysis of

Gram-negative bacteria with lysozyme, and it has been in general use since then. However, the effect of EDTA upon lysozyme-lysis of streptococci was found to be variable, being inhibitory in some species while accelerating lysis in others (Brown, Sandine and Elliker, 1962). The age of cells proved to be a critical factor in the lysis of Escherichia coli by p-chloromercuribenzoate (Schaechter and Santomassino, 1962). Most Gram-positive cells were refractory, and only Gram-negative species growing exponentially were sensitive. The ability of cysteine to cause partial reversion of the effect led the authors to propose that lysis resulted from inhibition of disulfide-bridge formation in structurally important proteins.

The role of ions in lysis of bacteria was considered by several workers who revealed the ionic make-up and environment of cells as important factors in growth and division. Brown and Turner (1963) examined the membrane stability and salt tolerance in Gram-negative bacteria and found a significant relation between the percent lysis/percent envelope protein and salt tolerance; presence of calcium chloride inhibited envelope lysis in buffered saline. The mucopeptide content of Gram-negative bacterial walls was related to the salt concentration of their natural habitat, in a discussion of ionic environment by Brown (1964). MacLeod (1965) considered the maintenance of cellular integrity as a partial explanation for the special requirement for inorganic ions exhibited by marine bacteria, thus supporting the evidence obtained by Brown (1964) concerning stabilization of

halophilic membranes by high salt concentrations. Buckmire and MacLeod (1964) demonstrated the presence of mucopeptide constituents in a marine pseudomonad, and suggested that a cell wall whose rigid layer embodied metal-ion bridges would explain the lytic behaviour of this bacterium. Vincent and Humphrey (1963) found that calcium was more concentrated in the wall than in the rest of the cell, and their study of calcium-deprived cells led to their suggestion that calcium was involved in stabilizing the rigid mucopeptide layer by bonding with excess carboxyl groups or by linking the lipoprotein and rigid wall components. Unlike magnesium or barium, strontium was able to substitute for calcium in its wall function, though it was less efficient.

Many workers studied the behaviour and properties of protoplasts, spheroplasts, and L-forms, and compared them with the intact organism, in an effort to determine the role played by the wall components affected. Unfortunately, a great deal of incompatible data resulted, largely because the exact nature of the wall elements in the derived forms was not always known. Murray, Francombe, and Mayall (1959) identified the site of penicillin-action as the ingrowing septa taking part in cell division, and many of the penicillin-induced defective forms were known to have division problems. In a study of penicillin-induced L-forms of Proteus mirabilis, Altenbern and Landman (1960) suggested that mutant L-forms capable of growing in penicillin-containing broth differed from the parent L-form in being

more resistant to penicillin inhibition of septum formation. In 1964, Martin described a defective type of mucopeptide manufactured by Proteus mirabilis cells treated with penicillin. Fitz-James (1964b) observed the extrusion of mesosomes during the conversion of Bacillus megaterium to protoplasts, and Ryter and Landman (1964) studied the relationship between mesosome loss and the stable L-state (or protoplast state) of Bacillus subtilis. These latter authors found that the cell wall-protoplast membrane of the L-form envelope could not be identified with either the cell wall or cytoplasmic membrane; it had a distinctive composition with respect to the proportions of components found in the wall and membrane.

(E) Problems of Fixation

The highly complex and delicately balanced structures and mechanisms involved in bacterial walls and division indicate a special need for adequate preservation during fixation. A survey of fixation procedures reveals the development of methods designed to provide suitable environmental conditions during fixation.

Early workers (Porter, Claude and Fullam, 1945) were aware of the influence of the fixative on cell morphology as revealed in the electron microscope, and Palade (1952) introduced his acetate-veronal buffered fixative to eliminate the undesirable hypotonic effects of unbuffered osmium tetroxide. In a comparative study with unbuffered osmium tetroxide, Palade confirmed

the improved fixation with his buffered fixative, and also made the important observation that the quality of fixation varied with different types of specimens. Changes in pH and tonicity were included in his procedures and, again, he noted the range of responses by different cell types. De Robertis (1956) used a slight modification of Palade's standard buffered osmium and discovered that the presence of calcium ions in solution insured better preservation of the macromolecular structure of chromosomes. This indicated the possibility that, in addition to cell-type, each cell component may require a specific fixing environment to effect optimum preservation.

Evidence of better preservation of structures was obtained by Sjöstrand (1953) when he adjusted the fixing and washing solutions to isotonicity. The contribution made by isotonicity was stressed again (1956) when isotonic non-buffered osmium solutions produced satisfactory results while Palade's buffered fixative proved strongly hypotonic for some cells. However, the question of the importance of pH in the fixing process puzzled him when he read of the wide pH range used in fixation by different investigators without considerable differences in results. Caulfield (1957) substituted non-ionic sucrose for the commonly used salts as the tonicity-adjusting agent in fixation. Employing Palade's basic fixative, he compared animal and plant tissues and concluded that no single fixative satisfied the requirements of all specimens.

In 1958, there appeared in the literature a fixing procedure devised by Kellenberger, Ryter and Séchaud as a result of their systematic study of the response of bacterial nuclei to fixation. Now a standard method among bacteriologists, this process, consisting of a veronal-acetate buffer with added calcium chloride used in the osmium fixative, the washing fluid and the embedding agar, produced a fine-stranded nucleoplasm in several bacterial species. Previously, Whitfield and Murray (1956) had shown the effects of the ionic environment on bacterial chromatin structures as seen by light microscopy.

The value of the veronal-acetate buffer in fixation was questioned by Bennet and Luft (1959) who concluded that this buffer system was not fully effective at the pH range used. They recommended the use of s-collidine buffer which had maximum buffering capacity at the pH range used in fixation. Millonig (1961a) decided to test sodium mono- and di-phosphate buffer as a vehicle for osmium tetroxide in fixation of biological tissues since this buffer system existed in body-fluids of animals. Comparative studies with veronal-acetate buffered osmium on different animal tissues revealed improved preservation of some elements when treated with the isotonic phosphate-buffered fixative. Tahmisian (1964) advised the use of the freezing-point depression method for adjusting the tonicity of fixing solutions to approximate physiological conditions. Tonicity adjustments based on calculations of ionic strengths were said to be subject to error because

of possible interactions among the various components of a fixative. It was also pointed out that a true evaluation of the hydrogen ion concentration as an important property of fixatives could be made when tonicity was accounted for. A comprehensive study was undertaken by Wood and Luft (1965) to determine the influence of buffer systems on fixation with osmium tetroxide. The quality of fixation was estimated in terms of penetration of fixative, stainability, appearance by light microscopy, sectioning properties, acceptance of heavy metal stains, and general morphology in the electron microscope. The results were not easily explained on the basis of tonicity differences alone, and were interpreted as evidence in favour of the concept of specific ion effects. They, too, concluded that a single "best" fixative was not likely, and recommended choice of fixatives suitable for specific purposes.

MATERIALS AND METHODS

Wherever possible, reagent grade chemicals were employed. All aqueous solutions were prepared with distilled water. Concentrations represent w/v unless designated otherwise (v/v).

(A) Biological(1) Cultures

Spirillum serpens VH was isolated by Dr. C. F. Robinow of this Department in 1957. This culture possessed a surface layer of hexagonally arranged units similar to the external "macromolecular monolayer" of a Spirillum species described by Houwink (1953). A paralysed mutant, S. serpens VHX, also with the surface pattern, was derived from S. serpens VH by X-irradiation. An extensive account of the pattern in S. serpens VH was published by Murray (1963a). However, since the time of publication, the strain selected by subculture in this laboratory, S. serpens VHL, permanently lost the surface pattern. A culture maintained by Dr. S. Maier in Athens, Ohio, S. serpens VHA, still possesses the surface component.

Escherichia coli B was received from the culture collection in this Department. Lineola longa 1741 was obtained from Dr. C. F.

Robinow who received it from Dr. E. G. Pringsheim's laboratory in April, 1963. Simonsiella crassa and Alysiella filiformis were isolated from sheep saliva (Steed, 1963).

(2) Media

The following media were prepared in distilled water, sterilized by autoclaving at 121°C for 20 minutes, and used at neutral pH values.

(i) Yeast-acetate-peptone (YAP)

0.3%	yeast extract (Difco)
0.3%	Bactopeptone (Difco)
0.05%	Sodium acetate

(ii) Yeast-peptone-acetate (YPA)

0.1%	yeast extract (Difco)
0.1%	Bactopeptone (Difco)
0.1%	sodium acetate
0.005%	L-cystine (Difco)

(iii) Tryptone

1%	Bactotryptone (Difco)
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(iv) Calcium carbonate

2%	calcium carbonate powder
2%	glucose
1%	yeast extract (Difco)

(v) Penassay (Hirokawa, 1962)

0.3%	beef extract (Difco)
0.15%	yeast extract (Difco)
0.5%	Polypeptone (Difco)
0.1%	glucose
0.35%	sodium chloride
0.368%	potassium phosphate
0.132%	potassium dihydrogen phosphate

(vi) Sucrose-penicillin (S-P) (Hirokawa, 1962)

Penassay medium supplemented with 20% sucrose, 0.2% magnesium sulphate and 1,000 units/ml potassium penicillin "G" (from Nutritional Biochemicals Corporation, Cleveland, Ohio).

(vii) Serum

Oxoid nutrient medium supplemented with 10% (v/v) horse serum (Difco).

Solid media were prepared by adding 1.2% Bactoagar (Difco).

(3) Conditions of Growth(i) Maintenance

Stock cultures of Spirillum serpens, Escherichia coli and Lineola longa grew readily at room temperature and were maintained by monthly subculturing on YPA media. Both Simonsiella crassa and Alysiella filiformis required subculturing in serum broth at 37°C two to three times each month. All stock cultures were refrigerated

(at 4°C) between subcultures.

(ii) Experimental

For experimental purposes liquid cultures were grown under stationary, shaken, or aerated conditions. Shaking was carried out at room temperature in a reciprocating shaker (Model 6000, Eberbach Corporation, Ann Arbor, Michigan). Aerating consisted of bubbling air through pasteur pipettes in tubes of broth fitted with cotton wool plugs. Broth cultures were grown in 20 to 25 ml. aliquots distributed in 25 x 200 mm. test tubes. It was noted that freshly inoculated broths usually failed to grow when aerated or shaken immediately after inoculation. Subsequently, inoculated broths were allowed to grow as stationary cultures for several hours before aeration or shaking was initiated. The various incubation temperatures used for broth and agar cultures were room temperature (20°C to 25°C), 26°C, 30°C, 37°C and 45°C. Turbidity values of cell suspensions were measured in a Bausch and Lomb Spectronic 20 Spectrophotometer at 520 m μ wave length.

(B) Electron Microscopy

(1) Routine

(i) Grids

A formvar film prepared from 0.175% formvar in chloroform was floated on the surface of distilled water. Onto this film were placed 200-mesh copper grids which were picked up on paper, dried, then lightly coated with evaporated carbon in a Speedivac Coating Unit

(Model 12E6/1252, Edwards High Vacuum Limited) or Philips Shadow Casting Unit (Type 39980/13).

(ii) Negative staining

Phosphotungstic acid (PTA), adjusted to neutrality (with sodium- or potassium-hydroxide), as a 1% solution or as a 4% solution containing 0.004% sucrose, and 1% uranyl acetate (UA) at pH 4.5 or pH 2.2, were the reagents used as negative stains. Preparations were made by following the loop-film technique developed by Murray (1963a).

(iii) Shadowing

Grids carrying bacterial samples were shadowed with tungsten oxide (TO) at an angle of 1:3. The tungsten oxide was formed on a tungsten wire "basket" by heating briefly to redness in air.

(iv) Sectioning

The routine preparation of bacterial specimens for sectioning involved a procedure of prefixing, enrobing, fixing, staining, dehydrating and embedding developed by Kellenberger, Ryter and Séchaud (1958). Details of the methods as they were carried out for this study are presented in Appendix I. Unless stated otherwise, osmium tetroxide (O_5O_4) was the fixative used. The polyester medium, Vestopal W, obtained from Martin Jaeger, Vésanaz, Geneva, was employed for all embeddings.

(v) Staining of sections

Grids carrying sections were placed section-side down on drops of various staining and rinsing solutions, excess fluid being drained by

minimal contact with bibulous paper. The most commonly used stains were 1% solutions of uranyl acetate, thallium sulphate, and lanthanum nitrate, and the lead hydroxide stain described by Millonig (1961b). A method combining uranyl acetate and the lead citrate stain of Reynolds (1963) consisted of 1% uranyl acetate-lead citrate-0.01N sodium hydroxide (1:1:3 by volume) the stained sections being rinsed with 0.01N sodium hydroxide. Staining times varied from 20 seconds to 5 minutes.

(vi) Photography

Electron micrographs were taken on Kodak fine-grain positive film using a modified Philips EM-100 at 60 kV. The microscope was fitted with pole pieces of 1.8mm bore, a Ladd anode, mechanical astigmatism correction, and accessory stabilization of the input.

(2) Variations

Several variations of the reagents and methods used in the routine preparation for sectioning were carried out to observe the effect of these variations on the ultrastructural appearance of cells. Osmotic values of several different fixing media were estimated with an Osmette Precision Osmometer (Precision Systems, Framingham, Massachusetts) using freshly prepared 2 ml. samples chilled in ice.

(i) Buffer

YPA agar cultures of S. serpens VH were harvested in various buffers containing 0.1% OsO₄ for prefixation. The Noble agar (Difco)

and fixative (with 1% O_5O_4) were prepared with these same buffers which included veronal-acetate (VA) buffer (Kellenberger, Ryter and Séchaud, 1958), 0.05M sodium cacodylate-HCl, 0.05M 2:4:6 collidine-HCl, and 0.05M phosphate (KH_2PO_4 - Na_2HPO_4) buffer, all at pH 6.4.

(ii) Sucrose

Different concentrations of sucrose were substituted for a buffer in the prefixative, Noble agar and fixative. YPA agar cultures of S. serpens VH were prefixed with 0.1% O_5O_4 in distilled water alone and in 2%, 5% and 10% sucrose solutions.

(iii) No prefixation

YPA agar cultures of S. serpens VH were harvested in dilute (1/5) VA buffer without added O_5O_4 . The Noble agar and fixative were prepared with the dilute buffer.

(iv) Alkaline pH

YPA agar cultures of S. serpens VH were processed with prefixative, Noble agar and fixative prepared from 1/5 VA buffer adjusted to pH 7.4, 8.4 and 9.4.

(v) Sodium lauryl sulphate

Sodium lauryl sulphate (SLS) (Fischer Scientific Company, Fair Lawn, New Jersey) at 0.4% or 4% concentration was used to harvest YPA agar cultures of S. serpens VH. Prefixation was carried out at the temperature of incubation. Both Noble agar and fixative contained 1/5 VA buffer.

(vi) Temperature

Prefixation of YPA agar culture of S. serpens VH was performed at the temperature of incubation. Dilute (1/6) VA buffer was included in the prefixative, Noble agar and fixative. The dilute buffer contained the full complement (0.01M) of calcium chloride present in full strength VA buffer. A companion culture was treated similarly with the dilute buffer at pH 7.0.

(vii) Dehydration

In addition to the prefixative, Noble agar and fixative, the 30%, 50% and 75% acetone solutions used for dehydration were prepared with 2.5% sucrose in deionized water (with and without 0.1% calcium chloride) and in VA buffer pH 6.1. YPA agar cultures of S. serpens VH were prefixed at the incubation temperature.

(viii) Hot

YPA broth cultures of S. serpens VH were aerated at room temperature for 11 hours and were prefixed with OsO₄ at various temperatures up to 70°C. The cultures were heated to the appropriate temperature within a matter of seconds by mixing with OsO₄ in a metal dish of large surface area placed on a "Temp-Blok" Module Heater (Lab-Line Instruments Incorporated, Melrose Park, Illinois).

(ix) Fixative

Cultures of E. coli were prefixed by addition of glutaraldehyde (Fischer Scientific Company, Fair Lawn, New Jersey) to give 7% (v/v) (Sabatini, Miller and Barnett, 1964). After 90 minutes, the cells were

washed three times with 0.05M tris (hydroxymethyl) aminomethane (Tris) buffer pH 7.0. Fixation with O_5O_4 and subsequent steps were carried out in the routine manner.

(3) Pretreatments

(i) Sonication

Bacterial samples in 2 to 3 ml aliquots were sonicated in an MSE Ultrasonic Apparatus at 20 kc using a 0.5 inch probe with an output of 1.75 amp. The samples were chilled in ice during treatment which lasted 5 to 60 seconds.

(ii) Exposure to reagents

Various bacterial preparations were exposed to certain reagents before examination in the electron microscope: urea, guanidine hydrochloride, disodium ethylenediaminetetraacetate (EDTA) adjusted to neutrality, sodium lauryl sulphate (SLS), from Fischer Scientific Company, Fair Lawn, New Jersey; petroleum ether (boiling point range 35°C to 60°C) with or without 2.5% sucrose; hexadecyltrimethylammonium bromide (CTAB) from Eastman Organic Chemicals, Rochester, New York; DL pantoyl lactone, trypsin (2x crystallized, salt free), pepsin (3x crystallized), lipase 448 (porcine) from Nutritional Biochemicals Corporation, Cleveland, Ohio; lysozyme (3x crystallized, egg white) from General Biochemicals, Chagrin Falls, Ohio.

(iii) Enzymes

The following buffered enzyme preparations were centrifuged, and undissolved material discarded before use. Trypsin: 0.1% in 0.05M

phosphate buffer pH 7.8; pepsin: 0.5% in 0.2M glycine-HCl-buffer pH 2.2; lipase: 0.5% in 0.05M phosphate buffer pH 7.3; lysozyme: 0.4 mg/ml in Tris buffer pH 7.2.

(iv) Spheroplast formation

Bacteria were converted to spheroplasts by exposure to the combined action of lysozyme and EDTA in the presence of a suitable osmotic environment.

The method used for Escherichia coli B was modified from that of Repaske (1956) and consisted of suspending young cells in 0.3M Tris buffer pH 8.0 containing 134 $\mu\text{g/ml}$ EDTA, 68 $\mu\text{g/ml}$ lysozyme and 10% sucrose (TELS mixture). Spheroplast formation was observed by microscopic examination and after several minutes magnesium sulphate, to give 0.05M, was added to the suspension.

Spirillum serpens VH and Simonsiella crassa were successfully transformed to spheroplasts by a method slightly modified from that of Karunairatnam, Spizizen and Gest (1958). The cells from young broth cultures were suspended in 0.01M phosphate buffer pH 7.0 containing 1.6 mg/ml EDTA, 0.5 mg/ml lysozyme and 10% sucrose (BELS mixture). After spheroplasts were produced, half volume of 40% sucrose and magnesium sulphate to give 0.05M were added.

Control specimens were prepared by suspending cells in mixtures without lysozyme (TES, BES). Both spheroplasts and controls were prefixed by addition of OsO_4 and processed for sectioning.

(v) Methyl ferritin

The use of methyl ferritin described by Nachmias and Marshall (1961) in their study of amoebae was adopted. Methyl ferritin was prepared by methylation of free carboxyl groups on ferritin (2x crystallized, horse spleen, from General Biochemicals, Chagrin Falls, Ohio), following the procedure for esterifying proteins described by Fraenkel-Conrat and Olcott (1945). An aliquot (0.1 to 0.2 ml) was suspended in a hundred-fold volume of methanol. To this was added 0.1 ml hydrochloric acid (concentrate). This mixture was allowed to stand at room temperature for one week with occasional shaking. The methylated product was isolated by evaporation after extensive dialysis against cold distilled water and kept in the frozen state.

(vi) Freeze-etching

The freeze-etched specimen of Spirillum serpens VHA was prepared by Mr. J. Marak of this Department.

(C) Serology

(1) Antigens

Somatic antigens of S. serpens VHA and S. serpens VHL were prepared from tryptone agar cultures grown at 26°C which were harvested in distilled water and boiled to inactivate flagellar antigens.

(2) Antisera

The somatic antigen preparations were suspended in 0.3% sodium chloride containing 0.75% formalin. Rabbits were given a series of

injections of 0.5 ml amounts through the loose skin behind the head. Test samples of serum obtained from ear-bleeds were checked for antibody levels and, when suitable, the animals were bled from the heart.

(3) Reciprocal adsorption test

The procedure adopted was that described by Carpenter (1956). Cells of S. serpens VHA and S. serpens VHL prepared as somatic antigens were incubated for one hour at 37°C with 1/20 dilutions of the reciprocal antisera. After centrifugation, the sera were recovered and reincubated with a fresh batch of reciprocal somatic antigens. The sera were again recovered after centrifugation and used in reciprocal adsorption agglutination tests in which whole cells (not boiled) were the test antigens.

(D) Isolation of Surface Pattern

It was observed that old agar cultures of S. serpens VHA contained an accumulation of cell-free surface pattern. Advantage was taken of this phenomenon in order to isolate the pattern material for chemical analysis.

Tryptone agar plates were flooded with young YPA broth cultures and excess inoculum withdrawn. Up to 100 plates at a time were inoculated and incubated at 26°C for one week. Growth was harvested in calcium solution (10^{-3} M calcium chloride in distilled water). The cell-free material was separated from cells by differential centrifugation at

2,000 g for 15 minutes. The supernatant was then centrifuged at 26,000 g for 30 minutes at 4°C and the pellet resuspended in a small volume of calcium solution. This pellet contained both flagella and pattern components so heat treatment of the suspension was carried out (60°C to 65°C for 1 to 2 hours) to destroy the flagella. The pattern material appeared to remain undamaged and was washed three to four times with calcium solution. Final resuspension of the cleaned, isolated pattern material yielded a milky, opalescent, birefringent suspension. This concentrated material was distributed in 1 to 2 ml amounts and stored in the frozen state.

The same procedure was applied to S. serpens VHL.

(E) Chemical Methods

All tests were carried out in duplicate. Optical density readings were made with an Hitachi Perkin-Elmer 139 UV-VIS spectrophotometer.

(1) UV absorption spectra

Samples (1 ml) of the surface material isolated from S. serpens VHA and S. serpens VHL were diluted to 3 ml with distilled water and sonicated for 30 to 60 seconds. The UV absorption spectra were obtained from optical density readings in the wavelength range 230 to 290 mμ.

(2) Dry weight determinations

Aluminium foil pans were dried to constant weight before and after addition of the sample by heating at 105°C overnight then storing

over phosphorus pentoxide in a desiccator. Weighings were made on a Mettler Model H16 analytical balance.

(3) Lipid analysis

(i) Extraction

Lipid was extracted by following the procedure outlined by Folch, Lees and Sloane Stanley (1957). A volume (1 ml) of an aqueous suspension of the sample was shaken with 4 ml of chloroform-methanol (2:1 by volume). After removal of the upper phase, the interface was washed with chloroform-methanol - 0.003M aqueous calcium chloride (3:48:47 by volume) to remove the interfacial fluff. Finally, methanol was added drop by drop until a single phase was produced. This extract was used for dry weight determinations and for lipid chromatography.

(ii) Lipid chromatography

Lipid chromatography was carried out under the direction of Dr. K. K. Carroll with the technical assistance of Mr. H. E. Pedersen in the Department of Medical Research. Standards used were phosphatidyl ethanolamine L-alpha-cephalin dipalmitoyl (Synth.) M.A. from Mann Research Laboratories Incorporated, New York, and a glycolipid derived from Listeria monocytogenes (a product of the Department of Medical Research).

The lipid extracts were evaporated to dryness under a stream of nitrogen and reconstituted in 0.2 ml of chloroform for application to chromatoplates. The general procedure for thin-layer chromatography

described by Skipski, Peterson and Barclay (1964) was followed. Glass plates were spread uniformly with a slurry of 44.4% silica gel H (E. Merck Ag. Darmstadt) in 0.001N sodium carbonate. Dry plates were activated at 120°C for 20 minutes before use. Ascending chromatography was performed in glass jars lined with solvent-saturated filter paper.

Solvent system (a)

Absolute acetone with 6% (v/v) water was the solvent used to determine the presence of glycolipids; sulphuric acid spray was employed to develop spots.

Solvent system (b)

After a run in a solvent consisting of chloroform-methanol-water (65:20:3 by volume), the chromatoplates were dried at room temperature, sprayed with sulphuric acid, then heated at 120°C for one hour to develop the spots. Alternative spray reagents were substituted for sulphuric acid in order to detect certain groups in the lipid samples.

(i) Presence of free amino groups of phospholipids was tested by spraying with 0.2% ninhydrin in butanol saturated with water then heating the chromatoplates for five minutes at 100°C to 105°C in a water-saturated atmosphere.

(ii) The modified Dragendorff reagent was used as described by Wagner, Hörhammer and Wolff (1961). Dry chromatoplates were sprayed with this reagent which reveals the presence of free choline (purple spot) and of choline-containing compounds (orange spots).

Solvent system (c)

Two-dimensional chromatography was performed by the method of Rouser, Siakotos and Fleischer (1966) modified by using chloroform-methanol-water (65:20:3 by volume) as the first solvent. This was followed by a second run in chloroform-acetone-methanol-acetic acid-water (10:4:2:2:1 by volume) after which the dried chromatoplates were sprayed with sulphuric acid or, preferably, with Rouser's reagent consisting of 0.6% potassium dichromate in 55% (by weight) sulphuric acid. The sprayed chromatoplates were developed by heating in an oven at 180°C for 30 minutes or overnight.

(4) Carbohydrate(i) Total

The anthrone test for carbohydrates was performed as described by Morris (1948). Test samples, blanks and glucose standards were prepared in 5 ml volumes with distilled water. To each tube were added 10 ml of freshly prepared reagent : 0.2% anthrone (Nutritional Biochemicals Corporation, Cleveland, Ohio) in sulphuric acid (95% v/v). The contents were mixed by thorough shaking immediately after addition of the reagent. Optical density values of the cooled samples were measured at 625 m μ wavelength.

(ii) Chromatography

Isolated surface pattern material from S. serpens VHA was hydrolysed in 2N hydrochloric acid at 100°C for two hours. Standards of glucose, arabinose, fructose, mannose, rhamnose, xylose, ribose

and galactose were prepared in distilled water at a concentration of 1 mg/ml. Applications were made with a platinum loop (3 mm diameter) the spots being placed 2 cm apart and 3 cm from the base of the sheet.

The method for rapid separation of monosaccharides described by Gabriel and Igals (1960) was used with ITLC media (Gelman Instrument Company, Ann Arbor, Michigan). Sheets of the medium type SG (silica gel combined with microglass filaments) were pretreated by immersing in 0.1M sodium phosphate (dibasic, anhydrous) adjusted to pH 5.0 with orthophosphoric acid (85%), and were subsequently dried by hanging at room temperature. Ascending chromatography was carried out for 90 minutes (to a distance of 10 to 12 cm) with a solvent consisting of pyridine-water-butanol (2:3:6 by volume). The chromatograms, dried at room temperature, were sprayed with the cysteine-sulphuric acid reagent (Gabriel and Igals, 1960) then dried in an oven at 70°C to develop the coloured spots.

(iii) Polysaccharide

Polysaccharide material was separated from the surface pattern isolated from S. serpens VHA using a method described by Kwapinski (1965). The procedure involved removal of proteins by extraction for six hours with an equal volume (5 ml) of 0.5N sodium hydroxide in 45% (v/v) ethanol after the pattern material had been suspended in benzene. The benzene phase, recovered in a separatory funnel, was evaporated and the residue dissolved in 5 ml of ether mixed

with an equal volume of 10% (v/v) acetic acid. Extraction was continued by shaking in a separatory funnel for four hours. The aqueous phase was removed and acetone-ethanol (3:1 by volume) added to precipitate the polysaccharide material. A fluffy, white precipitate settled out after standing overnight.

(5) Protein

(i) Total

Protein was estimated by the method of Lowry, Rosebrough, Farr and Randall (1951) using standards of albumin (Crystallized bovine from Nutritional Biochemicals Corporation, Cleveland, Ohio).

(ii) Amino acid analysis

Protein precipitated from isolated S. serpens VHA pattern material by 10% trichloroacetic acid was dialysed and concentrated before hydrolysis. Samples were hydrolysed for 22 hours at 110°C in 0.5 ml aliquots of 6N hydrochloric acid sealed under vacuum in pyrex test tubes. After centrifugation to remove insoluble material the hydrolysates were dried in an evacuated desiccator containing sodium hydroxide pellets.

Amino acids were analysed in a Beckman Model 120C Amino Acid Analyzer under the supervision of Dr. D. B. Smith and with the technical assistance of Miss M. S. Kennedy in the Department of Biochemistry.

(6) Phosphorus determination

The procedure of Ernster, Zetterström and Lindberg (1950)

was used for estimation of phosphorus. Test samples, blanks and phosphate standards prepared with B.D.H. Standard Phosphate were washed for 20 to 30 minutes in 1 ml quantities with 1.2 ml 60% perchloric acid.

RESULTS

(A) Profiles of Cell Surfaces

Sections of Gram-negative bacteria examined in this present study regularly revealed a dense-light-dense triplet-layer and an underlying electron-dense wall element outside the cytoplasmic membrane. The former corresponded to the wall profile identified in Escherichia coli by Kellenberger and Ryter (1958) and the latter to the intermediate layer observed in sections of Acetobacter suboxydans cells by Claus and Roth (1964) who suggested that it might represent the rigid mucopeptide layer of the wall. Lautrop, Reyn and Birch-Andersen (1964) and Reyn, Lautrop and Birch-Andersen (1964) carried out a comparative electron microscope study of Gram-negative bacterial walls in which they demonstrated that these two components represented a "typical" wall structure among Gram-negative bacteria.

(1) Spirillum serpens(i) Untreated

Figure I illustrates the "typical" wall profile in a lead-stained section of S. serpens VH cells from a YPA broth culture aerated at 45°C for 6 hours. It can be seen that the inner wall element is taut

FIGURE 1

Spirillum serpens VH: a lead-stained section showing the "typical" wall profile consisting of an inner, taut, electron-dense element and an outer, loose, dense-light-dense triplet-layer lying outside the cytoplasmic membrane X 156,500.

FIGURE 1



and has a smooth contour while the outer triplet-layer is loose and wavy. This kind of picture was obtained with most of the sections examined in this and other embedded preparations of S. serpens.

Measurements of the different components at the cell surface provided information about their dimensions. In all instances, the estimated figures represent the average value derived from at least 10 measurements of regions showing the most clearly defined profiles. A collection of dimensions is presented in Figure 26 at the end of this section.

(ii) Plasmolysed

That the cell wall confers both strength and shape on bacteria has been aptly demonstrated in plasmolysed cells examined by light microscopy (Robinow and Murray, 1953) and in sections (Cota-Robles, 1963). Knowledge that Gram-negative bacteria possess complex walls with several organized layers posed the problem of identifying the component(s) responsible for rigidity.

Plasmolysis of S. serpens VH was effected by harvesting cells grown on YPA agar at 37°C for 6 hours in cold veronal-acetate (VA) buffer (Kellenberger, Ryter, and Séchaud, 1958). The suspension was prefixed with osmium tetroxide (O_5O_4) for 10 minutes at 4°C. Figure 2 demonstrates the effect of plasmolysis at the electron microscopic level. At a higher magnification (Figure 3) it can be seen that both elements of the wall remain at the surface without any sign of collapse

FIGURE 2

Spirillum serpens VH: a section of plasmolysed cells stained with lead X 32,500.

FIGURE 3

Spirillum serpens VH: detail of the cytoplasmic membrane and wall profile in a lead-stained section of a plasmolysed cell X 110,000.

FIGURE 2



FIGURE 3



into the inner cell space. It was not possible to deduce from these sections which of the wall components maintained cell shape and rigidity.

This preparation also served to emphasize the fact that VA buffer is hypertonic for S. serpens VH cells. Under normal conditions, that is when treatment with this buffer took place at room or incubation temperatures, the plasmolysed cells underwent reversion. In the present experiment, a decreased rate or, perhaps, inhibition of the reversal process occurred at the lower temperature and the cells were fixed in the plasmolysed state. Figure 3 also demonstrates that numerous cytoplasmic masses, both large and small, produced as a result of plasmolysis, are membrane-bound. This indicates a marked degree of disruption and a corresponding degree of reorganization upon reversion, involving both cell membranes and cytoplasm. Further aspects of this effect are dealt with in Section B (4)(ii).

(iii) Partially disrupted

The section shown in Figure 4 demonstrates a distinct difference in behaviour of the inner and outer components of the wall. In this experiment, an aerated YPA broth culture of S. serpens VH incubated at 20°C for 11 hours was rapidly heated and prefixed at 70°C. This treatment disrupted the triplet-layer of the wall although the dense-light-dense constituents were still identifiable. In dramatic contrast, the inner wall component remained intact and lay closely

FIGURE 4

Spirillum serpens VH: partial disruption of the wall profile in a cell which was rapidly heated and prefixed at 70°C. Lead stained section X 57,000.

FIGURE 5

Spirillum serpens VH: absence of the inner, taut, electron-dense layer from the wall profile of a spheroplast induced by the combined action of lysozyme and EDTA (BELS solution). Section not stained X 156,500.

FIGURE 4

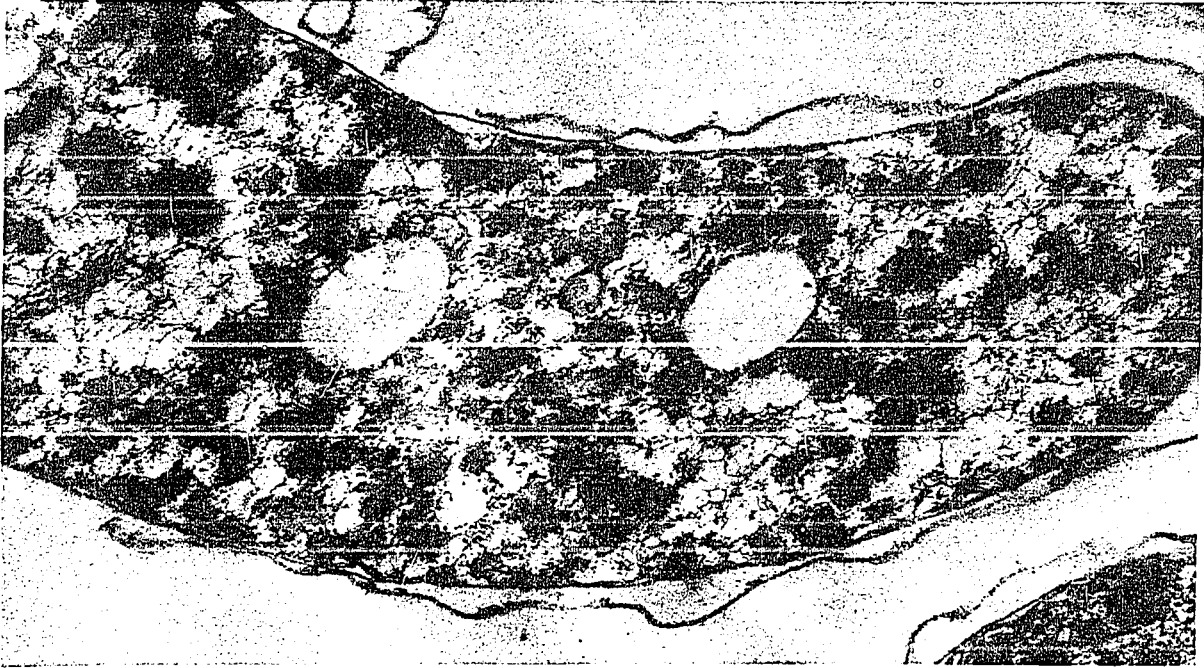


FIGURE 5



FIGURE 4

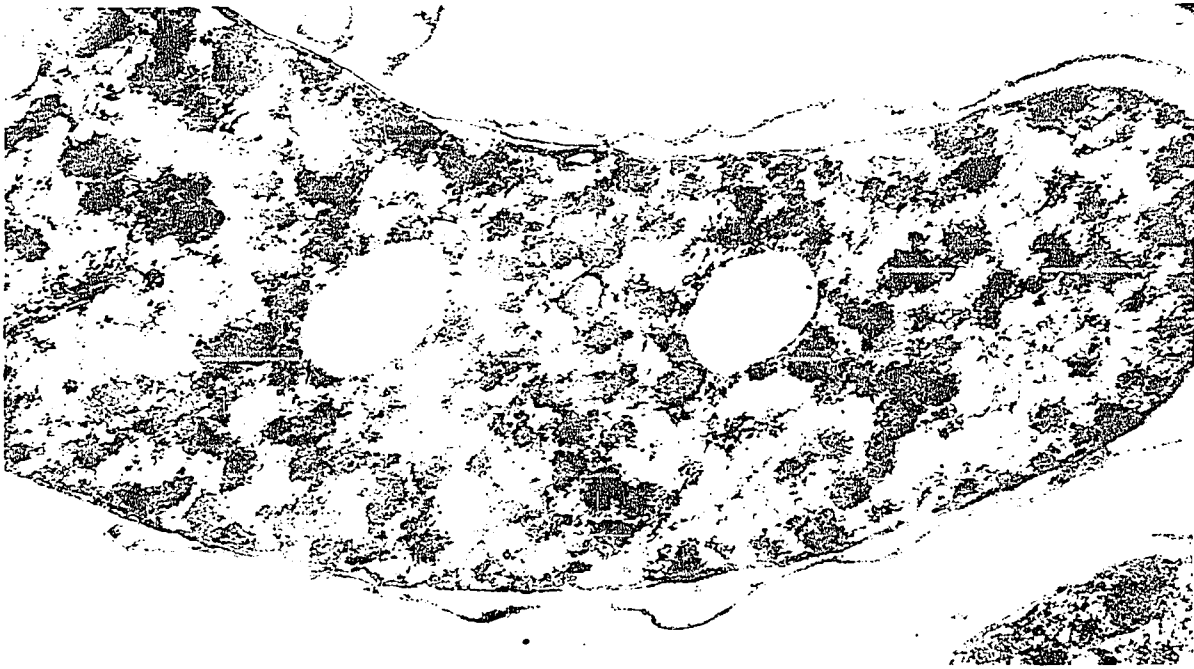


FIGURE 5



applied to the cytoplasmic membrane. These results indicate preservation of cell strength and shape by the taut layer in the absence of the outer triplet-layer. However, some coagulation by the heat treatment may have been involved in the apparent rigidity afforded by this inner wall structure.

(iv) Spheroplasts

Kern, Kingkade, Kern and Behrens (1951) demonstrated that exposure to the action of lysozyme may result in destruction of bacterial shape and rigidity. When this treatment is carried out in a suitable osmotic environment, Gram-positive bacteria assume a spherical shape and lyse when the medium is diluted (Weibull, 1953). Repaske (1956) discovered the effectiveness of the chelating agent, ethylenediaminetetracetic acid (EDTA) in enabling lysozyme to act upon and destroy the rigid component in Gram-negative bacterial walls. However, the osmotically-sensitive spheres produced by such a combined attack differ from protoplasts in that they retain residual wall components incapable of maintaining shape or strength of the cell. These forms are termed spheroplasts (McQuillen, 1960).

Spheroplasts of S. serpens VH cells were prepared from a YPA broth culture aerated overnight at room temperature. Cells from the centrifuged culture were suspended in BELS solution which consisted of 0.01 M potassium-sodium phosphate buffer pH 7.0 containing EDTA (1.6 mg/ml.), lysozyme (0.5 mg/ml.), and sucrose (10% w/v).

When production of spherical cells was complete as observed by light microscopy (after 30 minutes), magnesium sulphate (to give 0.05M) and additional sucrose (20%) were introduced. The suspension of spheroplasts was then prefixed with O_5O_4 .

Figure 5 provides a picture of the sectioned surface of a spheroplast. The loose, outer wall layer is the only visible component outside the cytoplasmic membrane. Absence of the inner electron-dense entity from the wall profile with the concomitant loss of cell shape and rigidity was taken as evidence for identifying the missing constituent as the layer embodying these lost properties. Thus, the taut, inner moiety in wall profiles was taken to represent the rigid mucopeptide element known to be a substrate for lysozyme activity (Salton, 1952).

(v) Effect of ionic detergents

Weidel, Frank and Martin (1960) extracted the outermost layer, lipoprotein, from walls of E. coli B using 1% sodium lauryl sulphate (SLS). This study formed part of their investigation of wall structure which involved serial chemical extraction of the different layers; shadowed preparations of the residual wall were examined after each extraction.

S. serpens VHA cells were exposed to SLS then processed for sectioning in order to determine what effect this treatment would have upon the wall profile. Figure 6 shows the result obtained when cells

FIGURE 6

Spirillum serpens VHA: cells exposed to an anionic detergent (SLS) for 60 minutes. Both the cytoplasmic membrane and the wall triplet-layer are missing. Section stained with lead-uranium X 110,000.

FIGURE 7

Spirillum serpens VHA: effect of treatment with a cationic detergent (CTAB) for 60 minutes. The triplet-layer can be seen in some areas of the wall-profile. Section stained with lead-uranium X 110,000.

FIGURE 6

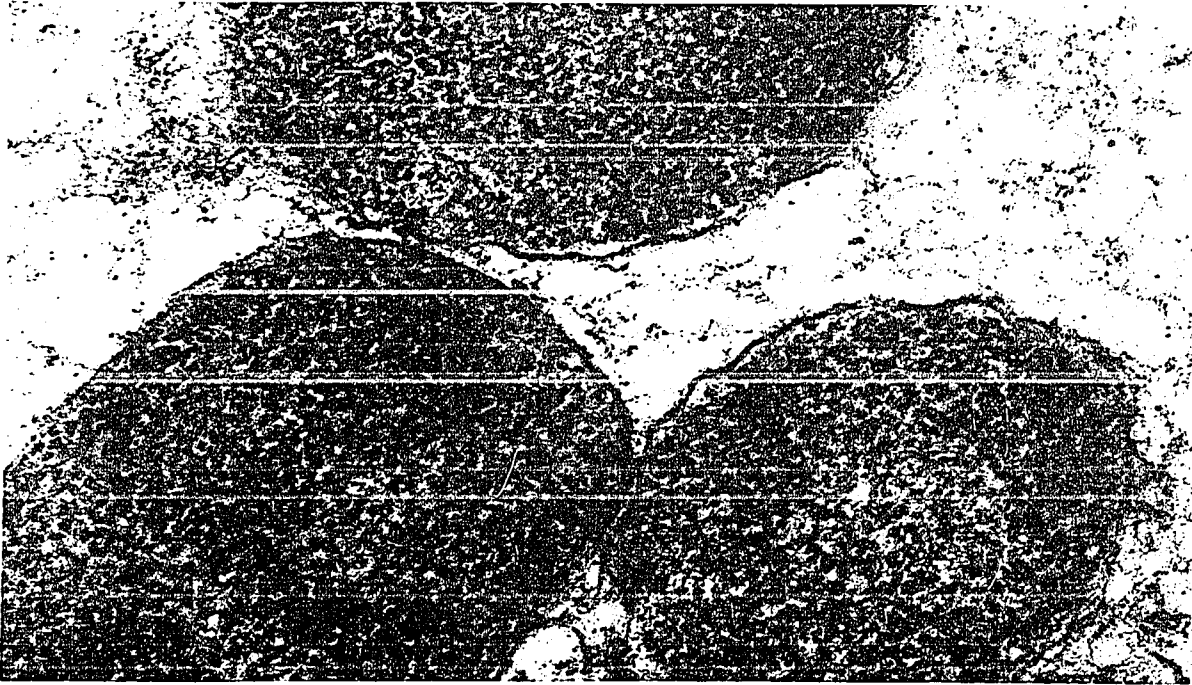
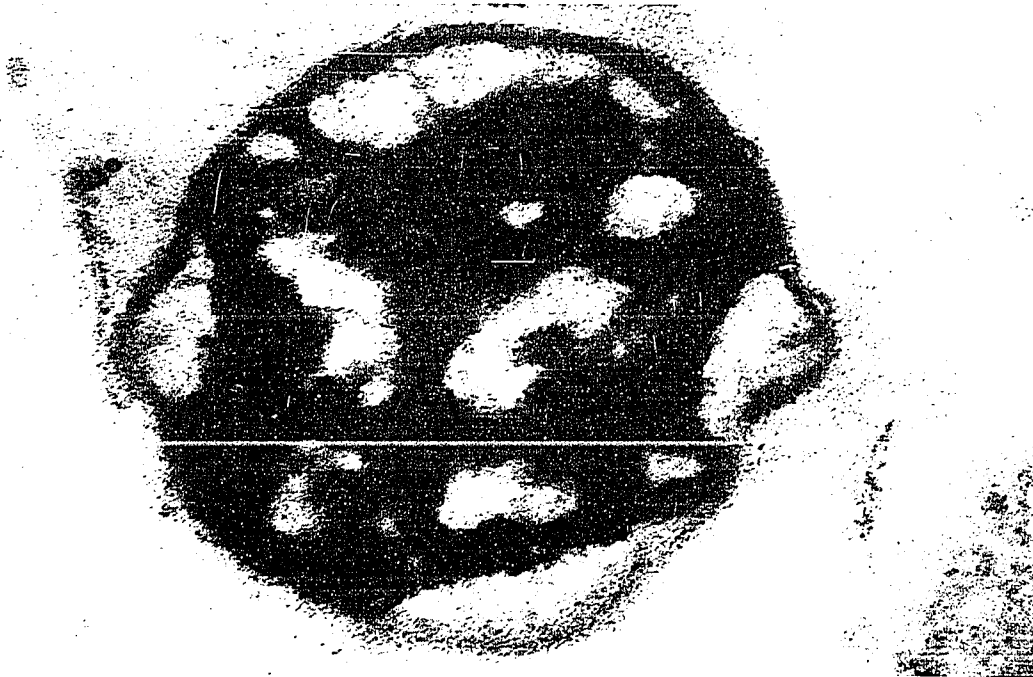


FIGURE 7



grown on YPA agar for 36 hours at 26°C were suspended in 0.25% (w/v) SLS aqueous solution and shaken at room temperature for 60 minutes. It is immediately obvious that the triplet-layer has completely disappeared from the wall profile. The cytoplasmic membrane also is absent and the only identifiable component remaining at the cytoplasmic surface is the rigid mucopeptide layer maintaining cell shape and retaining the ribosome-packed cell contents. Presumably, small molecular weight constituents have escaped with disruption of the cytoplasmic membrane while large cytoplasmic ingredients unable to penetrate the mucopeptide layer have remained. This would account for the densely packed ribosomes and lack of normal cytoplasmic detail. A markedly different effect was observed when a companion culture was treated with cationic detergent, hexadecyltrimethylammonium bromide (CTAB). Figure 7 shows a sectioned cell after shaking for 60 minutes with 0.25% (w/v) aqueous CTAB. The cytoplasmic material is grossly distorted but close examination of the surface layer reveals some residual elements with the dense-light-dense profile.

Since the SLS preparation showed extensive disruption of surface organization, a second experiment was planned to enable examination of an earlier stage in SLS action. S. serpens VHA cells grown on YPA agar for 36 hours at 26°C were suspended in 0.25% SLS and shaken at room temperature for periods varying from 1 to 30 minutes.

Figure 8, which shows the effect after 1 minute, clearly indicates rapid extraction of the cytoplasmic membrane and wall triplet-layer by the anionic detergent. Some fragments of the disrupted outer wall, still attached to the cell, or lying free, can be recognized by their dense-light-dense profile.

The appearance of whole cells of S. serpens VHA negatively stained with phosphotungstate (PTA) is depicted in Figure 9; a shadowed preparation is illustrated in Figure 10. Comparison of these pictures with sectioned SLS-treated cells permits their interpretation as cytoplasmic material trapped within a residual surface layer, namely, the mucopeptide component. This view is supported by results obtained by Weidel, et al. (1960) with a species of Spirillum.

(vi) Methyl ferritin treatment

Both S. serpens VHA and its pattern-less mutant S. serpens VHL were examined in section on many occasions. A disturbing observation made from these studies was the fact that their wall profiles appeared to be identical, being composed of the inner rigid mucopeptide component and outer triplet-layer. This implied that the wall pattern material was unidentifiable in sectioned cells. A paper published by Nachmias and Marshall (1961), describing the use of methyl ferritin in their study of pinocytosis in amoebae, suggested the possible application of this reagent to help solve the problem at hand. Ferritin has found considerable use in electron microscopy by virtue

FIGURE 8

Spirillum serpens VHA: cells after brief exposure (1 minute) to SLS. Fragments of the wall triplet-layer can be identified by their dense-light-dense profile. Section stained with lead-uranium X 110,000.

FIGURE 8

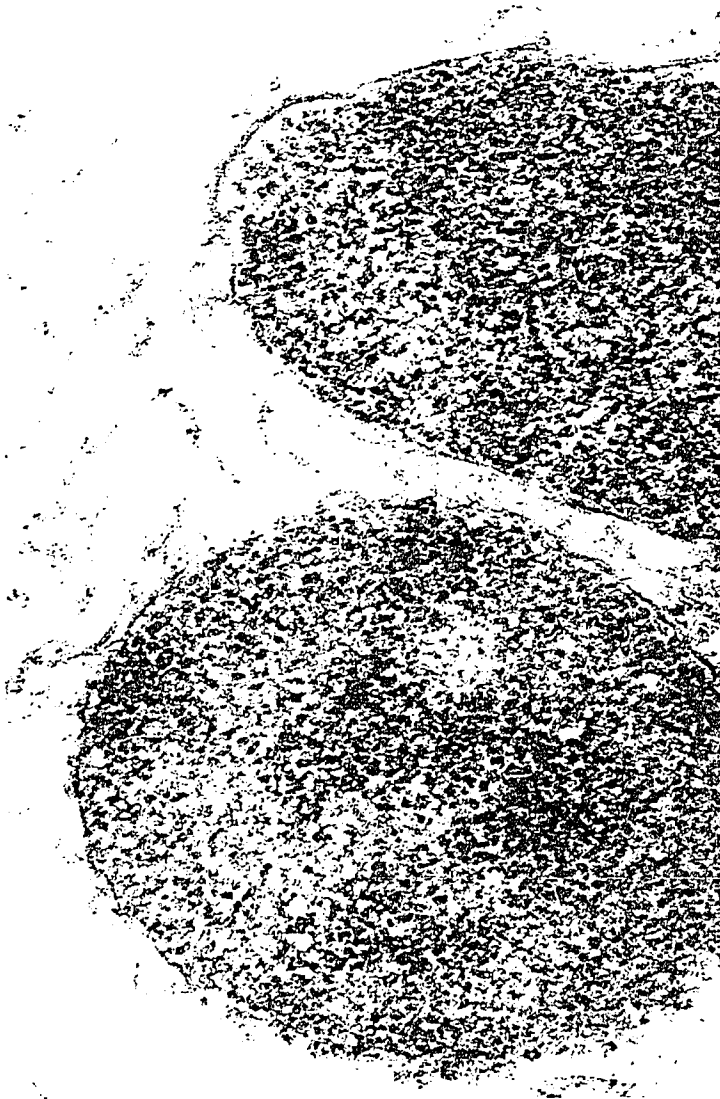


FIGURE 9

Spirillum serpens VHA: a cell negatively stained with phospho- tungstate after exposure to SLS X 83,000.

FIGURE 10

Spirillum serpens VHA: a cell treated with SLS and shadowed with tungsten oxide X 83,000.

FIGURE 9

Spirillum serpens VHA: a cell negatively stained with phospho-
tungstate after exposure to SLS X 83,000.

FIGURE 10

Spirillum serpens VHA: a cell treated with SLS and shadowed
with tungsten oxide X 83,000.

FIGURE 9

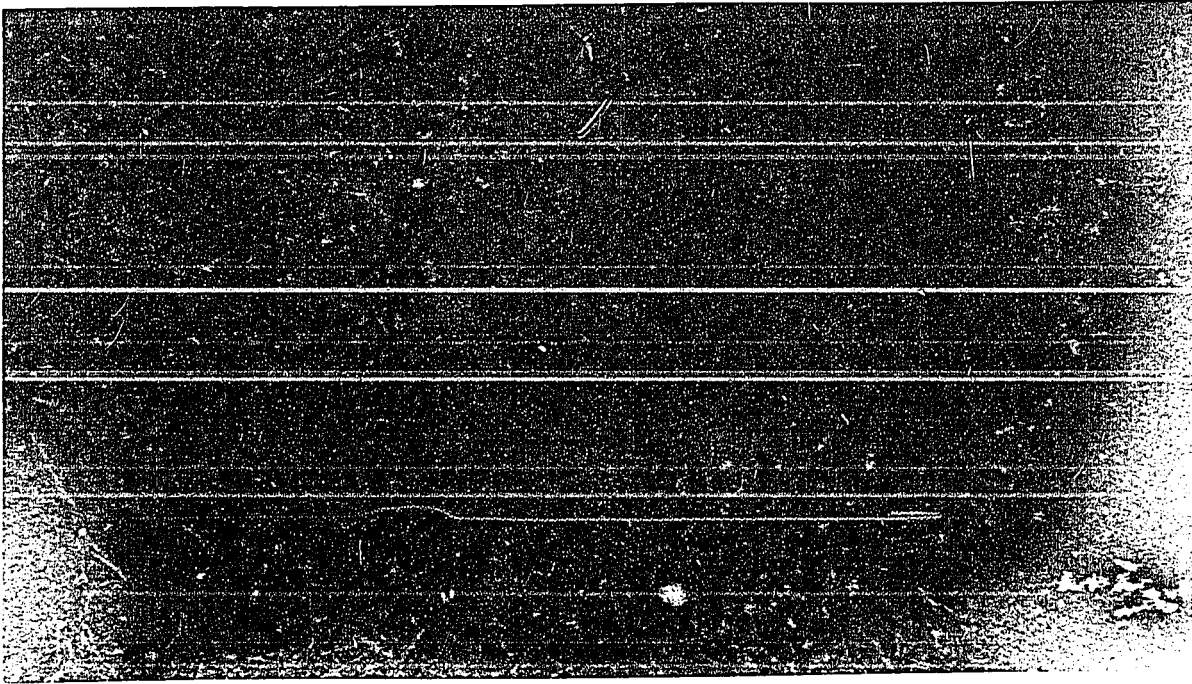
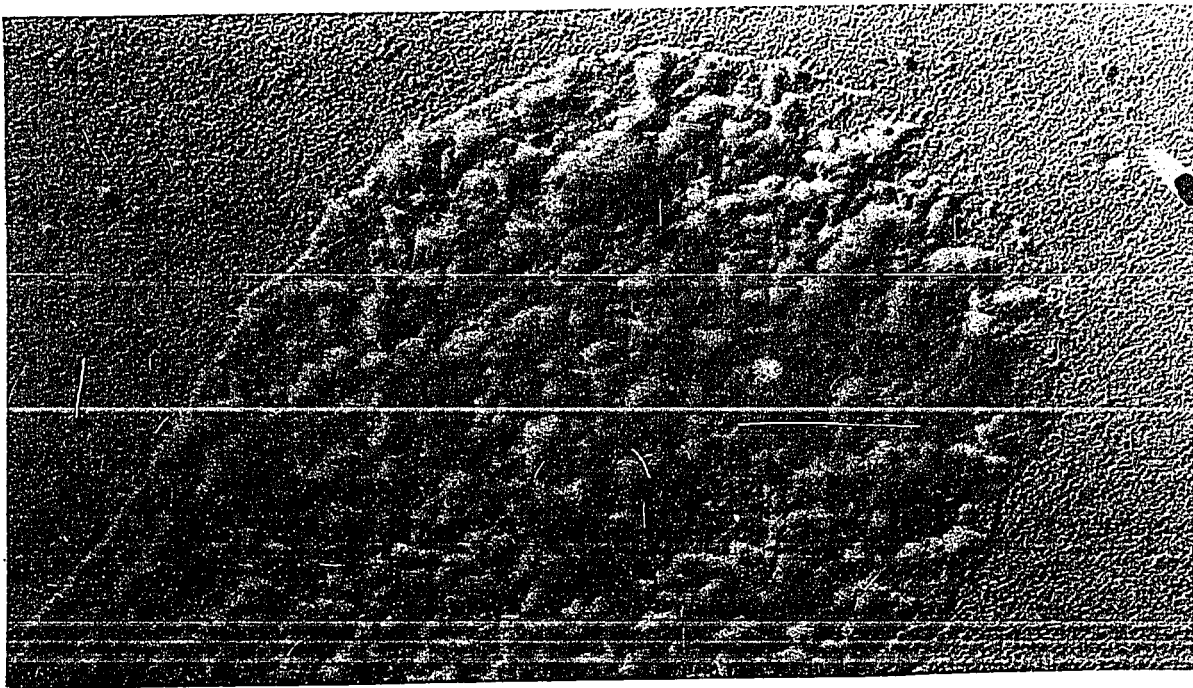


FIGURE 10



of its electron density which allows its observation in sectioned material. It has been most commonly employed as a conjugate with antibodies to provide the anatomical location of specific antigenic components. As a nonspecific marker ferritin itself was found to be of limited value because of its inability to remain adsorbed to substrates when washed with buffers used in fixatives (due to the pH). Nachmias and Marshall compared ferritin with its methylated derivative and their results clearly demonstrated the superior adsorption and retention of methyl ferritin.

This observation was confirmed with cells of S. serpens simply by examining whole cells washed with VA buffer after suspension in the buffer containing these reagents. Very few ferritin particles were present while cells were completely covered with methyl ferritin. Cultures of S. serpens VHA and of S. serpens VHL grown on YPA agar at 26°C for 54 hours were suspended in 1/5 dilution of VA buffer with added methyl ferritin. After 30 minutes, the cells were washed once with the dilute buffer then prefixed. The results obtained on sectioning were not disappointing. Figure 11 shows a section of S. serpens VHA in which the adsorbed methyl ferritin defines the actual limit of the cell surface. An otherwise unidentifiable layer was thus shown to exist beyond the wall triplet-layer. This surface material follows the irregular contour of the underlying layer suggesting a close association of the two components. A similar profile was not

FIGURE 11

Spirillum serpens VHA: an additional layer (electron-transparent) outside the "typical" wall profile is outlined by the electron-dense methyl ferritin adsorbed to the cell surface. Lead-stained section X 110,000.

FIGURE 12

Spirillum serpens VHL: methyl ferritin preparation. An additional layer is not evident. Section stained with lead X 110,000.

FIGURE 11

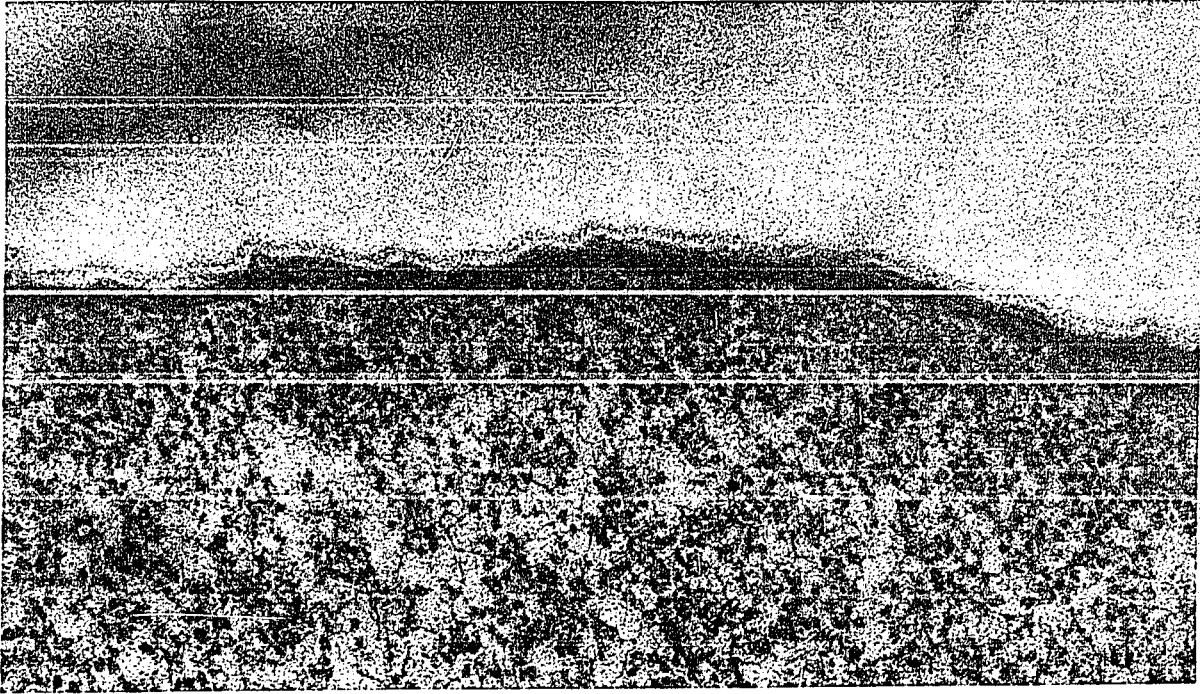


FIGURE 12



observed in sections of S. serpens VHL (see Figure 12), indicating absence of the surface constituent in this mutant. On the basis of these results, the additional stratum at the surface of S. serpens VHA cells was tentatively identified as the surface pattern material.

(vii) Surface pattern in section

Cells from a culture of S. serpens VHA grown on calcium carbonate agar for 24 hours at 26°C revealed a structure outside the wall triplet-layer. Figure 13 shows a sectioned cell that had been processed with 1/6 dilution of VA buffer in the prefixative and fixative. The section was stained with lead and uranium, and, in some areas of the surface layer, regularly-spaced electron-transparent structures can be seen. These were interpreted as repeating units in the pattern.

(viii) Compact profiles

On odd occasions sections were observed in which the constituent layers of the cell wall were closely applied thus presenting a compact profile. Such a case, shown in Figure 14 was seen in a preparation of S. serpens VH aerated in YPA broth for 6 hours at 45°C. An extremely rare wall profile of S. serpens VHX appeared in the section depicted in Figure 15. This paralyzed mutant, which possessed the surface pattern, was grown in YPA broth aerated at room temperature for 8 hours. Apart from the omission of tryptone from the fixative, this preparation was processed in the routine manner. It can be seen that an area of the wall profile consists of a compact structure

FIGURE 13

Spirillum serpens VHA: an additional layer outside the "typical" wall profile appears as an electron-dense region with some electron-transparent areas. Section is stained with lead-uranium X 110,000.

FIGURE 13

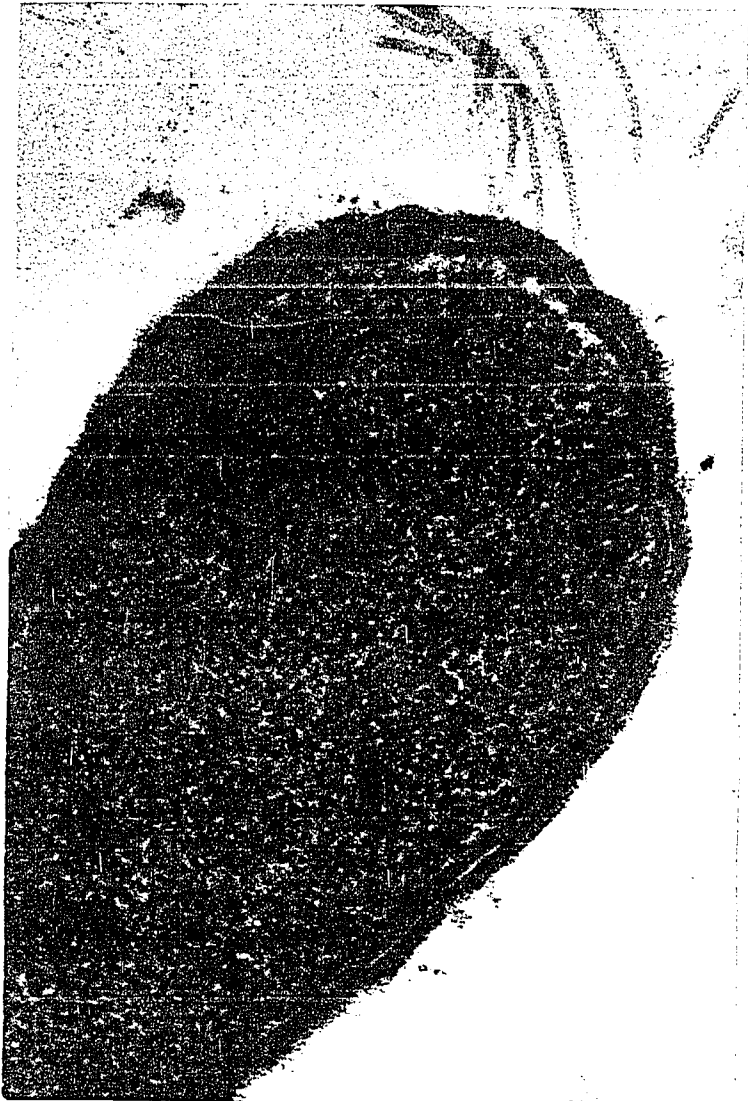


FIGURE 14

Spirillum serpens VH: thallium-stained section of a cell with a compact wall profile presenting a five-layered dense-light-dense-light-dense structure X 156,500.

FIGURE 15

Spirillum serpens VHX: complex compact arrangement of the "typical" wall profile together with an additional surface layer. Section stained with uranium X 156,500.

FIGURE 14

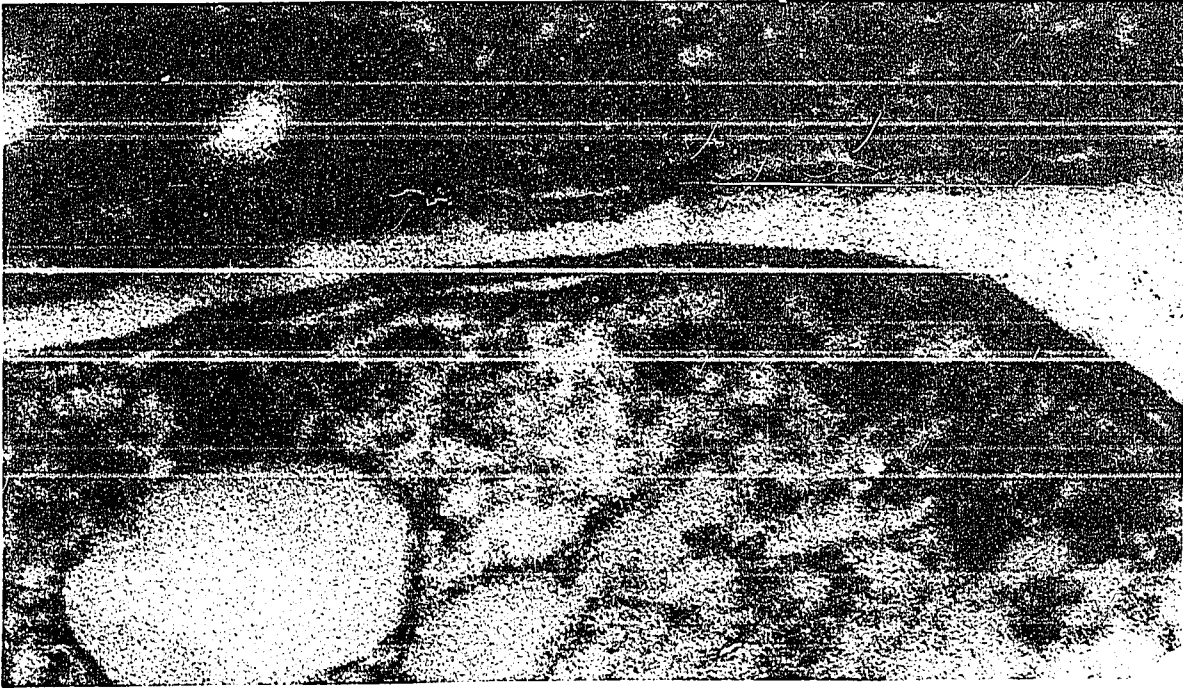
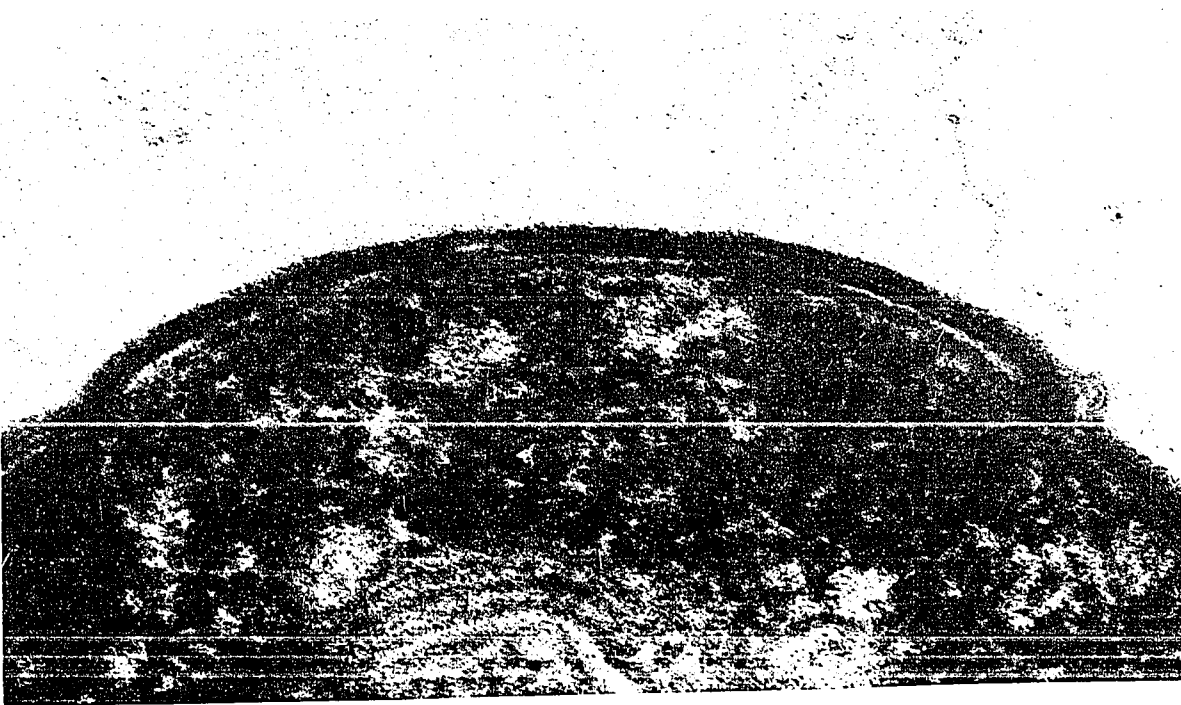


FIGURE 15



embodying all three elements, the mucopeptide, the triplet-layer, and the surface pattern. The remarkable feature is the close association of all these strata in contrast to the loose, wavy profile observed in most sections of this and other preparations. The overall thickness of this wall structure measured approximately 220\AA .

Thus, the complex multi-layered wall of S. serpens VH consists of at least five elements which can be seen in sectioned preparations. When the surface pattern is present, six or possibly more layers constitute the wall profile. Improved resolution is required for identification of components in the pattern layer.

(2) Escherichia coli B

(i) Untreated

(a) Osmium-fixed

Although some preparations of sectioned E. coli cells presented the "typical Gram-negative" wall profile (see Figure 16), the more usual picture revealed a thickened electron-dense component adhering to the inner surface of the wall triplet-layer. A profile of this latter type is depicted in Figure 17 which shows a cell from a logarithmic YAP broth culture aerated at room temperature. This particular preparation was, in fact, a control in a spheroplast experiment. The nature of the uniform electron density of the inner wall observed in most sections was not determined though, undoubtedly, the rigid mucopeptide component resides in both types of inner wall

FIGURE 16

Escherichia coli B: uranium-stained section showing the cytoplasmic membrane and "typical" wall profile which appears as a compact structure in some areas X 156,500.

FIGURE 17

Escherichia coli B: a more usual appearance in which the wall profile consists of a thick, electron-dense component adhering to the inner surface of the triplet-layer. Thallium-stained section X 156,500.

FIGURE 18

Escherichia coli B: a cell prefixed with glutaraldehyde showing a surface structure with closely applied constituent layers. Lead-stained section X 118,000.

FIGURE 16

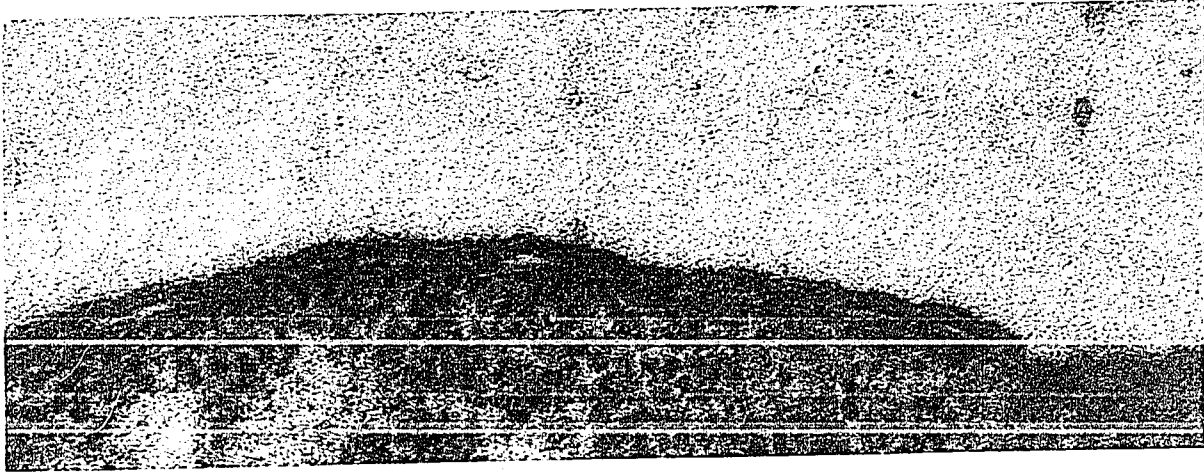


FIGURE 17

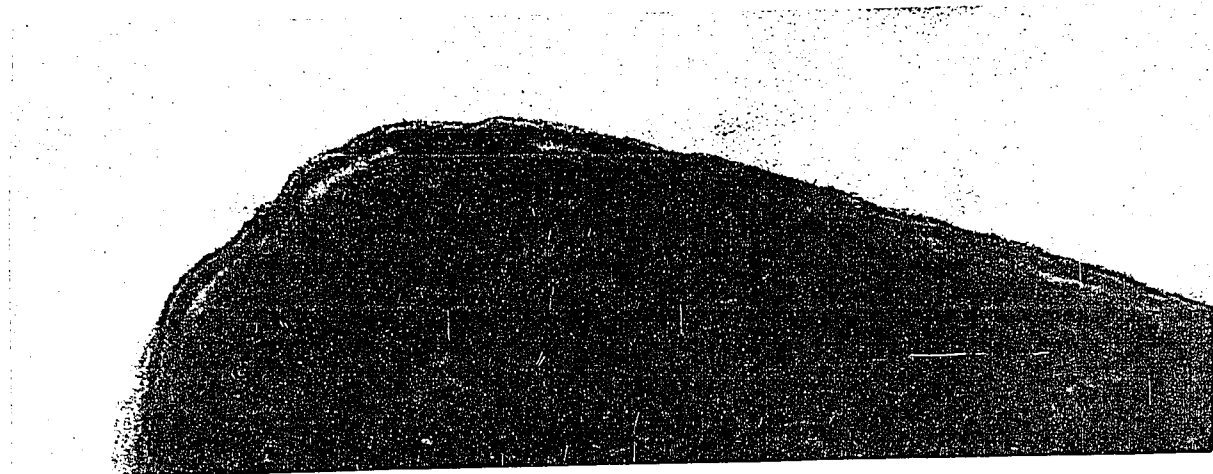
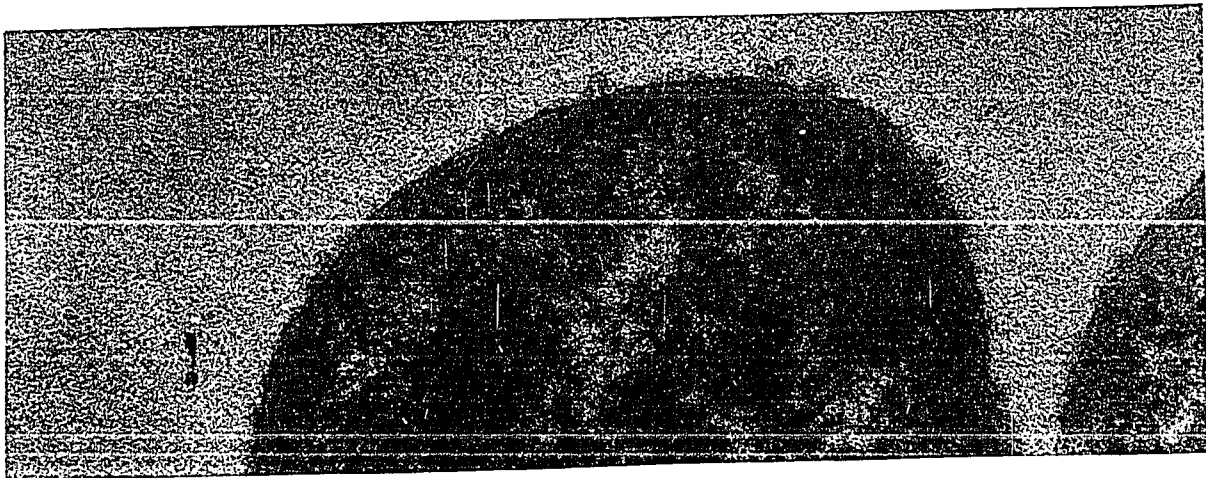


FIGURE 18



profile.

(b) Glutaraldehyde-fixed

Cells prefixed with glutaraldehyde (Sabatini, Miller and Barrnet, 1964) were compared with the routine osmium-fixed preparations. Figure 18 illustrates a sectioned cell from a logarithmic phase Penassay broth culture aerated at 37°C. The culture was prefixed for 90 minutes at room temperature by adding glutaraldehyde to give 7% (v/v). After three washes in 0.05M trishydroxymethyl aminomethane (Tris) buffer pH7.0, the cells were fixed with OsO₄ and processed in the usual manner. As Figure 18 clearly shows, the constituent layers at the cell surface, though less distinct than in osmium-fixed preparations, are closely applied and present a compact profile. In contrast with osmium fixation, compact wall profiles proved to be the rule rather than the exception in glutaraldehyde-fixed specimens.

(ii) Spheroplasts

(a) Lysozyme - EDTA

When logarithmic phase cells of E. coli were suspended in the BELS solution used to prepare spheroplasts of S. serpens, a most unexpected result was obtained: spheroplasts were not produced. When the experiment was repeated with a higher concentration (15 mg/ml.) of lysozyme in the mixture, once again spheroplasts failed to develop and the wall profile shown in Figure 19 still contained the thick electron-dense component underlying the triplet-layer. Following the method described in the original application of lysozyme and EDTA for

producing E. coli spheroplasts (Repaske, 1956), the phosphate buffer in BELS solution was replaced by 0.3 M Tris buffer pH8.0. In this mixture (TELS) there was almost 100% conversion of cells to spherical forms within 5 minutes. Figure 20 demonstrates the loss of the thick electron-dense element from the wall profile when cells from a logarithmic phase YAP broth culture aerated at room temperature were suspended in TELS solution. Magnesium sulphate (to give 0.05M) was added to the suspension followed by introduction of O_5O_4 for pre-fixation.

E. coli spheroplasts resembled those produced from S. serpens when seen in section and the loss of cell shape and strength accompanying the disappearance of the inner element from E. coli wall profiles was taken as evidence for identifying the missing constituent as the rigid mucopeptide layer. However, the inability of BELS mixture to produce spheroplasts of E. coli points to some basic difference in wall structure and behaviour displayed by S. serpens and E. coli.

(b) Penicillin

Spheroplasts of E. coli were also produced by the action of penicillin on cells growing in a protective osmotic medium. Figure 21 presents the surface profile of a spheroplast produced in a logarithmic phase S-P broth culture aerated at 37°C in the presence of penicillin (8 mg/ml). After 3 hours, the culture was prefixed. Loss of the inner wall element was found to be incomplete in these sphero-

FIGURE 19

Escherichia coli B: surface profile of a cell exposed to lysozyme and EDTA (BELS solution) without spheroplast formation.

Thallium-stained section X 110,000.

FIGURE 20

Escherichia coli B: loss of the inner, dense layer in a spheroplast induced by lysozyme and EDTA (TELS solution).

Section stained with uranium X 156,500.

FIGURE 21

Escherichia coli B: surface profile of a penicillin-induced spheroplast showing remnants of the inner, dense layer of the wall. Uranium-stained section X 156,500.

FIGURE 19

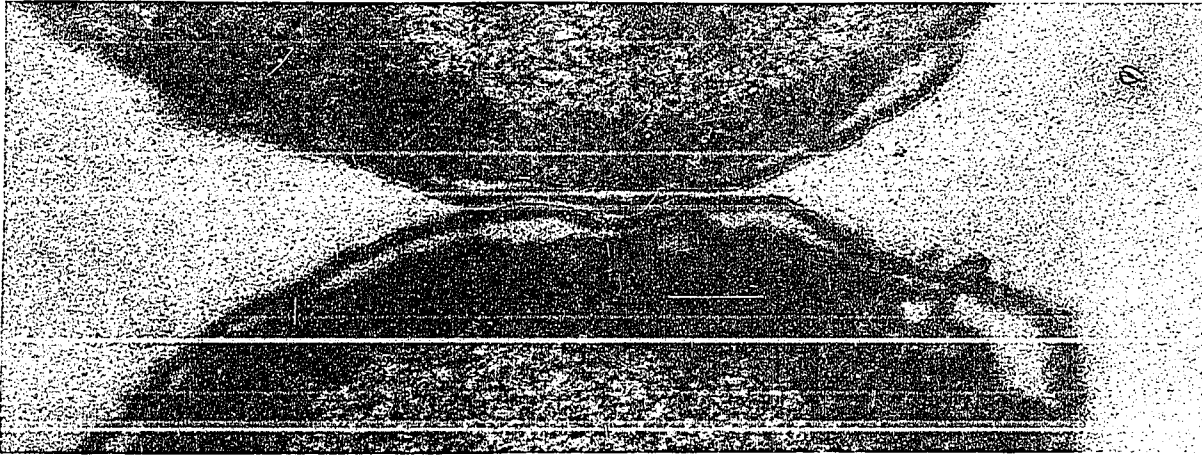


FIGURE 20



FIGURE 21



plasts as small surface areas revealed the wall profile observed in untreated cells. Since penicillin acts only on mucopeptide undergoing synthesis, the residual inner wall possibly represents that part of the mucopeptide layer which remained inert, and therefore resistant to penicillin, during the exposure period.

(3) Gliding bacteria

There are several genera of Gram-negative bacteria which lack flagella, the conventional organelles of motility, and exhibit a type of locomotion known as gliding. Simonsiella crassa and Alysiella filiformis are filamentous gliding bacteria remarkable in their pronounced degree of asymmetry (Steed, 1963). These thin, flat, ribbon-like bacteria exhibit gliding motility only when their flat surface is in contact with the substrate. It was thought that there might be some specialization of these flat surfaces so a study of their wall profiles was undertaken.

(i) Alysiella filiformis

Figure 22 shows a lead-stained section of A. filiformis strain Al grown on serum agar for 24 hours at 37°C. The organism consists of paired disc-shaped cells loosely associated as a filament. Extrusion of fragile, hair-like strands, resembling fimbriae, is evident along one surface of the filament. This was the only feature observed that was indicative of unilateral specialization in these organisms. Presence of fimbriae in other gliding bacteria is not

FIGURE 22

Alysiella filiformis strain A1: section of a filament with fine strands projecting from one surface. Stained with thallium X 57,000.

FIGURE 23

Alysiella filiformis strain A4: partially disrupted filament in which the wall triplet-layer is disocciated from the inner, dense component. Lead-stained section X 57,000.

FIGURE 22



FIGURE 23



known, and their significance in A. filiformis remains undetermined.

Upon close examination of the wall profile shown in Figure 22, it can be seen that it consists of the inner electron-dense component and outer triplet-layer typical of Gram-negative bacteria. A section of strain A4, grown under similar conditions, is shown in Figure 23. Because of some disruption of the integrity of this filament, the triplet-layer enclosing the constituent cell pairs has become dissociated from the inner wall. This effect demonstrates that shape and strength of the individual cells are determined by the inner rigid mucopeptide layer which is somewhat thicker in this strain.

(ii) Simonsiella crassa

(a) Untreated

The fimbriae seen in sections of A. filiformis were not observed in sectioned filaments of S. crassa. Figure 24 illustrates the organization of S. crassa in which individual cells of a filament are more closely associated than the paired elements of A. filiformis. Here again, the wall profile is of the "typical Gram-negative" type.

(b) Spheroplasts

Cells from a serum broth culture aerated for 12 hours at 37°C were suspended in the BELS solution described previously. After 2 to 3 hours magnesium sulphate (0.05M) and additional sucrose (20%) were introduced followed by prefixation. A sectioned filament from this preparation is shown in Figure 25. The enveloping triplet-layer

FIGURE 24

Simonsiella crassa: filament of cells depicting the outer, triplet-layer and inner, dense component of the wall profile. Section stained with lead X 57,000.

FIGURE 25

Simonsiella crassa: absence of the inner, dense layer in the wall profile of a filament exposed to lysozyme and EDTA (BELS solution). Uranium-stained section X 83,000.

FIGURE 24

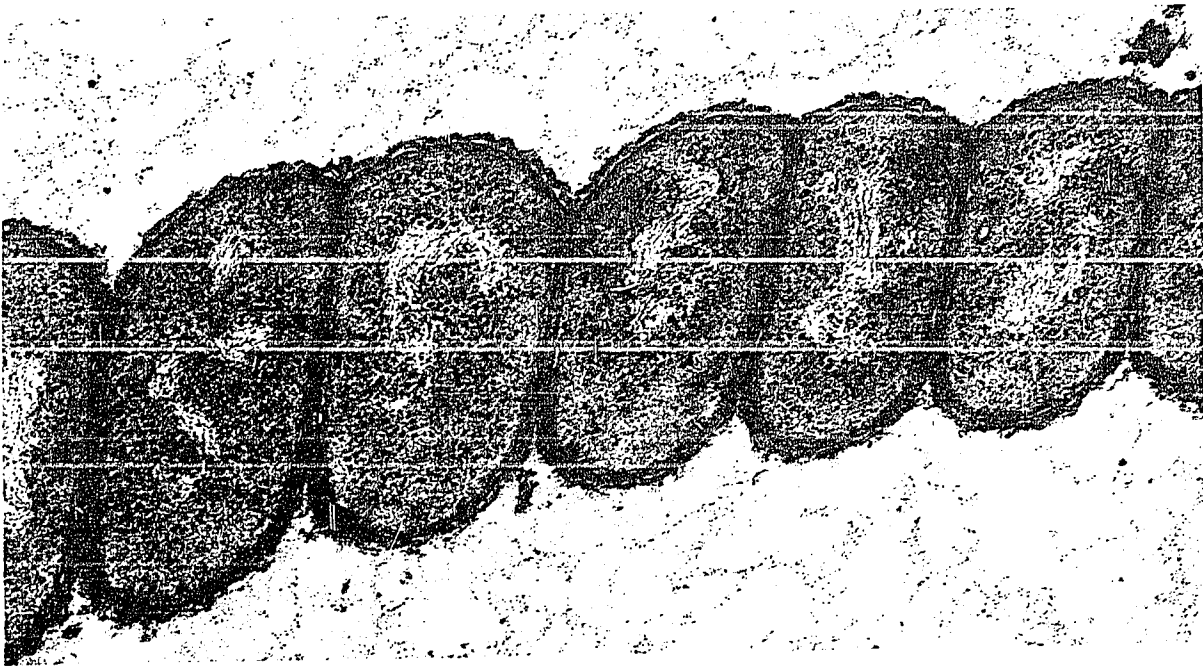


FIGURE 25

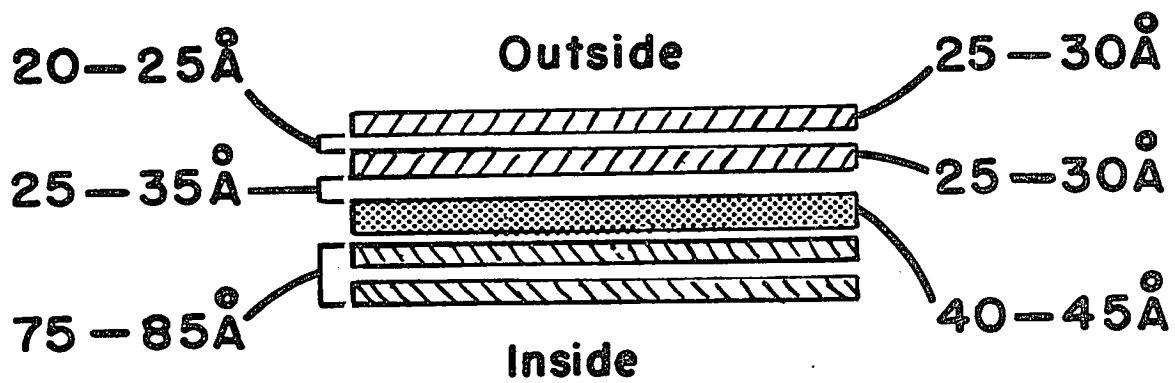
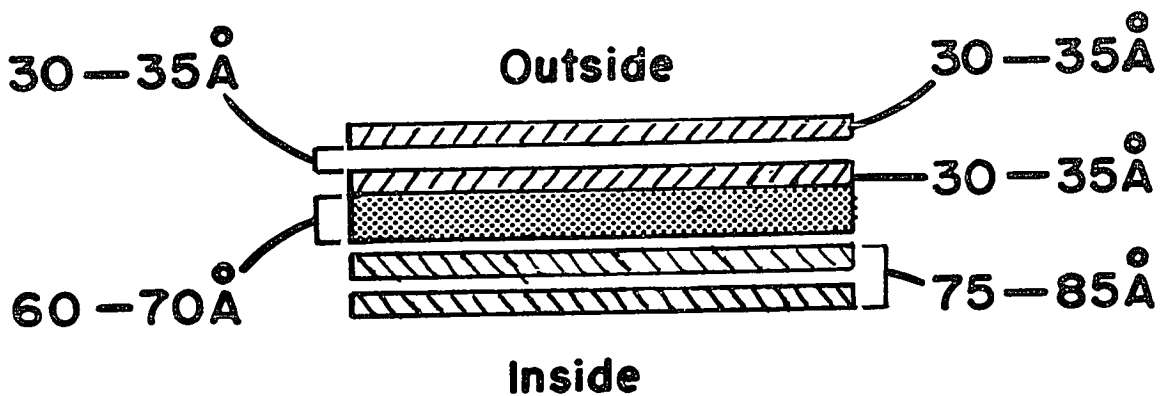


FIGURE 26

Ranges in the dimensions of the various electron-dense and electron-transparent surface layers observed in sections of Spirillum serpens and Escherichia coli are given.

FIGURE 26

DIMENSIONS OF PROFILES

A. SPIRILLUM SERPENS.B. ESCHERICHIA COLI.

encloses the individual cellular elements which have become distorted in the absence of the rigid mucopeptide layer. Whole filaments exhibited a spectrum of sensitivity to lysozyme ranging from resistance at one end to increasing susceptibility toward the opposite end. This effect reflects a parallel increase in resistance to lysozyme with cell maturity such as that observed by Chaloupka, Řichová and Křečková (1964). Consequently, it was inferred that these filaments reproduced from one pole, the least and most mature cells of a filament being terminal.

(B) Division

(1) Role of Cell Wall

Bacteria divide by binary fission in which cytokinesis, the mechanism of cellular division, can take one of two forms, shown diagrammatically in Figure 27. Septation involves preliminary partitioning of the cytoplasm by a "cross-wall" or septum consisting of cytoplasmic membrane-bound wall material while division by constriction occurs by centripetal indentation of all surface layers. In general, Gram-positive bacteria divide by septation and constriction is the rule for many Gram-negative species.

Routine examination of Escherichia coli and Spirillum serpens revealed relatively few cells in the process of division which was always of the constrictive type (see Figures 28 and 29). Therefore, it was surprising to find distinct septa in sectioned S. serpens cells which

encloses the individual cellular elements which have become distorted in the absence of the rigid mucopeptide layer. Whole filaments exhibited a spectrum of sensitivity to lysozyme ranging from resistance at one end to increasing susceptibility toward the opposite end. This effect reflects a parallel increase in resistance to lysozyme with cell maturity such as that observed by Chaloupka, Řichová and Křečková (1964). Consequently, it was inferred that these filaments reproduced from one pole, the least and most mature cells of a filament being terminal.

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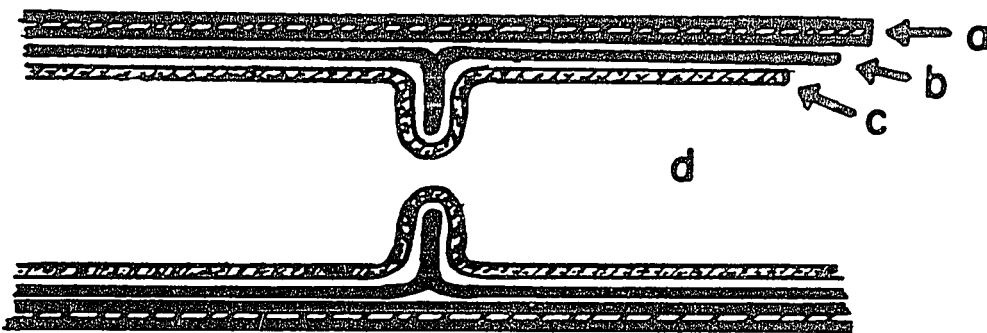
FIGURE 27

Diagrammatic representation of the multilayered surface profile of dividing Gram-negative bacteria.

- a: triplet-layer.
- b: rigid layer.
- c: cytoplasmic membrane.
- d: cytoplasm.

FIGURE 27.

SEPTATION



CONSTRICTION

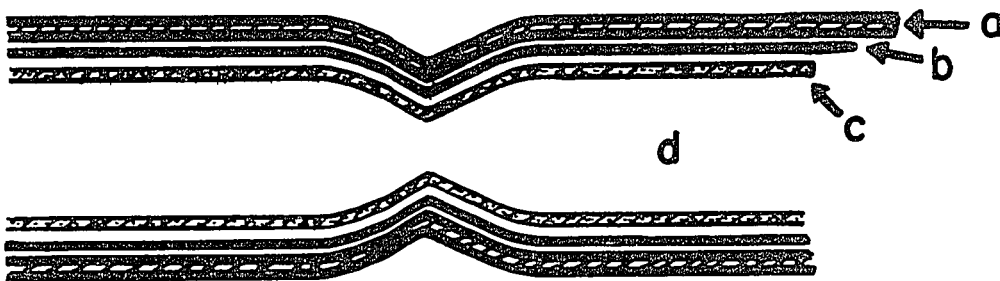


FIGURE 28

Escherichia coli B: section of a cell dividing by constriction.

Lead stained X 63,000.

FIGURE 29

Spirillum serpens VH: constrictive appearance of a dividing

cell. Lead stained section X 94,000.

FIGURE 28

Escherichia coli B: section of a cell dividing by constriction.

Lead stained X 63,000.

FIGURE 29

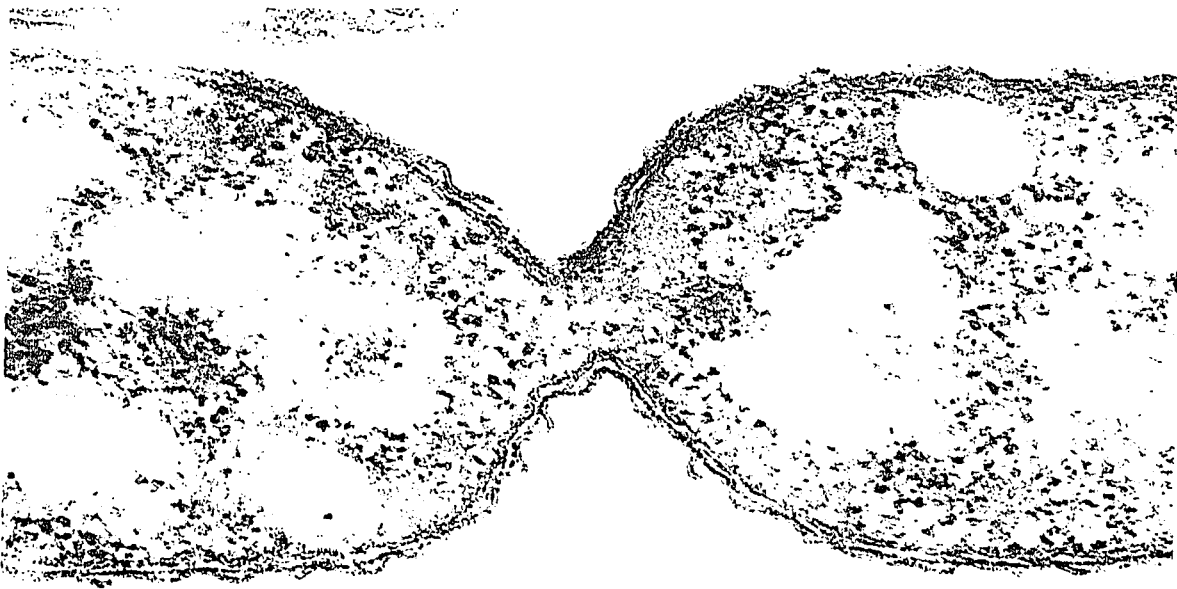
Spirillum serpens VH: constrictive appearance of a dividing

cell. Lead stained section X 94,000.

FIGURE 28



FIGURE 29



had been grown at 45°C instead of the usual lower temperatures. (The experiments carried out at 45°C were originally designed for an unrelated investigation but were subsequently performed for this study of cell division). Furthermore, these septa consisted of cytoplasmic membrane and only the innermost layer of the wall complex (see Figure 30). Examination of numerous sections from similar preparations of S. serpens demonstrated the regular appearance of septa and allowed identification of different stages in the cytokinetic sequence.

(i) The nascent septum was formed by a projection into the cytoplasm of the inner wall (mucopeptide) layer lying within an invaginated pocket of cytoplasmic membrane (see Figure 31). Apparently, centripetal growth of the septum ensued until the second identifiable stage was reached.

(ii) The wall material in the completed septum was seen to separate the now independent protoplasts. This dividing structure appeared as a single unit with a thickness 1 1/2 to 2 times greater than that of the peripheral elements from which it was derived (see Figures 30 and 32).

(iii) A prelude to cell separation was the equatorial splitting of the septal mucopeptide into two layers constituting the newly cleaved surfaces of the divided protoplasts. Each cell now possessed individual cytoplasmic membrane and mucopeptide layers but still shared the outer wall components (Figure 33).

FIGURE 30

Spirillum serpens VH: cell, incubated at 45°C, dividing by septation. Note the single, thick layer of the inner wall component which forms the septum. Lead-stained section X 110,000.

FIGURE 31

Spirillum serpens VH: early stage in septum formation in a cell incubated at 45°C. Section stained with thallium X 110,000.

FIGURE 30

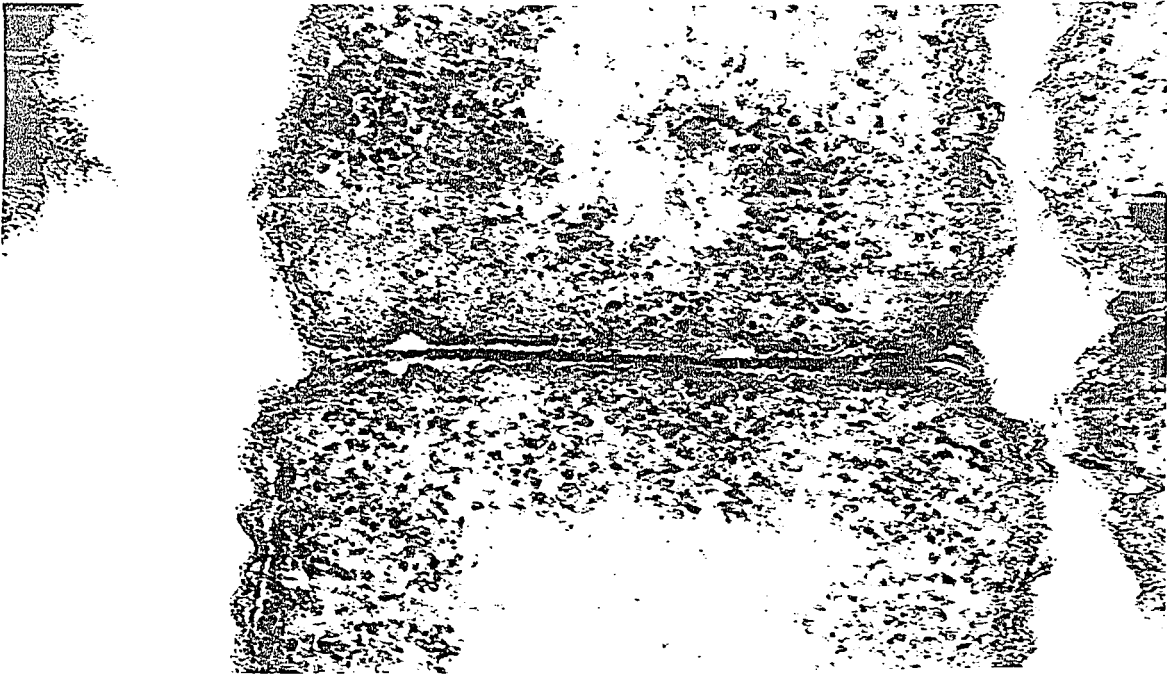


FIGURE 31

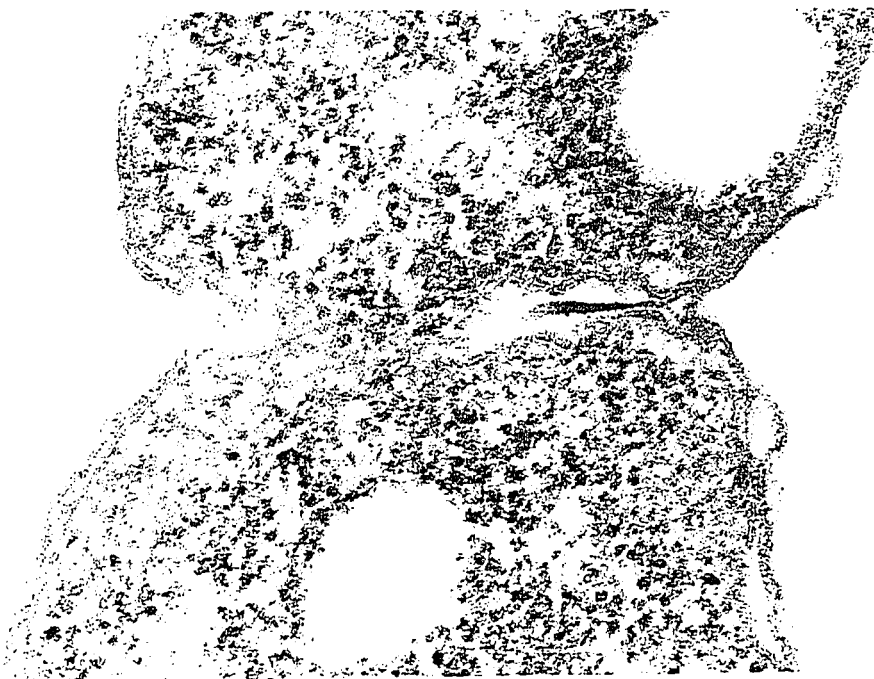


FIGURE 32

Spirillum serpens VH: detail of the septum in a sectioned cell from a 45°C culture. Stained with lanthanum
X 110,000.

FIGURE 33

Spirillum serpens VH: section of a dividing cell incubated at 45°C. Note the membranous structure (mesosome) and the "split" septum. Lead-stained X 78,000.

FIGURE 32

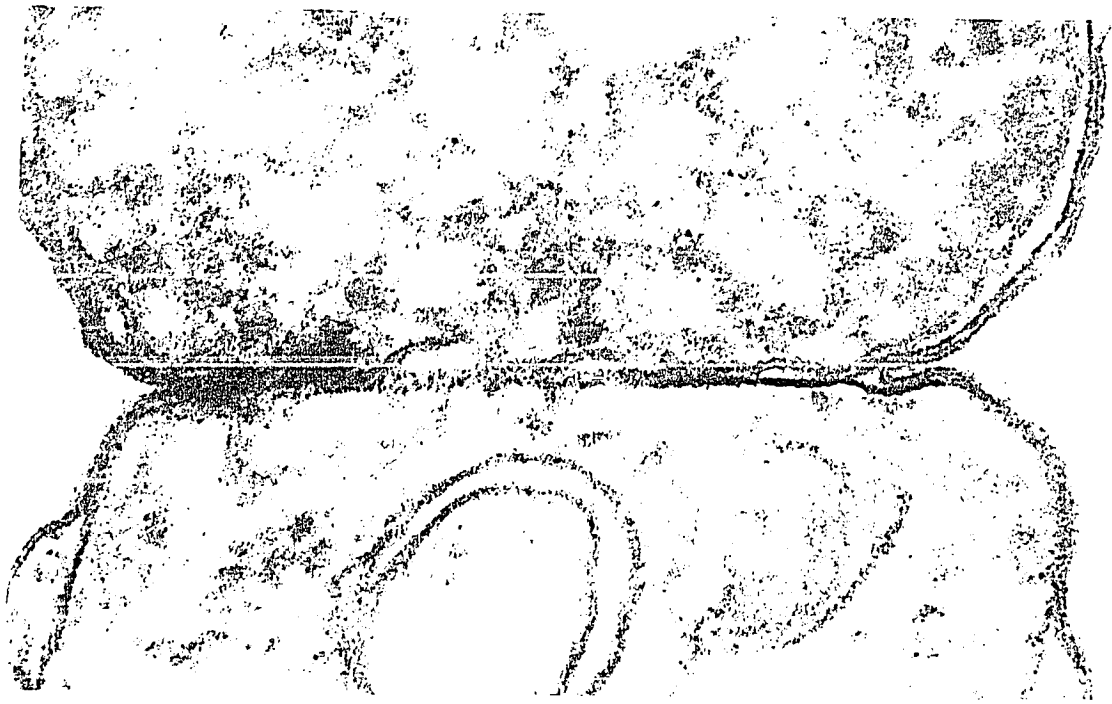


FIGURE 33



(iv) Final separation of completely independent cells awaited involvement of the outer wall. Progressive separation of the split septal mucopeptide layers began at the cell periphery and was accomplished by centripetal synthesis of the outer wall elements (Figure 34).

Cultures of E. coli also grown at 45°C were sectioned and examined in the electron microscope. Observations revealed a high proportion of dividing cells with septa. Essentially the same process as that described for S. serpens was observed and representative stages are illustrated in Figures 35 and 36. Of special interest is the involvement of only the innermost portion of the thick, electron-dense rigid layer in septation.

Analysis of the division processes exhibited during constriction and septation as demonstrated in S. serpens and E. coli can be expressed in terms of the relative rates at which the different surface layers were synthesized. In septation the centripetal growth of cytoplasmic membrane and mucopeptide preceded that of the remaining wall, while synthesis of all surface layers appeared to proceed in unison when constriction was observed. Further comparison was made with the cytokinetic behaviour of filamentous bacteria.

As illustrated in Figure 37, Simonsiella crassa consists of cells with individual cytoplasmic membrane and mucopeptide layers and a common outer wall envelope forming the filament. Complete

FIGURE 34

Spirillum serpens VH: late stage in division by septation (from a 45°C culture). Cell separation by centripetal synthesis of the wall triplet-layer is evident. Section stained with lead
X 106,000.

FIGURE 35

Escherichia coli B: division by septation in a cell incubated at 45°C. Note the thin septal component derived from the inner edge of the thick, rigid layer of the wall. Lead-stained section X 106,000.

FIGURE 34

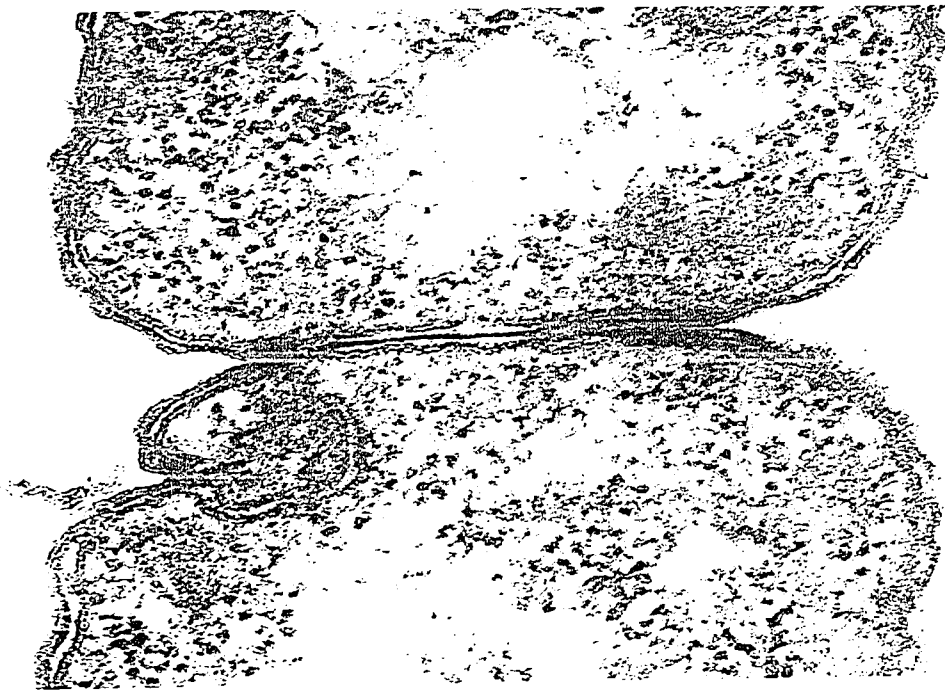


FIGURE 35



FIGURE 36

Escherichia coli B: septation in a dividing cell incubated at
45°C. Lead-stained serial sections X 106,000.

FIGURE 36

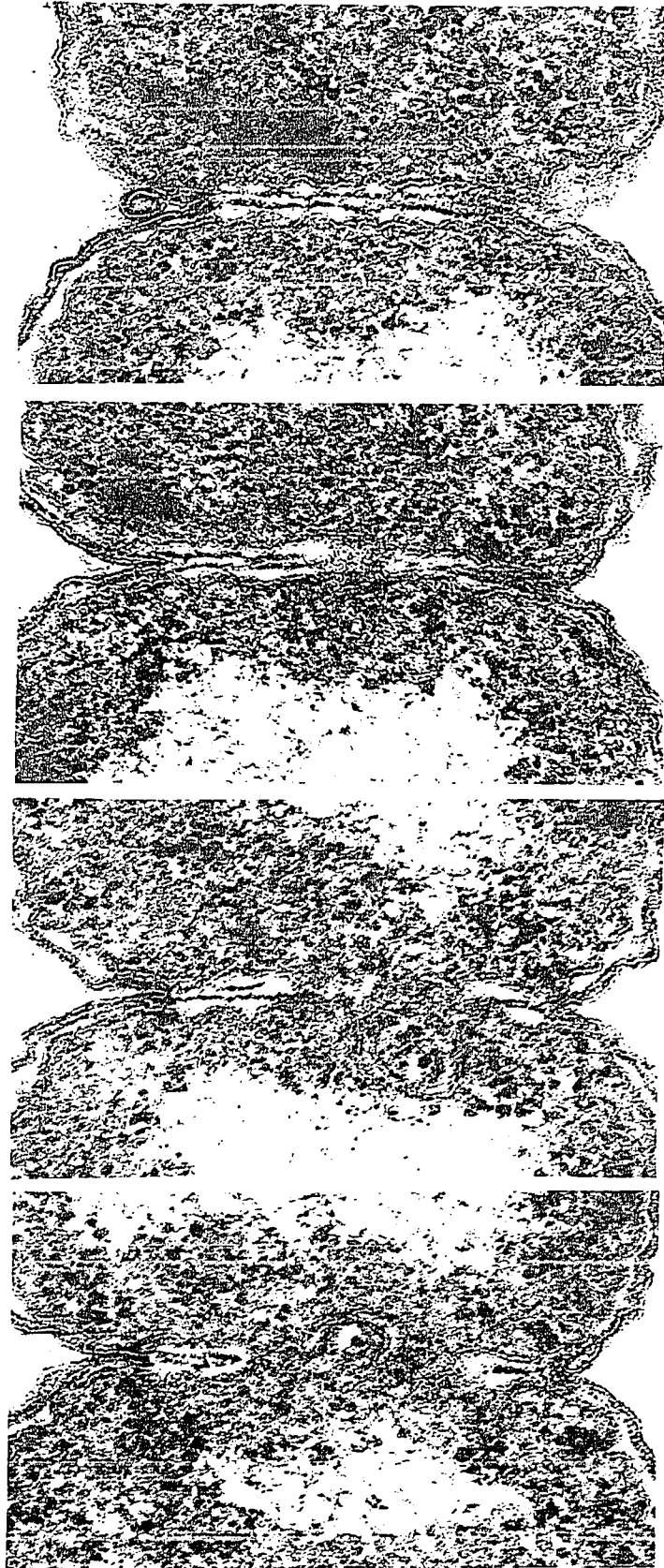
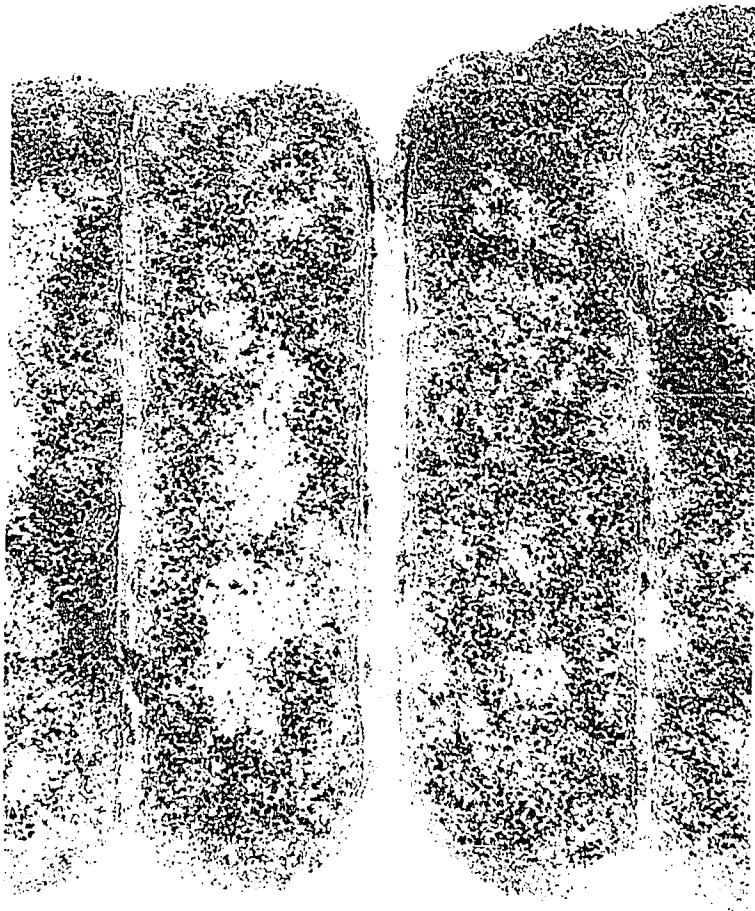


FIGURE 37

Simonsiella crassa: division leading to separation of filaments.

Lead-stained section X 65,000.

FIGURE 57



division is a less frequent event, and results in the separation of filaments instead of individual cells. Thus, the appearance of septation in Spirillum serpens and Escherichia coli can be interpreted as a manifestation of limited "multicellularity" which is usually bicellular in nature. Some instances of septation resembling the "supernumerary" septa described in Bacillus cereus (Chapman and Hillier, 1953) were observed in Spirillum serpens (Figure 33); these were considered as an abnormal trend toward "multicellularity."

(2) Mesosomes

Gram-positive bacteria possess prominent membranous bodies, termed mesosomes (Fitz-James, 1960), which have been assigned a role in division. Equivalent structures are not regularly found in Gram-negative species. However, membranous elements of a less complex nature have been identified in the cytoplasm of several Gram-negative bacteria (Vanderwinkel and Murray, 1962). Examples of simple membranous intrusions seen in S. serpens and E. coli are presented in Figures 38 and 39. It was a most rewarding experience, then, to observe complex mesosome-like membranes in dividing cells exposed to the conditions providing septa. The location of these structures at the site of septum formation strengthened the impression that they were, in fact, equivalent in both structure and function to the mesosomes originally described in Gram-positive bacteria (see Figures 33 and 36).

FIGURE 38

Spirillum serpens VH: example of a simple intrusion of cytoplasmic membrane. Section stained with lead X 118,000.

FIGURE 39

Escherichia coli B: sectioned cell showing intrusive membrane in the cytoplasm. Lead-stained section X 156,500.

FIGURE 38

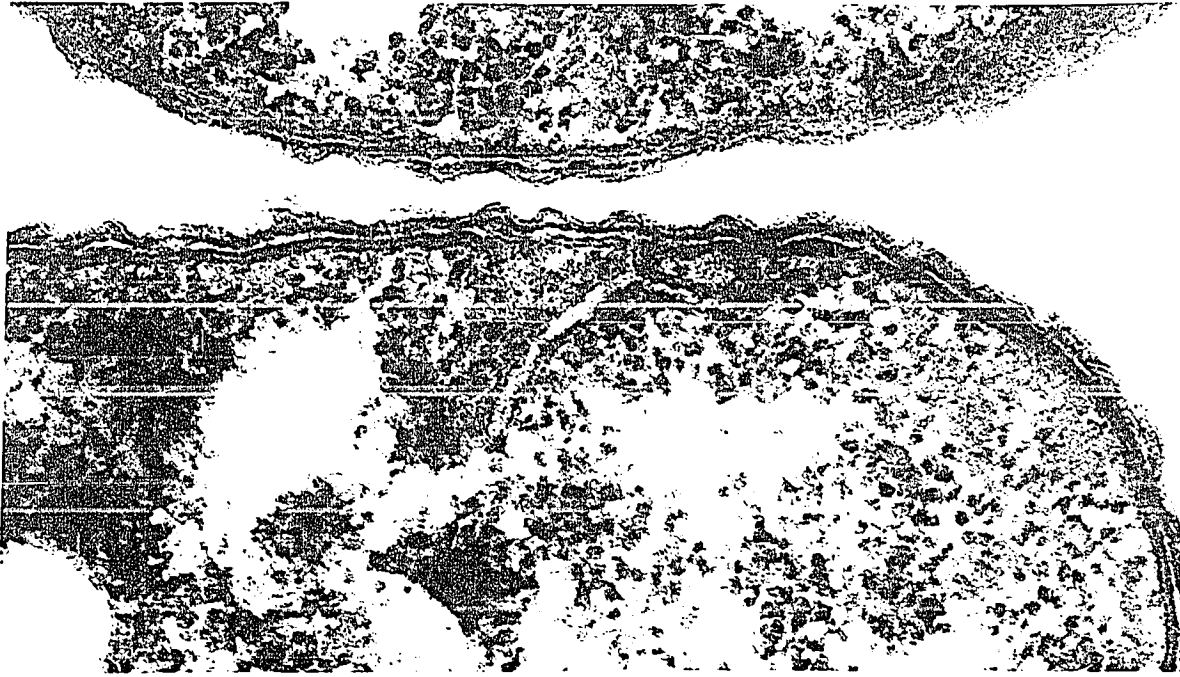
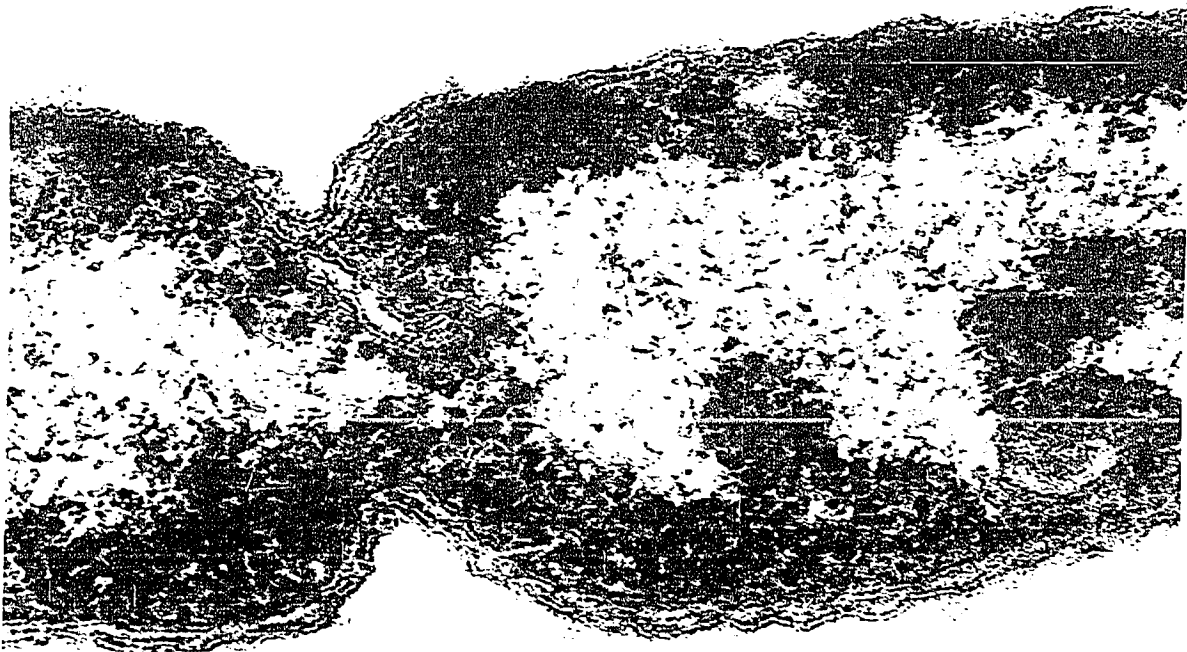


FIGURE 39



(3) Effect of Pantoyl Lactone

An extensive investigation of cell division in a species of Erwinia demonstrated the ability of pantoyl lactone to reverse several types of division inhibitions (Grula and Grula, 1962 a, b, c). These authors' suggestion that pantoyl lactone was active at the terminal stage of division prompted the use of this reagent for electron microscopic observations.

DL-pantoyl lactone (at 0.08M concentration), was added to an actively growing culture of Spirillum serpens VH at 37°C. After incubation for a further 30 minutes, the culture was prefixed. Sections revealed cells dividing by septation, often with a modest display of membranes adjacent to the septa. Similar septa and membranes were not observed in a control culture without added pantoyl lactone; only constrictive divisions were seen.

Sections of a similar culture incubated at 45°C presented the usual picture of dividing cells with septa and associated mesosomes. Comparison with sections of a companion S. serpens VH culture exposed to 0.08M pantoyl lactone for the final 30 minutes of incubation demonstrated a pronounced proliferation of mesosomal membranes (see Figure 40).

Incubation of S. serpens VH cells with 0.08M pantoyl lactone for 6 hours at 45°C produced pictures such as that shown in Figure 41. Despite considerable cytoplasmic disruption, the septum has been

FIGURE 40

Spirillum serpens VH: dividing cell from a 45°C culture exposed to pantoyl lactone for 30 minutes. Note the proliferation of mesosomal membranes. Section stained with thallium X 110,000.

FIGURE 41

Spirillum serpens VH: dividing cell from a 45°C culture incubated for 6 hours with pantoyl lactone. Uranium-stained section X 83,000.

FIGURE 40

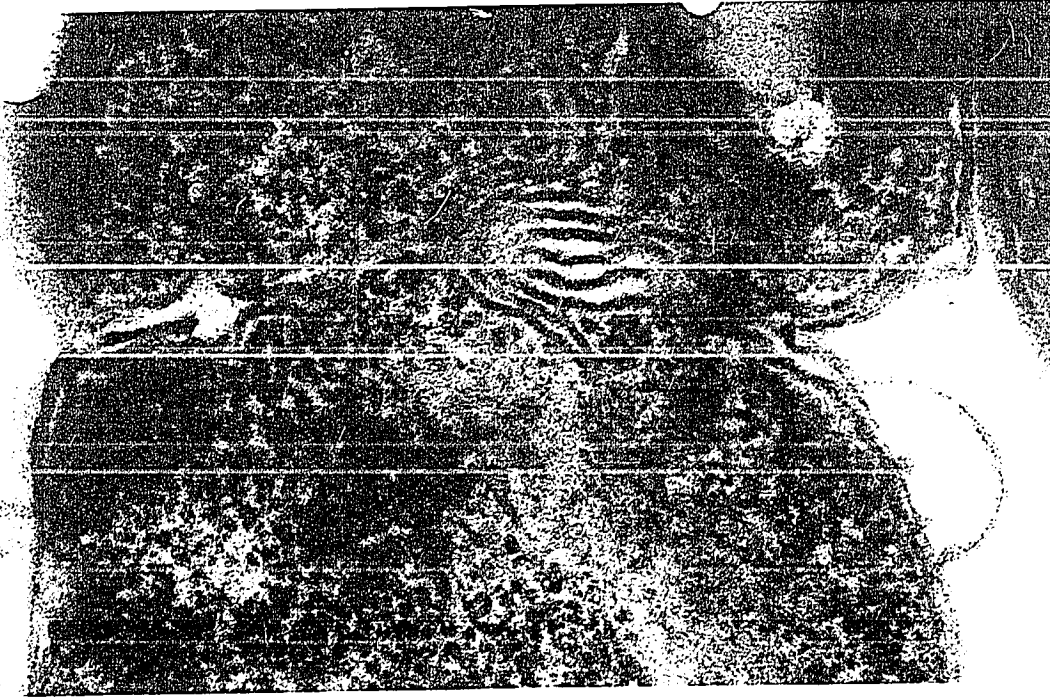


FIGURE 41



well preserved. Convolutions of membranes within the cytoplasm point to an excessive production of mesosomal material under these conditions.

(4) Factors Affecting the Appearance of Division

(i) Incubation temperature

The regular appearance of septa in dividing cells of E. coli and S. serpens incubated at 45°C contrasted with the few constrictive divisions observed in cells from 30°C cultures suggesting that the incubation temperature affected the mechanism involved. Experimental variations of the fixing environment were applied to pairs of S. serpens cultures grown at these two temperatures and sections were examined for dividing cells. Quantitative estimates made by calculating the percentage of cells with septa among fifty longitudinal sections are shown in Table I. Fisher's "Student's" t test was applied to the data and a 'p' value $<0.02>0.01$ was obtained when the percent of septa seen in the 45°C group was compared with that in the 30°C group. Thus, the incubation temperature proved to be statistically significant at the 2% level.

(ii) Tonicity of the fixing environment

Choice of 1/5 or 1/6 dilution of the standard veronal-acetate (VA) buffer (Kellenberger, Ryter and Séchaud, 1958) and of 2.5% (w/v) sucrose as fixing media was based upon direct light microscopic observation of S. serpens cells suspended in solutions with

TABLE I

EFFECT OF INCUBATION TEMPERATURE ON SEPTATION IN
SPIRILLUM SERPENS VH

Experi- ment	Fixing Environment	% with septa*	
		45°C	30°C
1	1/6 VA buffer, pH 6.1	34	8
2	2.5% (w/v) sucrose in VA buffer, pH 6.1	28	14
3	2.5% (w/v) sucrose with 0.1% (w/v) CaCl ₂	24	14
4	2.5% (w/v) sucrose	50	16
5	4% (w/v) SLS in 1/5 VA buffer, pH 6.1	14	0

* % of fifty longitudinal cell sections with septa;

VA: veronal-acetate buffer

SLS: sodium lauryl sulphate

ranging concentrations. Full strength VA buffer was found to be hypertonic for both S. serpens and E. coli cells, causing visible plasmolysis which reversed within 30 to 60 minutes. When osmium tetroxide (O_5O_4) at the prefixing (0.1% w/v) or fixing (1% w/v) concentration was included in the buffer, cells still showed signs of internal disturbance. Dilute (1/6 - 1/5) VA buffer and 2.5% sucrose were the highest concentrations of the series which failed to produce a visible osmotic effect in S. serpens VH cells when examined microscopically.

Several sets of experiments were performed with S. serpens VH in which the fixing environment was varied but always contained O_5O_4 as the fixing agent. The percentage of cells with septa or constrictions seen among fifty longitudinal cell sections was estimated in each preparation to determine the effect of environmental conditions on dividing cells. At a later date, the osmolarity was ascertained for each of the solutions, without O_5O_4 , employed in these experiments. The results are presented in Table 2. It was reassuring to note that 1/6 and 1/5 dilutions of VA buffer compared with 2.5% sucrose both in osmolarity and in preservation of septa. The limited degree of correlation between osmolarity of the fixing medium and preservation of dividing cells indicated by the results, suggested that osmolarity was merely one of several factors affecting the quality of fixation. It should be noted that cytokinesis was observed in no more than 16% of cells incubated at 30°C and up

TABLE 2

APPEARANCE OF DIVISION IN SPIRILLUM SERPENS VH IN RELATION TO THE OSMOLARITY
OF THE FIXING ENVIRONMENT

Cultural Conditions		Fixing Environment			% of divisions *	
Hours	Temperature	Composition	Milliosmoles	Septation	Constriction	
12	45°C	1/6 VA buffer pH 7.0	75	32	0	
12	45°C	1/6 VA buffer pH 6.1	75	34	0	
12	30°C	1/6 VA buffer pH 6.1	75	8	2	
<hr/>						
14	45°C	2.5% (w/v) sucrose in VA buffer				
14	30°C	pH 6.1	505	28	0	
14	45°C	2.5% (w/v) sucrose in VA buffer				
14	45°C	pH 6.1	505	14	0	
14	30°C	2.5% (w/v) sucrose with 0.1% (w/v) CaCl ₂	114	24	0	
14	45°C	2.5% (w/v) sucrose with 0.1% (w/v) CaCl ₂	114	14	0	
14	30°C	2.5% (w/v) sucrose	81	50	0	
14	30°C	2.5% (w/v) sucrose	81	16	0	

12	30°C	1/5 VA buffer pH 6.4	88	14	0
12	30°C	0.05M phosphate buffer pH 6.4	112	14	0
12	30°C	0.05M cacodylate buffer pH 6.4	109	4	2
12	30°C	0.05M collidine buffer pH 6.4	87	6	4
9	30°C	Distilled Water	0	0	8
9	30°C	1% (w/v) sucrose	34	0	6
9	30°C	2% (w/v) sucrose	68	2	6
9	30°C	5% (w/v) sucrose	163	0	16
9	30°C	10% (w/v) sucrose	318	0	10
9	30°C	1/5 VA buffer pH 6.1; no prefixation	88	0	16

* % of fifty longitudinal cell sections showing division by septation or constriction.

VA: veronal acetate buffer

to 50% of cells from 45°C cultures. Furthermore, the maximum number of dividing cells preserved in both 45°C and 30°C cultures, in which septate divisions alone were observed, resulted when the same fixing medium, 2.5% sucrose, was employed.

(C) Comparative Study of *S. serpens* VHA and *S. serpens* VHL

Spirillum serpens VHA and its pattern-less mutant Spirillum serpens VHL were studied to determine what properties might be associated with the presence of the surface pattern component. As a matter of convenience, *S. serpens* VHA and *S. serpens* VHL will be designated as VHA and VHL respectively throughout this section.

(1) Growth Curves

Growth curves of VHA and VHL broth cultures were obtained by plotting % turbidity against time on semi-logarithmic scales.

(i) Lysis

In YPA broth cultures aerated at 26°C, VHA cells lysed while VHL cells maintained almost constant turbidity in stationary phase. The maximum density of growth attained by VHL always exceeded that of VHA in this medium (see Figure 42A). Replacement of half of the culture supernatant by an equal volume of either fresh YPA broth or distilled water was accomplished by centrifuging half of the culture, resuspending the cells in the appropriate liquid substitute, and returning the suspension to the culture tube. Figure 42B demonstrates a second phase of growth which occurred after YPA broth was added

FIGURE 42

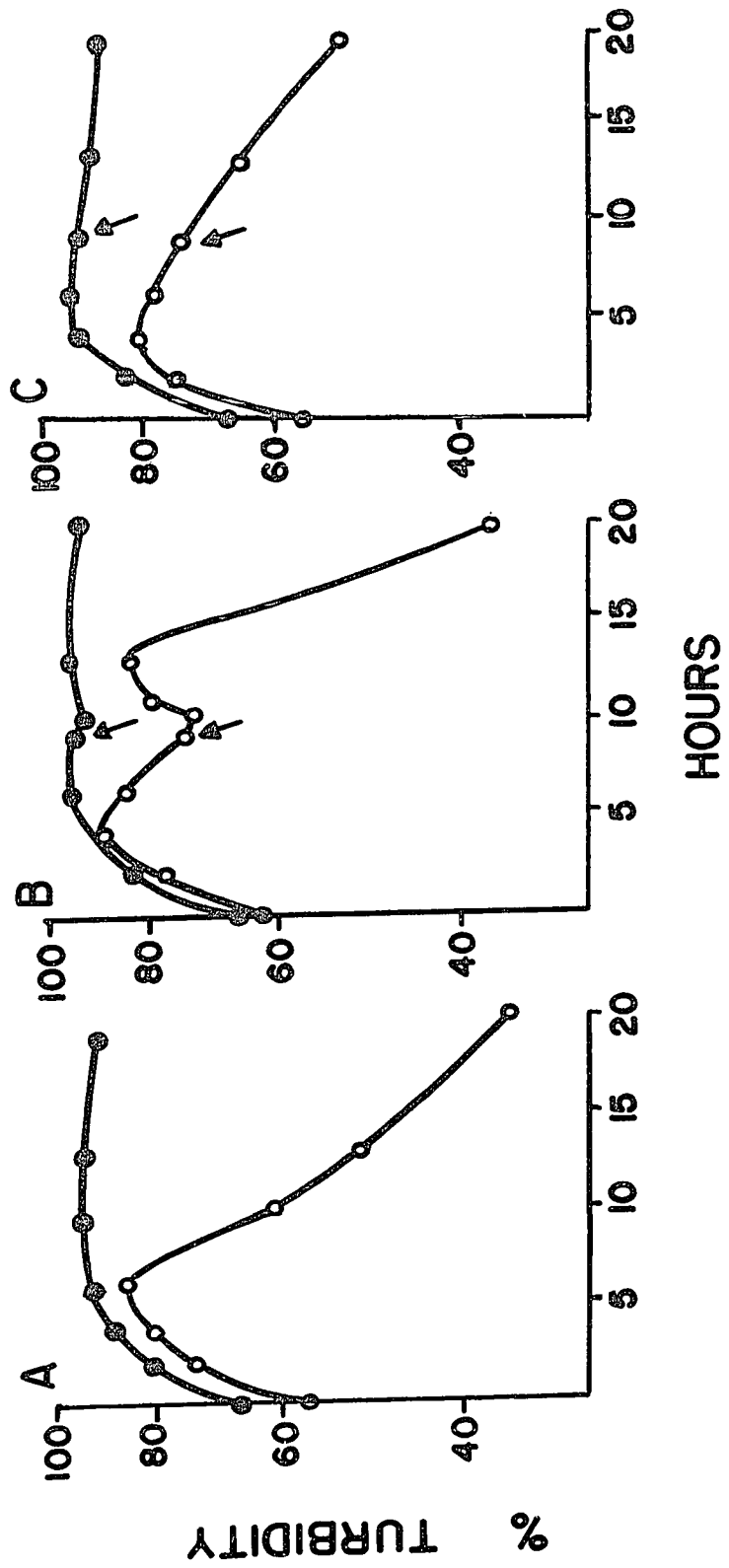
Lysis of Spirillum serpens VHA growing in YPA broth

- o Spirillum serpens VHA
- Pattern-less mutant Spirillum serpens VHL

YPA broth cultures were aerated at 26°C

- A: Controls (untreated)
- B: Replacement of half volume culture supernatants
with YPA broth
- C: Replacement of half volume culture supernatants
with distilled water.

FIGURE 42.



to a lysing VHA culture. Addition of distilled water failed to produce an effect on the progress of lysis (Figure 42C). The result obtained when a second replacement with YPA broth took place in a VHA culture after 71 hours is shown in Figure 43. Recovery from lysis began with a lag period and then proceeded at logarithmic rate. However, the growth peak fell short of the previous maximum, and lysis progressed to an even greater extent.

These results indicate the exhaustion of an essential factor in VHA cultures grown under these conditions. In the absence of this constituent cellular integrity was impaired and progressive lysis ensued. When several different cultures of Spirillum serpens were tested, lysis was found to occur only in those possessing the VHA type of surface pattern; pattern-less cultures behaved like VHL.

YPA broth cultures of VHA did not lyse when grown by shaking instead of aeration; nor did lysis occur in aerated tryptone broth cultures. Apparently, then, the YPA medium is depleted of some vital factor when VHA cultures are aerated by bubbling air.

(ii) Effect of metal cations

Figure 44 shows the effect of adding 10^{-2} M salts of magnesium, sodium and calcium to VHA cultures shortly after the onset of lysis. Magnesium and sodium were ineffective, but calcium immediately arrested lysis. Addition of calcium before the lytic phase (Figure 45A) prevented lysis of VHA cells and the maximum growth

FIGURE 43

Recovery from lysis of Spirillum serpens VHA

- Control (untreated)
- Replacement of half volume culture supernatant
with YPA broth

YPA broth cultures aerated at 26°C.

FIGURE 43.

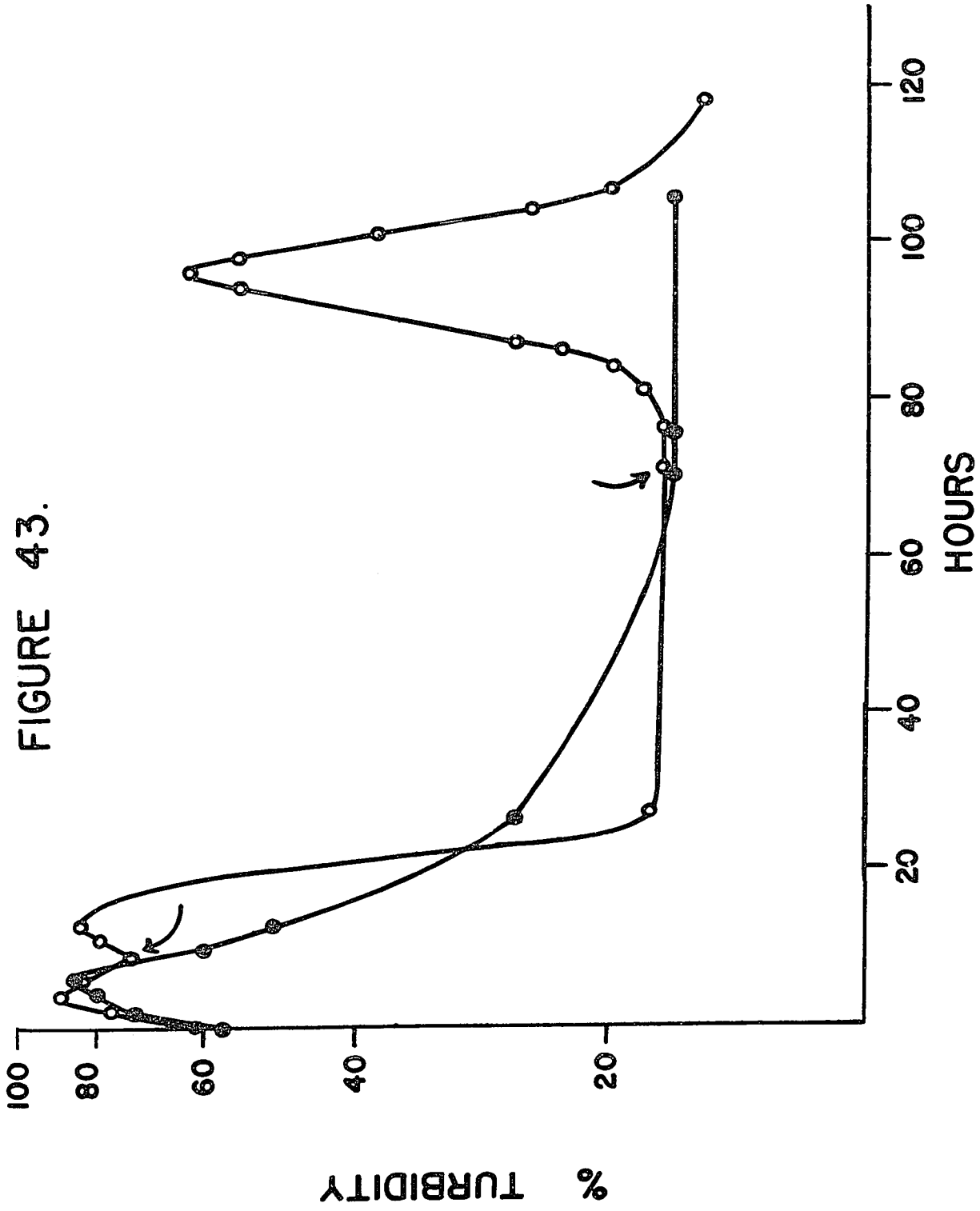


FIGURE 44

Effect of magnesium, sodium, and calcium salts added to lysing cultures of Spirillum serpens VHA.

YPA broth cultures aerated at 26°C

A: 10^{-2} M magnesium sulphate added

B: 10^{-2} M sodium chloride added

C: 10^{-2} M calcium chloride added.

FIGURE 44.

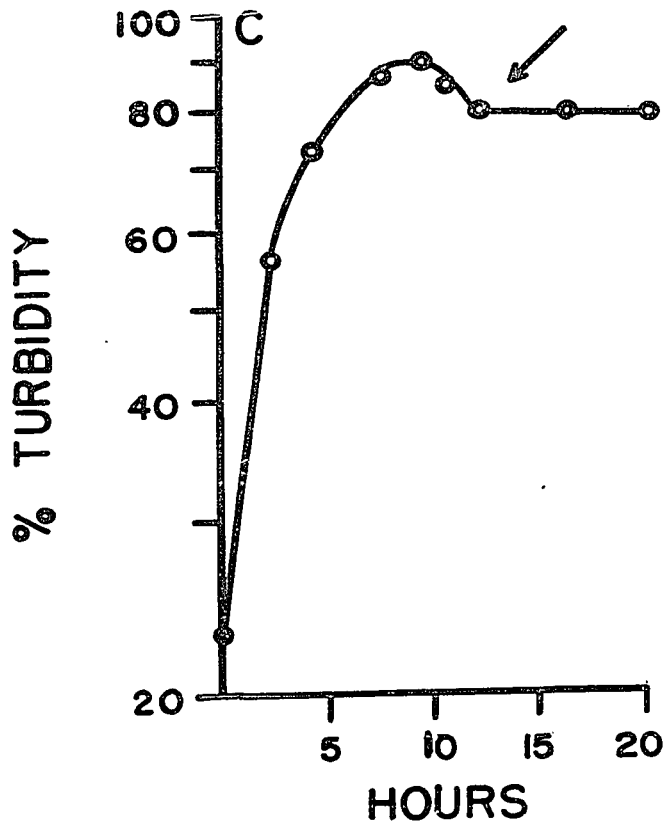
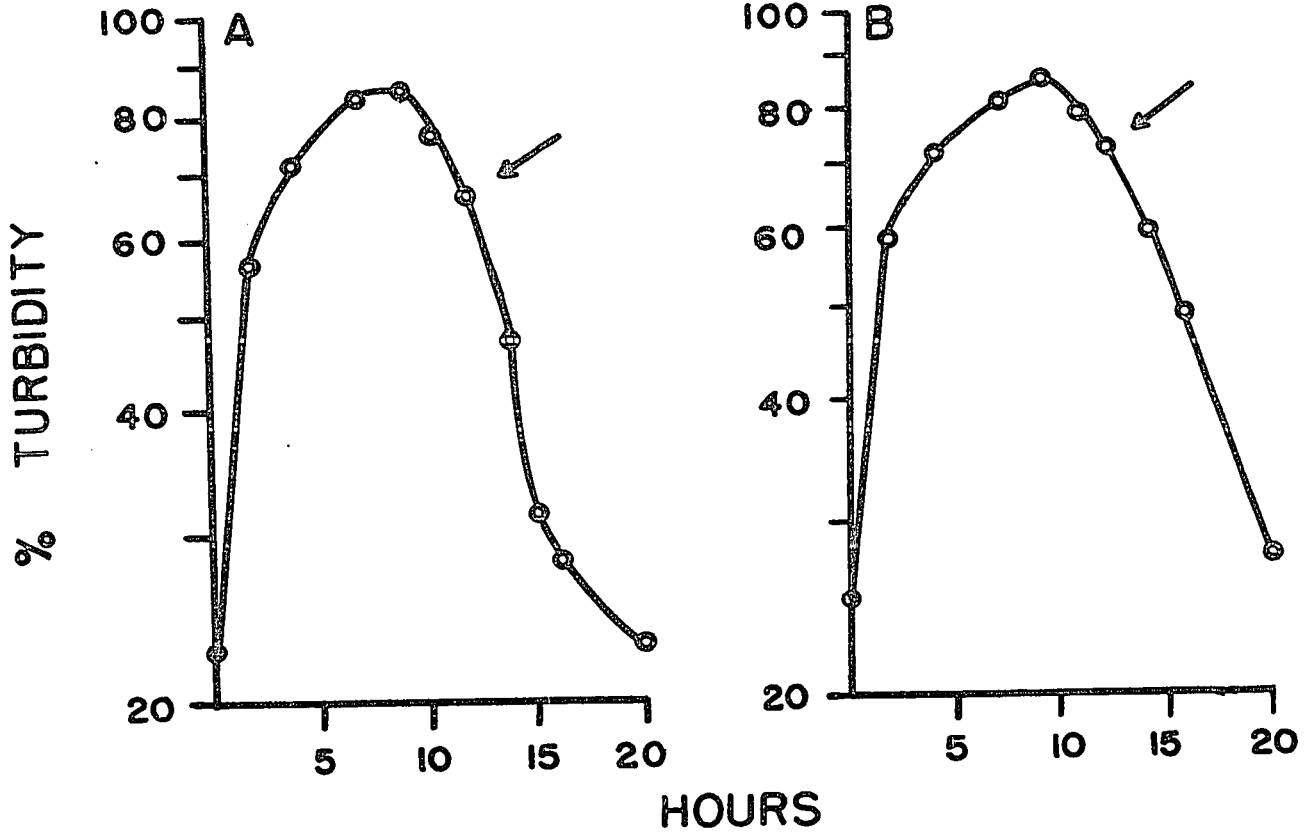
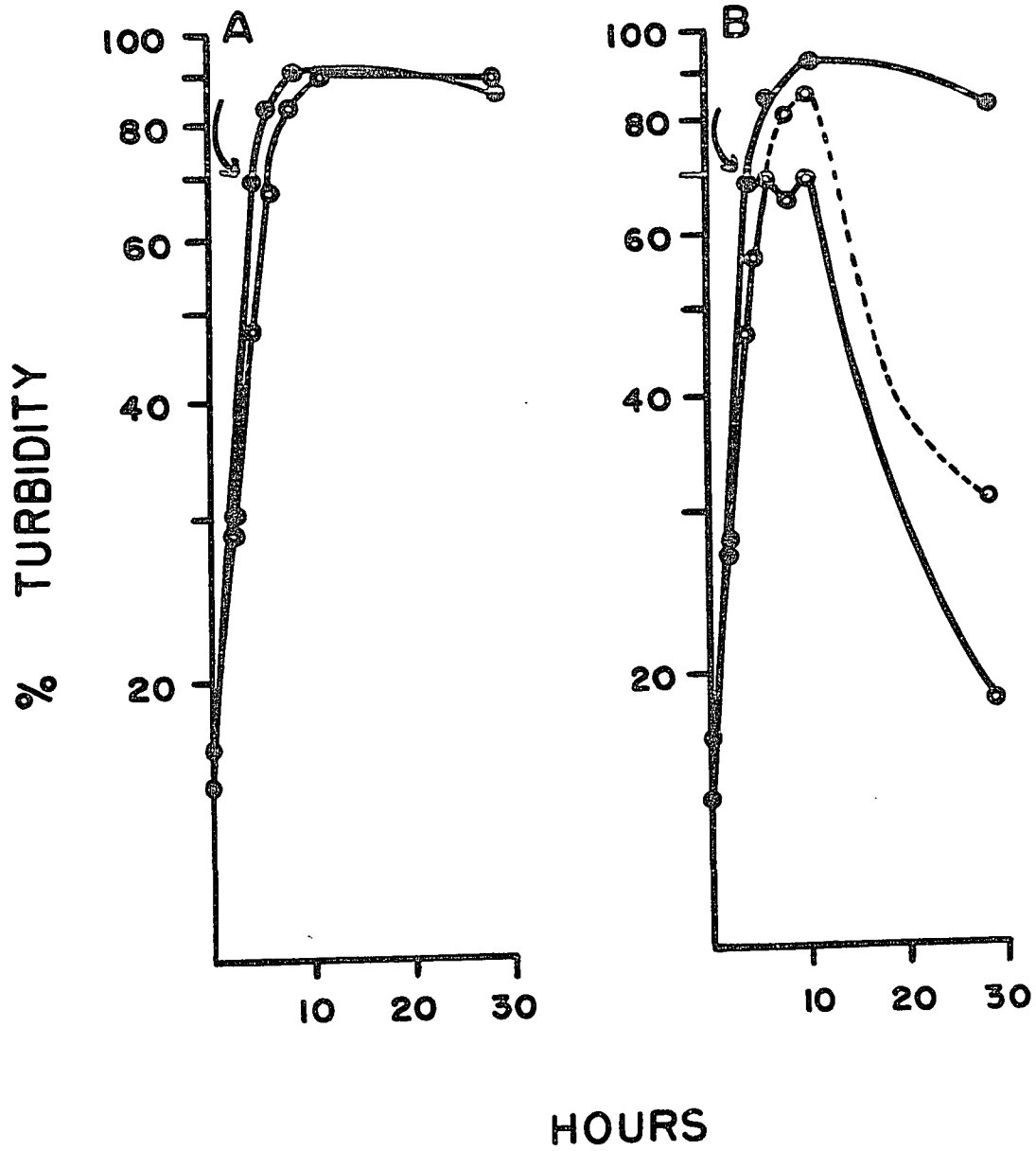


FIGURE 45.



attained equalled that of VHL cells. Thus, in the presence of calcium, the growth curves of VHA and VHL are identical.

Premature onset of lysis resulted when sodium was added to a VHA culture in logarithmic phase; the growth curve of a companion VHL culture was not affected (Figure 45B). Addition of magnesium, calcium, and strontium salts to logarithmic VHA cultures revealed that strontium also afforded protection from lysis (Figure 46). Although magnesium did not induce premature lysis, the rate of lysis was greater than that of the control culture.

Figure 47 depicts the growth curves of aerated VHA and VHL YPA broth cultures when 10^{-3} M salts of calcium, magnesium, and sodium were added prior to inoculation. Under these conditions, lysis in the presence of magnesium or sodium was more extensive than in the VHA control culture. All VHL growth curves were identical and resembled that of VHA with added calcium. Calcium chloride was found to be effective in preventing lysis of VHA cells when used at concentrations as low as 10^{-5} M. Lysis was also inhibited by 10^{-4} M calcium chloride in the presence of 10^{-2} M magnesium chloride.

These observations indicate that metal cations play a role in lysis of VHA cells. It would seem that under the conditions leading to lysis, YPA medium becomes deficient in cations essential for the integrity of VHA cells. This lytic response is not found in companion cultures of the pattern-less mutant, VHL.

FIGURE 46

Effect of calcium, strontium, and magnesium salts added
to logarithmic phase cultures of Spirillum serpens VHA

YPA broth cultures aerated at 26°C

A: ● Control (untreated)

○ 10⁻³ M calcium chloride added

● 10⁻³ M magnesium chloride added

B: ○ 10⁻³ M strontium chloride added.

FIGURE 46.

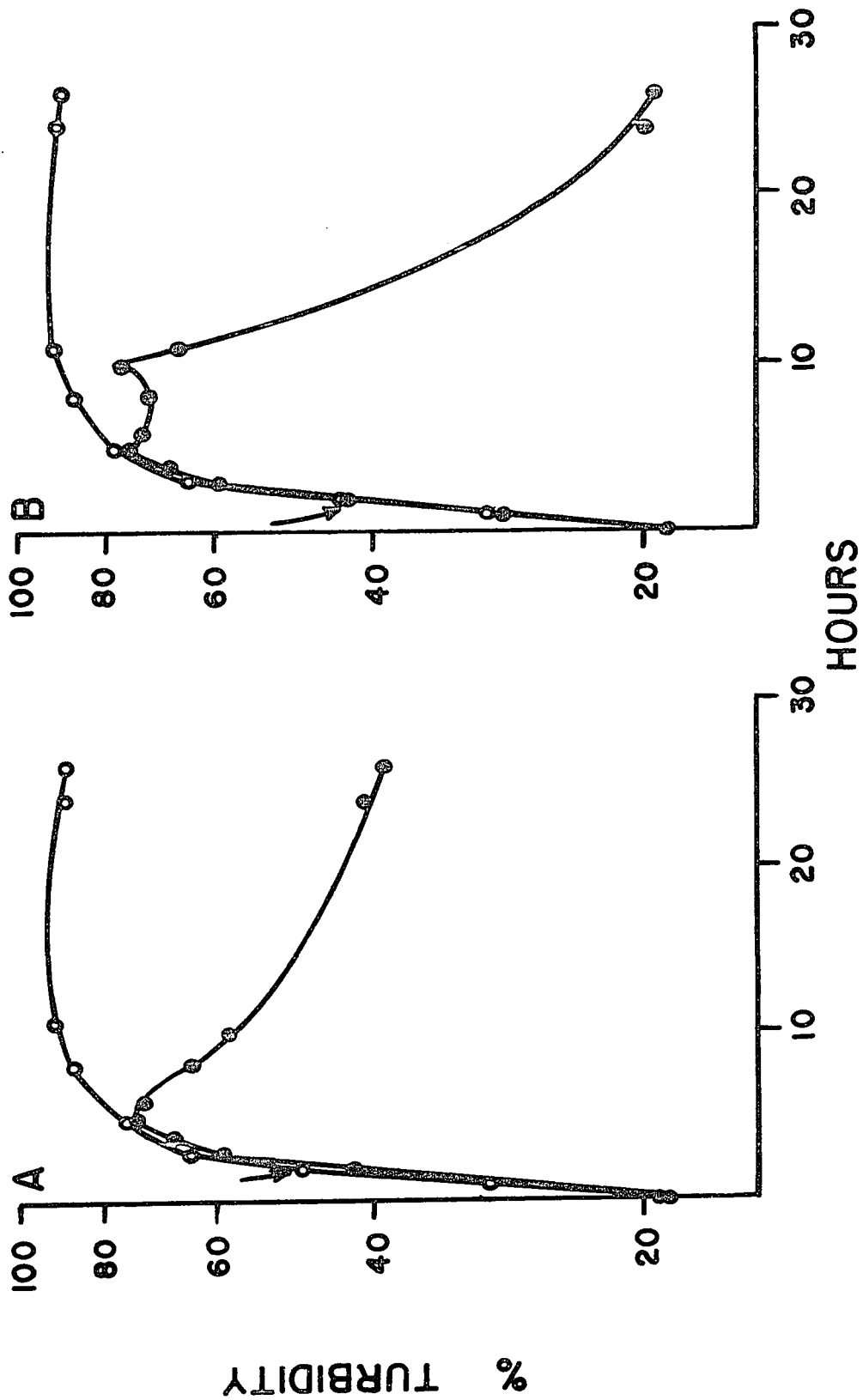


FIGURE 47

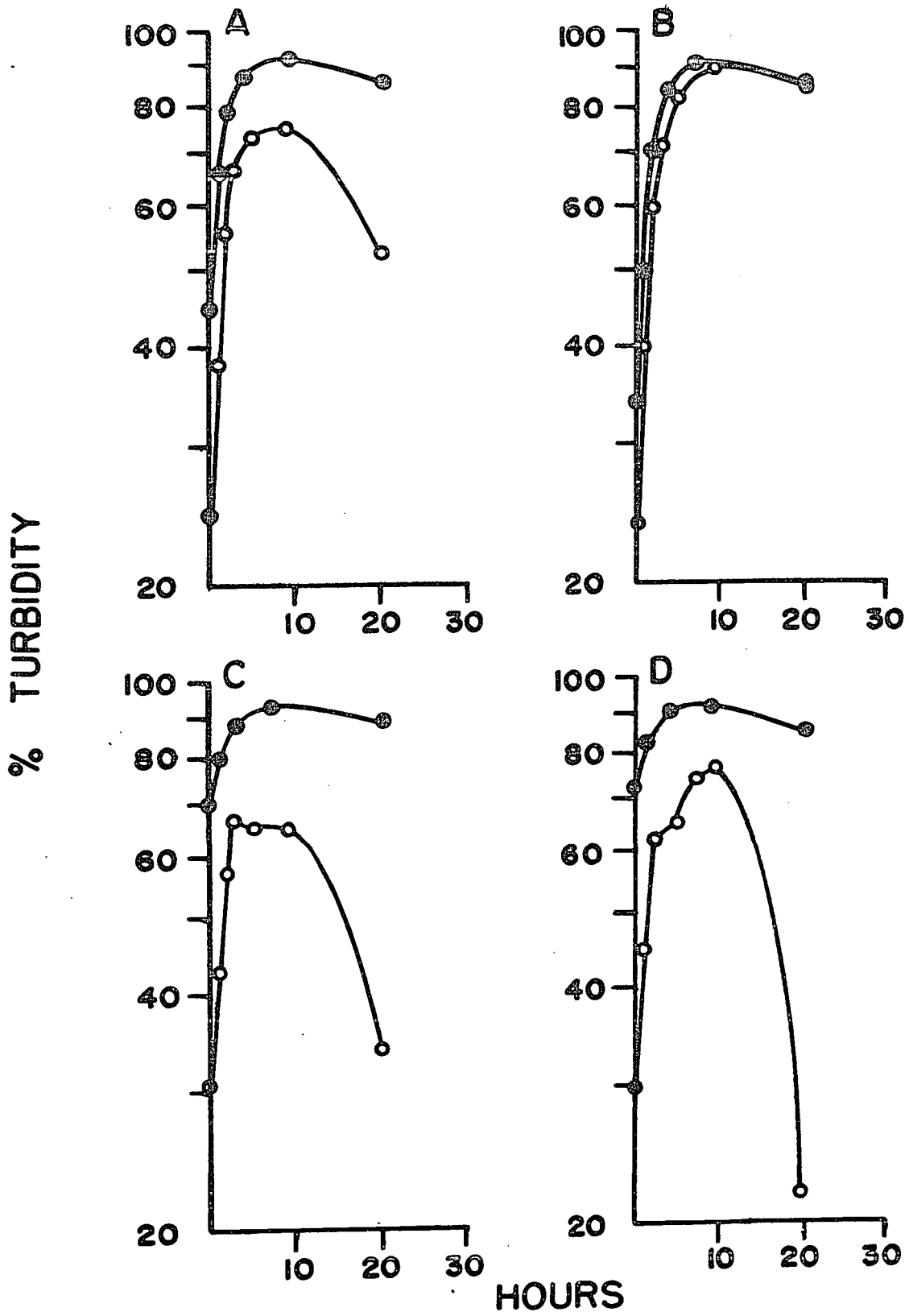
Effect of calcium, magnesium, and sodium salts added to broths prior to inoculation.

- o Spirillum serpens VHA
- Pattern-less mutant Spirillum serpens VHL

YPA broth cultures aerated at 26°C

- A: Controls (untreated)
- B: 10^{-3} M calcium chloride added
- C: 10^{-3} M magnesium chloride added
- D: 10^{-3} M sodium chloride added.

FIGURE 47.



(iii) Effect of ethylenediaminetetraacetate (EDTA)

Since metal cations were shown to be involved in lysis of VHA cultures, the effect of adding the metal-chelating agent, EDTA, was examined. As Figure 48A demonstrates, EDTA ($5 \times 10^{-3}M$) induced premature lysis of a logarithmic VHA culture. However, growth of the VHL culture was also retarded by this agent but the cells were less sensitive than those of the VHA culture (Figure 48B). This effect of chelation by EDTA differs from the highly specific nature of lysis in untreated VHA cultures.

(iv) Exchange of culture filtrates

The possibility that an active lytic factor might be produced in lysing VHA cultures was tested by exchanging the filtrates of VHA and VHL cultures. This exchange was accomplished by filtering the culture supernatants through membrane filters (porosity of 0.25μ) and returning the filtrates to the reciprocal pellet of cells. As shown in Figure 49A, the medium from a stationary phase VHL culture promoted growth of a lysed VHA culture. This indicates that the depletion which occurs in lysing VHA cultures does not progress to the same extent in VHL cultures. The absence of a lytic response by VHL cells when exposed to VHA culture filtrates (Figure 49) precludes the role of a lytic factor in causing lysis of VHA cultures.

The observations made from this study of VHA and VHL growth curves emphasize a vast difference in the behaviour of cells with and without the surface pattern.

FIGURE 48

Effect of EDTA on growing cultures

YPA broth cultures aerated at 26°C

A: Spirillum serpens VHA

B: Pattern-less mutant Spirillum
serpens VHL

- Controls (untreated)
- 5 x 10⁻³M EDTA added.

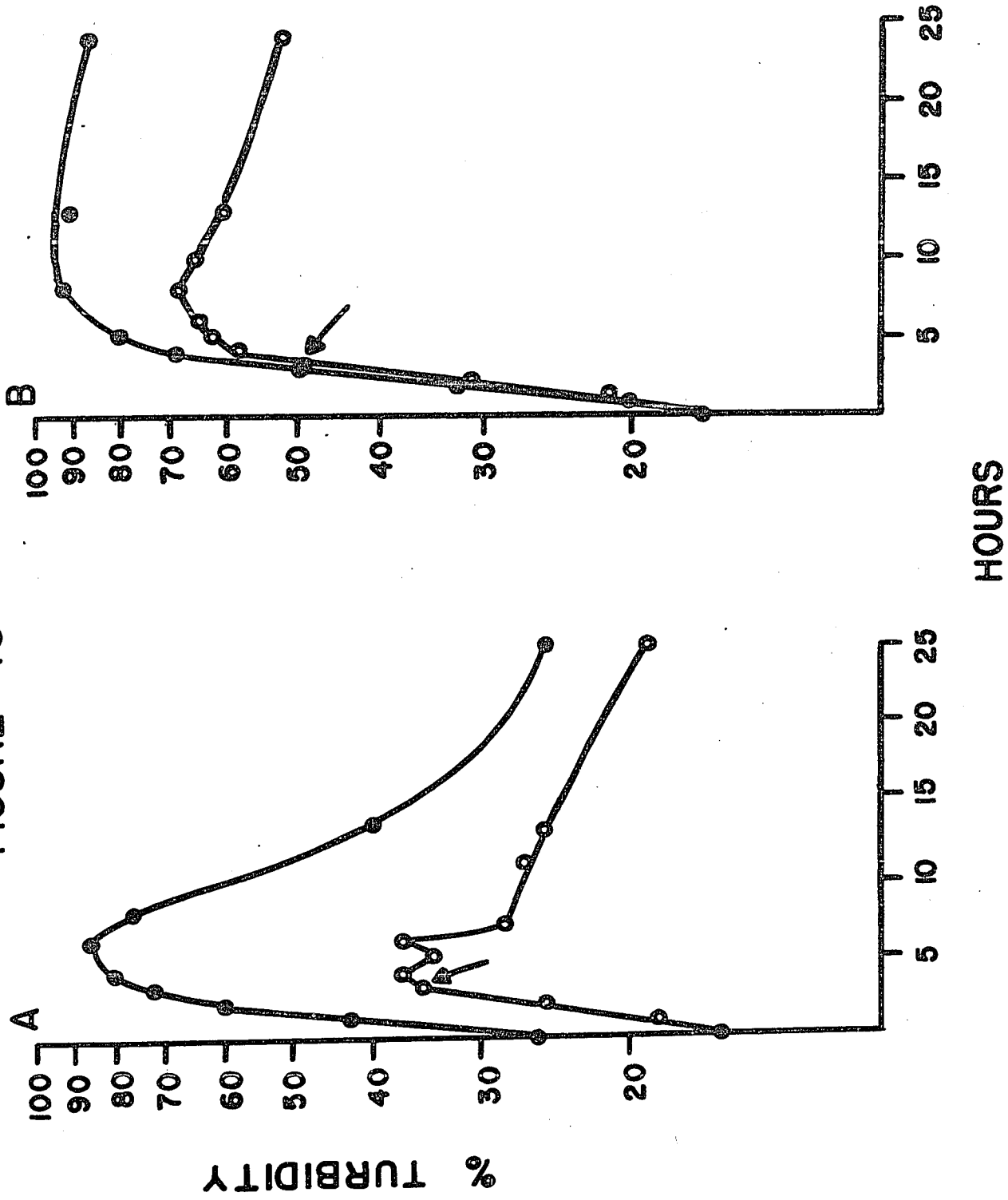


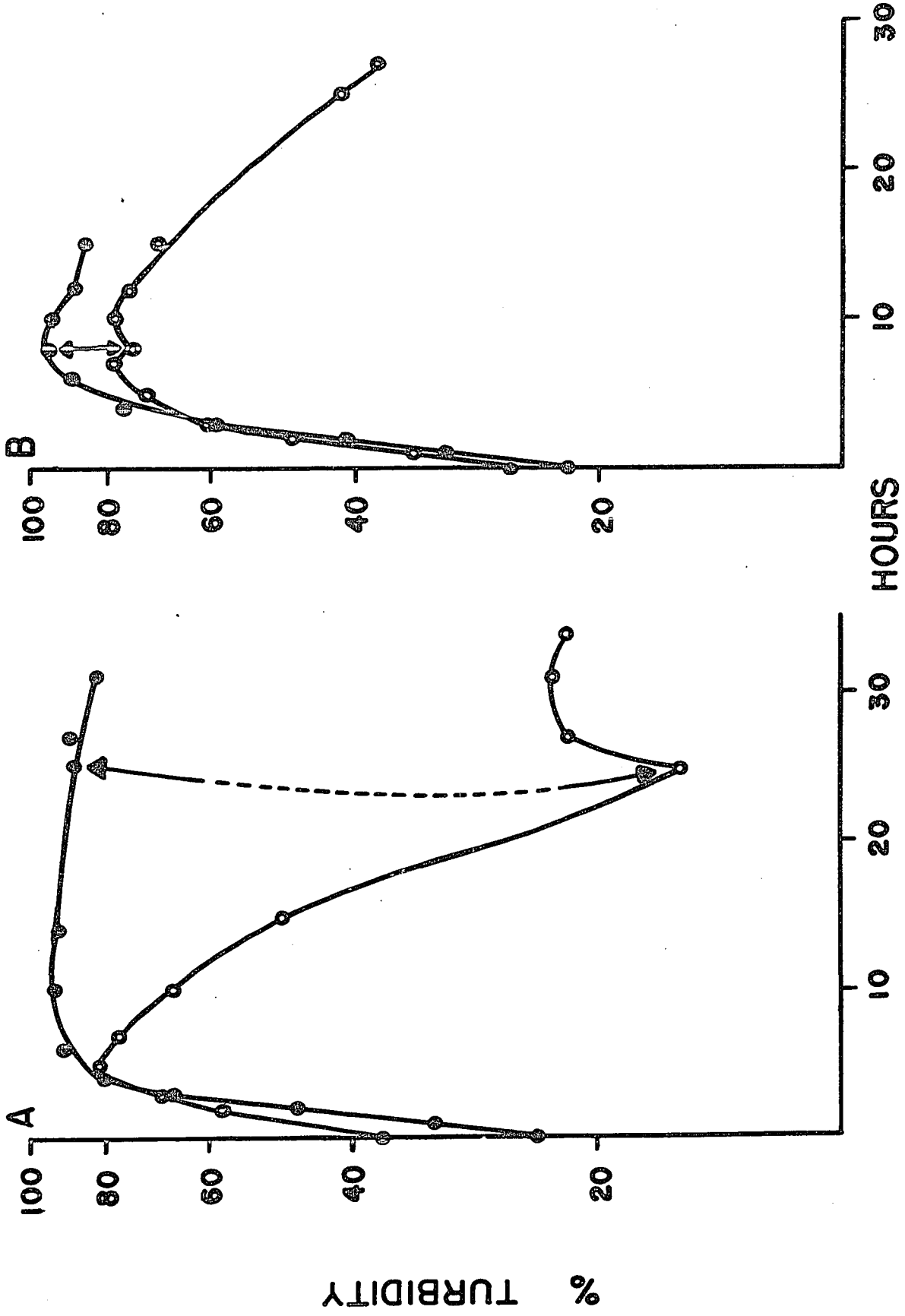
FIGURE 49

Effect of exchanging culture supernatants

YPA broth cultures aerated at 26°C

- Spirillum serpens VHA
- Pattern-less mutant Spirillum serpens VHL

FIGURE 49.



- (a) Under certain cultural conditions, VHA cells deplete the medium of certain divalent cations while VHL cells do not.
- (b) VHA cells are susceptible to lysis when growing in the depleted medium while VHL cells are not.
- (c) The depletion and sensitivity to lysis are prevented by adding calcium or strontium, and seem to be promoted by other cations such as sodium or magnesium; VHL growth curves remain unaffected by any of these added salts.

(2) Non-Proliferating Cells

Cells from YPA agar cultures of VHA and VHL incubated at 26°C were suspended in distilled water with and without added reagents. These suspensions were aerated at 26°C and turbidity values were plotted against time. Figure 50A shows that both VHA and VHL cells suspended in distilled water maintained constant turbidity. The cationic detergent CTAB, produced increases in turbidity which extended beyond the instrument's scale. Lysis of both cell types occurred in the presence of the anionic detergent, SLS, but was more pronounced in the VHA suspension. More extensive lysis resulted when VHA and VHL cells were aerated in distilled water for 6 hours before exposure to SLS (Figure 50B). Figure 50B also demonstrates that neither VHA nor VHL bacteria lysed in the presence of EDTA.

FIGURE 50

Behaviour of aerated non-proliferating cells.

Open symbols: Spirillum serpens VHA.

Closed symbols: Spirillum serpens VHL.

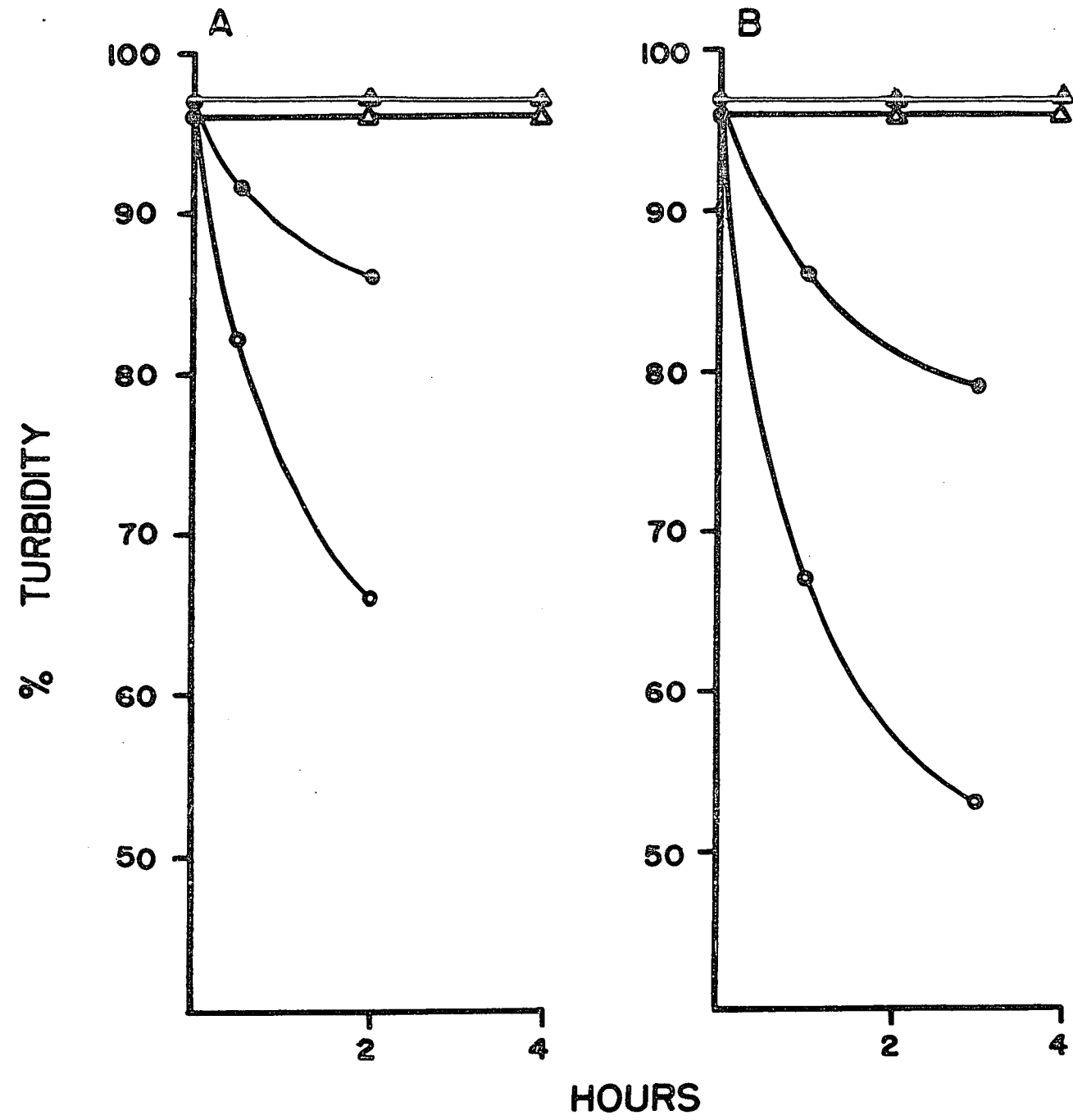
A. Δ/\blacktriangle distilled water.

\circ/\bullet SLS (0.25% w/v).

B. Δ/\blacktriangle EDTA ($5 \times 10^{-3}M$).

\circ/\bullet SLS after aeration in distilled water
for 6 hours.

FIGURE 50.



(3) Antigenic Relationship

Antisera were obtained from rabbits injected with somatic preparations of boiled, whole cells from tryptone agar cultures of VHA and VHL incubated at 26°C. Agglutination tests performed with VHA cells in the presence of 0.85% (w/v) sodium chloride produced non-specific agglutination, so subsequent tests with both VHA and VHL were carried out with dilute saline (0.2 to 0.3% w/v sodium chloride). Results of reciprocal adsorption tests are shown below.

Antigen	Antiserum from boiled cells	Titre
VHA	VHA	1/10,240
VHL	VHA	1/640
VHA	VHL	1/10,240
VHL	VHL	1/2,560
VHA	VHA adsorbed with VHA cells	<1/20
VHA	VHA adsorbed with VHL cells	1/10,240
VHL	VHL adsorbed with VHL cells	<1/20
VHL	VHL adsorbed with VHA cells	1/640

These titres reveal an antigenic relationship between VHA and VHL bacteria involving shared and unshared antigenic determinants. Discrepancies in the results may be due to the use of unboiled cells as antigenic material in these serological tests; (non-specific agglutination resulted when boiled cell preparations were employed).

(D) Nature of the Surface Pattern

(1) On the cell

The surface pattern of hexagonally arranged spheres was first observed by Houwink (1953) in a shadowed fragment preparation of Spirillum. In 1960, Weidel, Frank and Martin demonstrated the intact pattern on a shadowed cell of the same species. Negatively stained whole cell specimens of Gram-negative bacteria are generally of limited value due to lack of penetration by the staining reagent. Apart from some notable exceptions such as "Eubacterium" (Bladen, Nylen and Fitzgerald, 1964) and Caulobacter crescentus (Cohen-Bazire, Kunisawa and Poindexter, 1966), Gram-negative bacterial walls are impermeable to electron-scattering stains like phosphotungstate (PTA). In this respect Spirillum serpens behaves as a "typical" Gram-negative species in that PTA and uranyl acetate (UA) are arrested at the cell surface producing an electron-dense stain with little or no detail of surface and internal structure. Fragments from sonicated samples provide much more information and an extensive account of the pattern in sonicated preparations of Spirillum serpens VH was published by Murray (1963a).

A marked increase in detail of whole cells was obtained when S. serpens suspensions were pretreated with petroleum ether (Ivanov, Markov, Golowinsky and Charisanova, 1964) with or without added 2.5% (w/v) sucrose. An example is given in Figure 51 which shows both surface and internal detail. Presumably the ether treatment

extracts some surface lipid material which normally acts as a barrier to negative staining reagents. However, only a proportion of cells became permeable to PTA suggesting that some predisposing factor, possibly physiological in nature, is required for the extraction to proceed satisfactorily.

(2) Loss of pattern material

(i) Permanent

When a survey of several different Spirillum serpens VH cultures was undertaken, it was found that some, like S. serpens VHL, had lost the regular surface structure. This loss was permanent and probably resulted from a mutation.

(ii) Temporary

(a) Stationary broth cultures

The production of pattern was studied in S. serpens VHA cells grown at 26°C, 37°C, and 45°C in YPA or tryptone broth without aeration. It was found that cells from these liquid cultures gradually lost the surface component; however, very few fragments of pattern structure could be detected in culture supernatants. The loss at 26°C was noticeable after one to two days and proceeded more rapidly at higher temperatures. Examination of a YPA broth culture left standing at room temperature for two months revealed that 2 to 3% of the cells possessed the pattern, often only in patches; fragments or subunits of the surface structure were not observed in the medium.

FIGURE 51

Spirillum serpens VHA: surface and internal structure of a cell treated with petroleum ether. Phosphotungstate preparation X 57,000.

FIGURE 52

Spirillum serpens VHA: cell-free aggregates of pattern material from a YPA agar culture incubated at 26°C (flagella also present). Note the hexagonal arrangement of spherical subunits and the amorphous, irregular formations of "backing." Phosphotungstate preparation X 110,000.

FIGURE 51

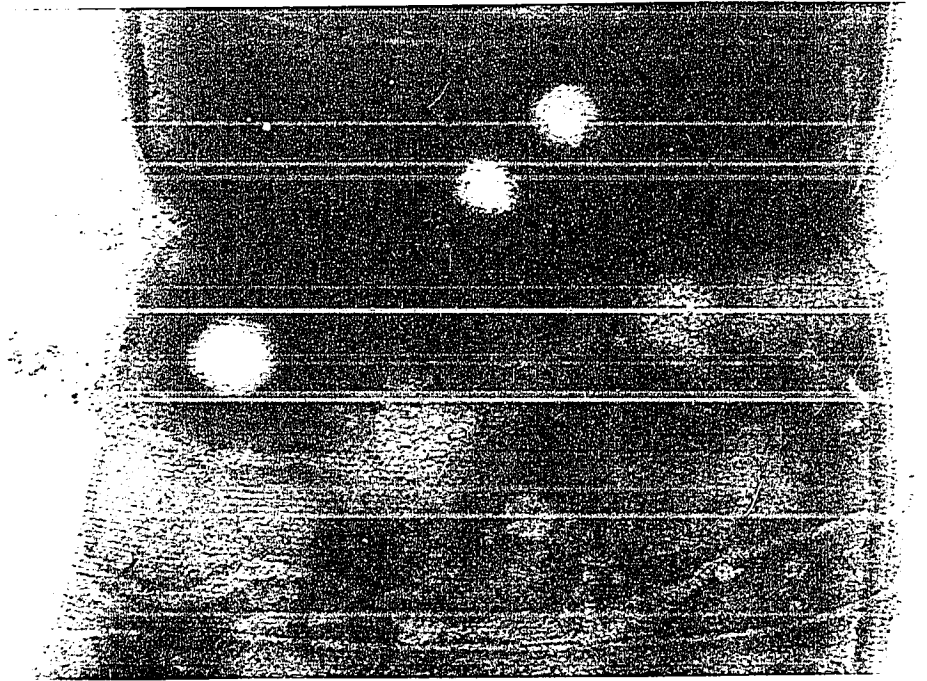


FIGURE 52

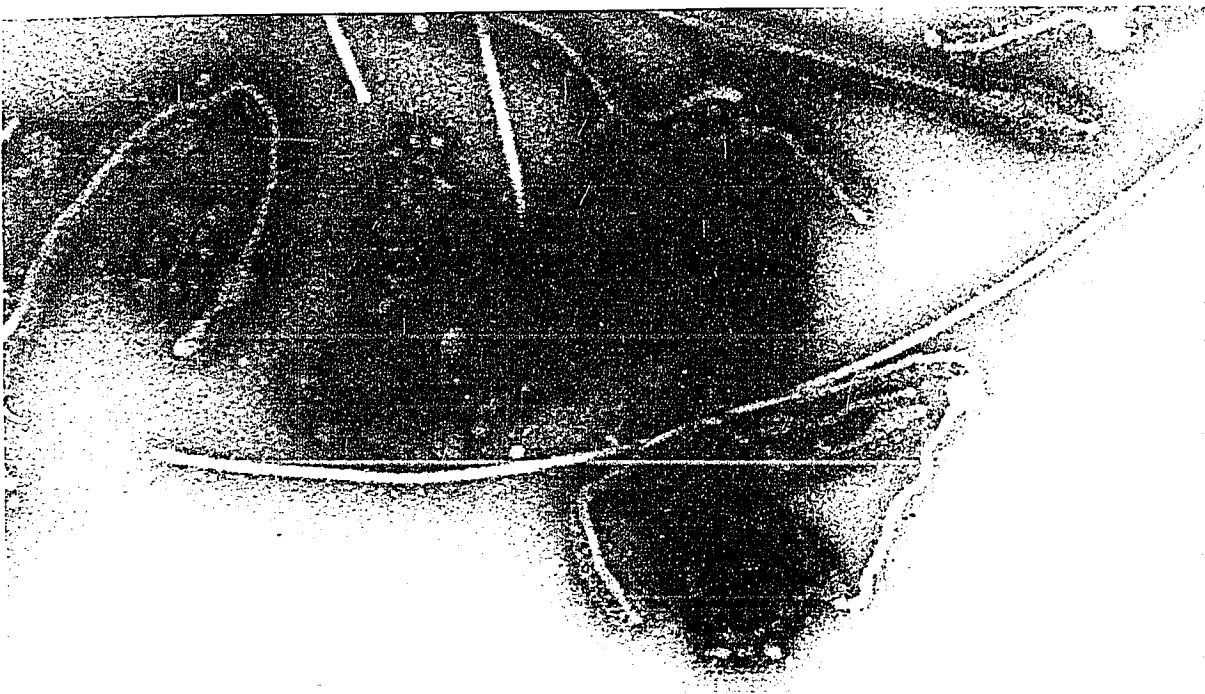


FIGURE 51

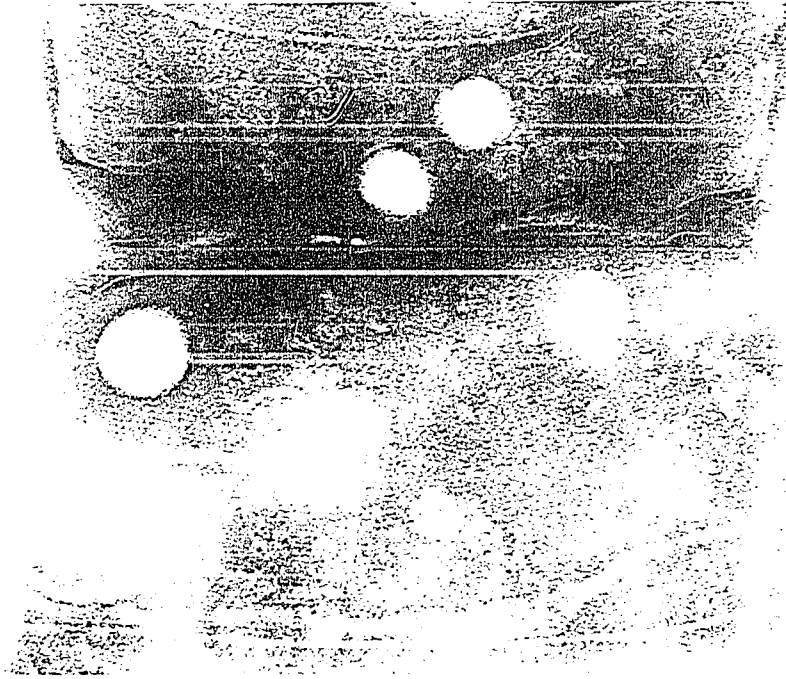


FIGURE 52



(b) Aerated broth cultures

A more rapid loss of pattern occurred in aerated broth cultures. Sonicated samples from different growth phases of a YPA broth culture aerated at 26°C were negatively stained and examined. The results demonstrated large amounts of pattern throughout logarithmic phase with reduced quantities and eventual absence of this material during the lytic stage. Presence of calcium in the medium prevented lysis and pattern was observed in the stationary phase samples examined. Addition of calcium to a lysing culture stimulated further growth and small quantities of pattern were seen. It should be noted that both S. serpens VHA and S. serpens VHL broth cultures produced a similar rise in pH from neutrality to pH 8.5 to 8.8.

(c) Agar cultures

Agar cultures behaved quite differently in that cells incubated at 26°C for up to 10 days were completely covered with intact pattern. A peculiar aggregation of cell-free pattern material as irregular fragments and cylindrical formations was observed in agar cultures at 26°C (see Figure 52). This effect could be detected after two days and was more pronounced in older cultures. Companion cultures incubated at higher temperatures showed signs of disintegration among the cell-free pattern aggregates. After two to three days at 45°C, cells on YPA agar appeared 'sick' (loss of normal cytoplasmic phase density), but were partially or completely covered with the regular surface structure.

Among the aggregated materials were many fragments showing partial or complete removal of the hexagonal network. The residual material, referred to as the "backing", appeared as irregular formations of an amorphous substance impermeable to PTA.

These observations revealed that under certain cultural conditions the hexagonal pattern of S. serpens VHA disappeared from the cell surface. However, this loss proved to be temporary for when such cultures were transferred to agar media or fresh broth at 26°C they invariably reproduced cells with the surface pattern.

(3) Effect of enzymes

Cells from tryptone agar cultures of S. serpens VHA incubated at 26°C for six days were suspended in buffered enzyme preparations. After incubation at 37°C for two hours the cells were washed then stained with PTA. The type of culture used for this experiment proved to be very useful in that it provided a preparation containing both intact pattern on the cell surface and considerable amounts of cell-free pattern material. Both trypsin and lysozyme failed to produce any change in the ultrastructural appearance of the preparation. Complete absence of pattern resulted from incubation with pepsin but this was found to be due to the acid buffer (pH 2.2). Cells exposed to lipase retained the intact pattern although a large proportion of the cells had been converted to spheroplasts. This result immediately revealed two properties of the surface network structure: its inability to maintain cell shape, and its plasticity in being able to accommodate to spheroplast expansion.

The cell-free pattern aggregates exhibited a rather quaint effect after lipase treatment (see Figure 53). This appearance had been observed occasionally in untreated samples from agar cultures grown at 37°C. However, the effect in this lipase-treated preparation was quite pronounced. It can be seen that the "backing" material has been displaced in certain areas while the repeating-structural component appears to be undamaged. This effect, resembling "pulled threads," sometimes occurred in parallel rows within the pattern "fabric". The displaced threads were in the form of continuous loops still attached to the undisturbed "backing" of the pattern, and free ends were not observed frequently.

(4) Effects of other reagents

(i) Effect of pH

The integrity of cell-free pattern material was found to be disrupted by exposure to acid solutions. Figure 54 illustrates the immediate effect produced when a sample of aggregated pattern from S. serpens VHA was stained with uranyl acetate at pH 2.2. The subunits which comprise the hexagonal array are destroyed and the "backing" substance becomes disorganized, and forms thread-like protrusions.

(ii) Other reagents

Aliquots from a preparation of cell-free and cell-bound pattern of S. serpens VHA were added to different reagents and shaken at room temperature for three to six hours. Samples were washed, then stained with PTA. The results, shown below, indicated all-or-none responses

FIGURE 53

Spirillum serpens VHA: effect of lipase on cell-free VHA
pattern material. Phosphotungstate preparation X 110,000.

FIGURE 54

Spirillum serpens VHA: disappearance of hexagonally-arranged
subunits and disorganization of the "backing" in cell-free VHA
pattern material prepared with uranyl acetate pH 2.2
X 110,000.

FIGURE 53

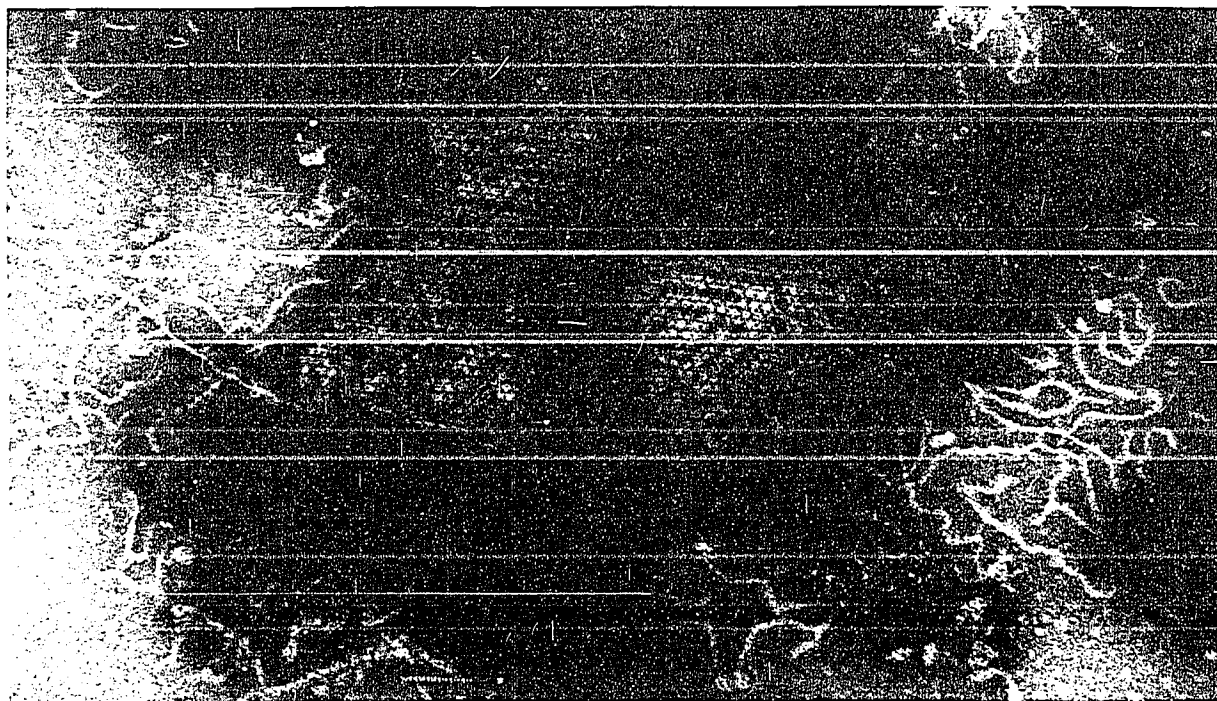
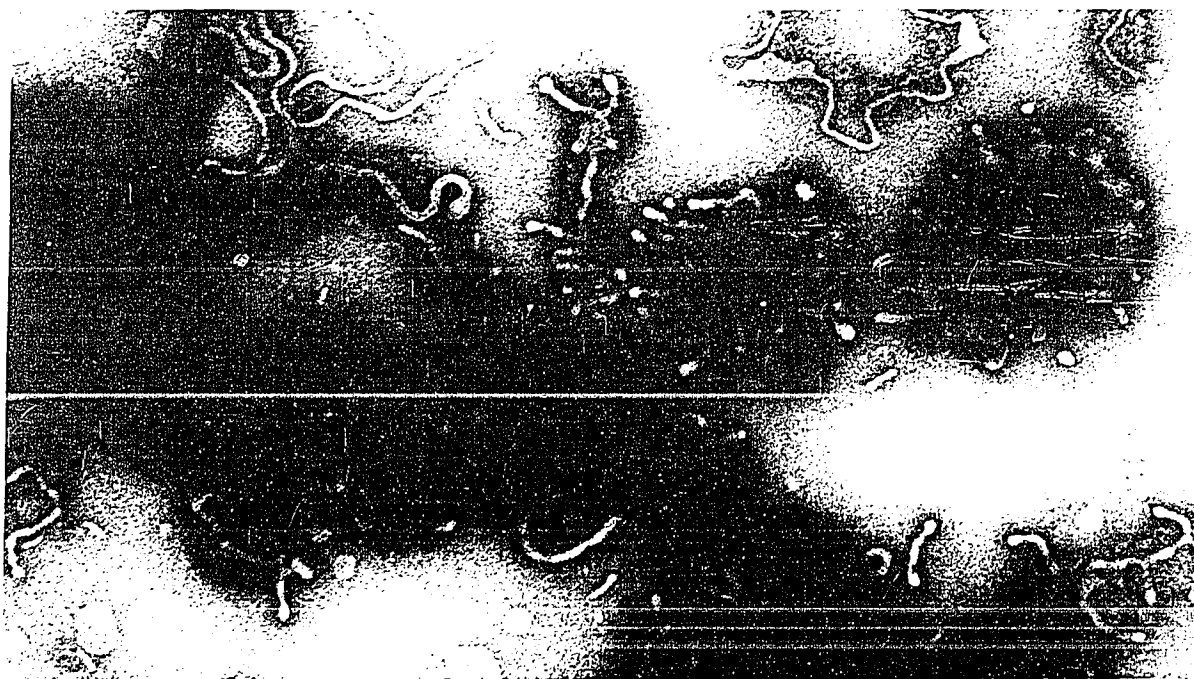


FIGURE 54



to the action of the reagents.

Reagent in Distilled Water	Cell-Free and Cell-Bound Pattern
None	Present
10^{-2} M EDTA pH 7.0	Present
0.1% (w/v) SLS pH 7.0	Absent
0.1% (w/v) SLS pH 9.8	Present
6M Urea	Absent
1M Guanidine Hydrochloride	Absent

(iii) Effect of SLS

The surface pattern on S. serpens VHA cells was not always removed by neutral aqueous solutions of SLS. Figures 55 and 56 reveal cells with residual patches of the hexagonal network after exposure to the anionic detergent. Cell-free material recovered after treatment with 0.25% (w/v) SLS as shown in Figure 57 which clearly demonstrates small islands of pattern.

(5) Isolation

(i) Electron microscopy

Surface pattern from S. serpens VHA was isolated by taking advantage of the natural accumulation of the cell-free aggregates in old agar cultures grown at 26°C. Growth after six to eight days was harvested in calcium solution (10^{-3} M calcium chloride in distilled water) and whole cells were removed by differential centrifugation. Heat treatment at 60°C to 65°C for one to two hours followed by three

FIGURE 55

Spirillum serpens VHA: patches of pattern at the surface of a cell treated with 0.01% SLS. Phosphotungstate preparation X 121,000.

FIGURE 56

Spirillum serpens VHA: as in Figure 55. Shadowed preparation with tungsten oxide (reversed) X 110,000.

FIGURE 55

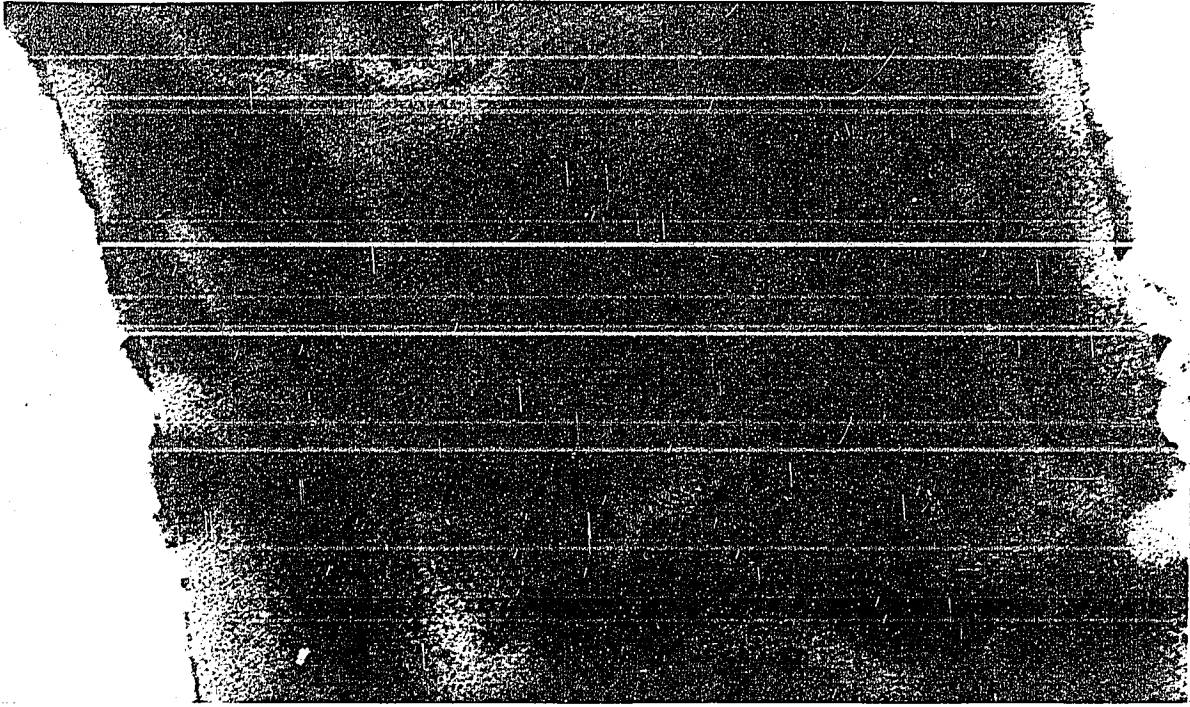


FIGURE 56

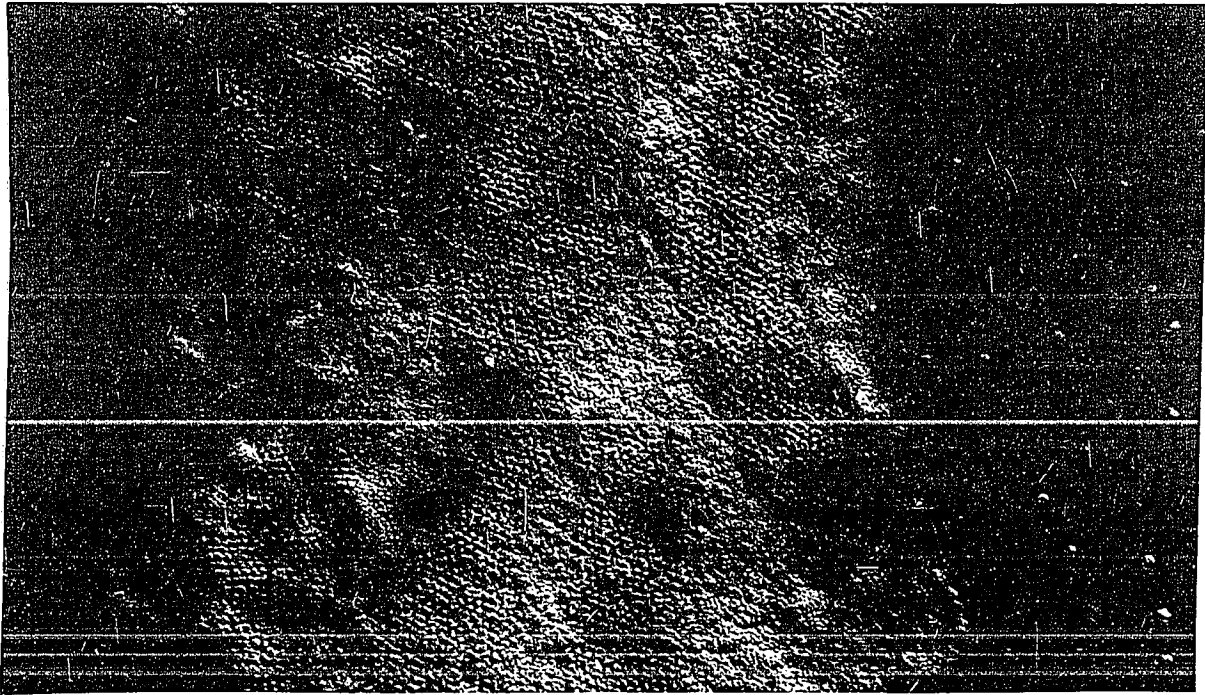


FIGURE 57

Spirillum serpens VHA: islands of pattern among the cell-free debris recovered from cells treated with 0.25% SLS. Phosphotungstate preparation X 83,000.

FIGURE 57



to four cycles of washing produced preparations such as that shown in Figure 58. The absence of flagella and other cellular constituents allowed this degree of homogeneity to be used as a criterion for purity.

Although S. serpens VHL cells no longer produce the surface pattern there was an accumulation of cell-free material in old agar cultures at 26°C. Figure 59 shows a sample of this material taken from a YPA agar culture incubated at 26°C for seven days. Flagella are readily distinguishable from the irregular formations of amorphous substance resembling the "backing" of VHA pattern material. Figure 60 depicts the VHL preparation after isolation and concentration.

(ii) UV absorption spectra

The UV absorption spectra of sonicated samples prepared from isolated VHA and VHL material are illustrated in Figure 61. Absence of absorption peaks at 260 m μ , indicating absence of nucleic acids, was taken as evidence for lack of cytoplasmic contamination and was used as an additional standard of purity.

(6) Chemical analysis

(i) Dry weight composition

The lipid, protein, polysaccharide and phosphorus contents of isolated cell-free aggregates are listed in Table 3.

(ii) Lipid chromatography

Further analysis of the lipid extracts derived from VHA and VHL isolated material was achieved by thin-layer chromatography.

FIGURE 58

Spirillum serpens VHA: isolated VHA pattern material. Note absence of flagella and cellular debris. Phosphotungstate preparation.

A: X 16,000

B: X 57,000

FIGURE 58 (A)

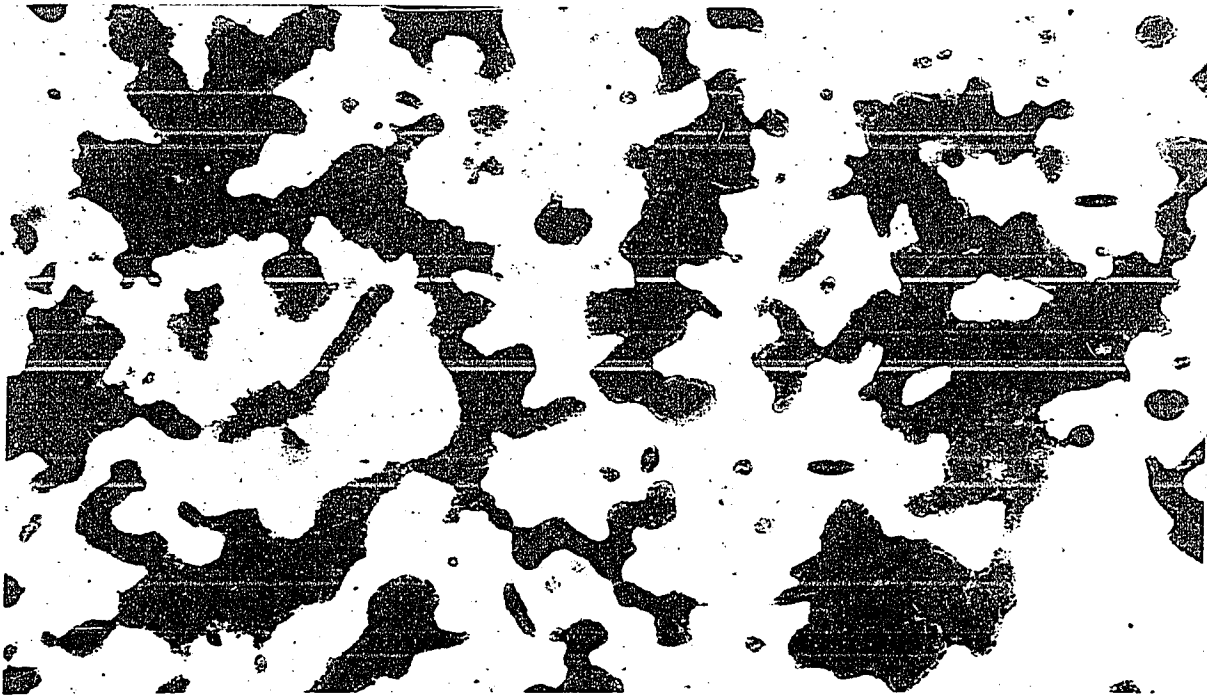


FIGURE 58 (B)



FIGURE 59

Spirillum serpens VHL: cell-free aggregates of VHL material resembling the "backing" of VHA pattern. Some flagella present. Phosphotungstate preparation X 110,000.

FIGURE 60

Spirillum serpens VHL: appearance of the isolated, concentrated VHL material. Phosphotungstate preparation X 83,000.

FIGURE 59

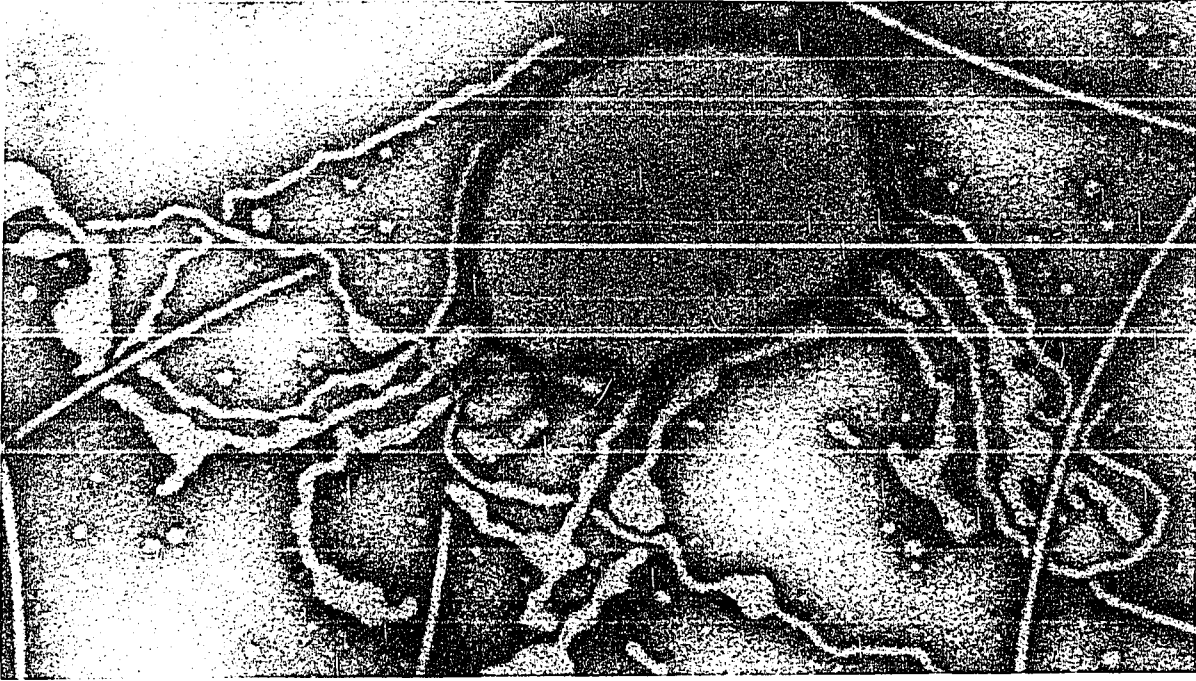


FIGURE 60



FIGURE 61

UV-absorption spectra of sonicated samples.

- VHA pattern material.
- VHL material.

FIGURE 61.

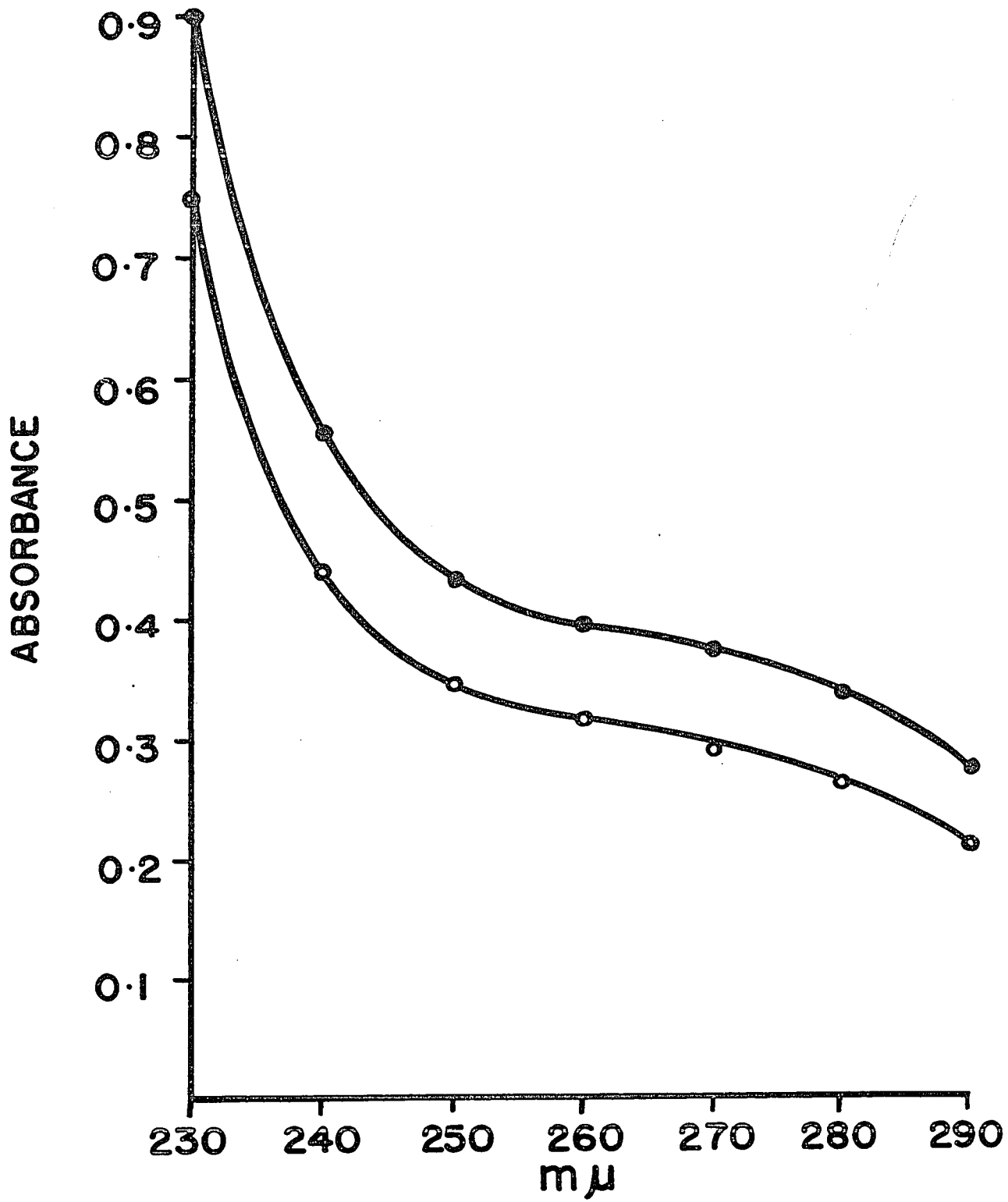


TABLE 3

CHEMICAL COMPOSITION OF ISOLATED CELL-FREE AGGREGATES*

Constituent	% Dry Weight (Mean \pm S.E.M.)	
	VHA	VHL
Lipid	10.6 \pm 0.16	42.0 \pm 0.71
Protein	55.5 \pm 4.44	14.7 \pm 0.69
Polysaccharide	6.9 \pm 1.3	4.2 \pm 0.42
Phosphorus	0.7 \pm 0.05	Not Tested

* These analyses do not account for total composition.

(a) Glycolipid

The results of chromatography in a solvent of absolute acetone with 6% water are shown in Figure 62B. In this solvent system glycolipids migrate while phospholipids remain at the origin. Thus, the absence of glycolipids in both VHA and VHL samples was indicated.

(b) Phospholipid

Both VHA and VHL lipid extracts produced positive reactions for free amino groups when sprayed with the ninhydrin reagent after chromatography in chloroform-methanol-water (65:20:3, by volume). Treatment with the modified Dragendorff reagent after a similar chromatographic run produced faintly positive reactions (orange spots) for choline-containing compounds. Presence of free choline, characterized by development of purple spots, was not demonstrated in either VHA or VHL samples.

Figure 62A depicts the chromatoplate developed with sulphuric acid spray after VHA and VHL samples and a phosphatidylethanolamine standard were run in the chloroform-methanol-water solvent. The VHA and VHL lipids appear to be identical and are identifiable with the standard. Presence of more than a single spot in the standard was most likely due to some breakdown product.

The identity of VHA and VHL lipid was confirmed by spotting both samples together prior to two-dimensional chromatography. A single spot developed (Figure 63) which compared with the standard phosphatidylethanolamine shown in Figure 64.

FIGURE 62

Thin-layer chromatoplates of lipid
samples

A: Solvent of chloroform-methanol-water (65: 20: 3 by volume)

(i) VHA sample

(ii) VHL sample

(iii) phosphatidylethanolamine

B: Solvent of absolute acetone with 6% (v/v) water

(i) VHA sample

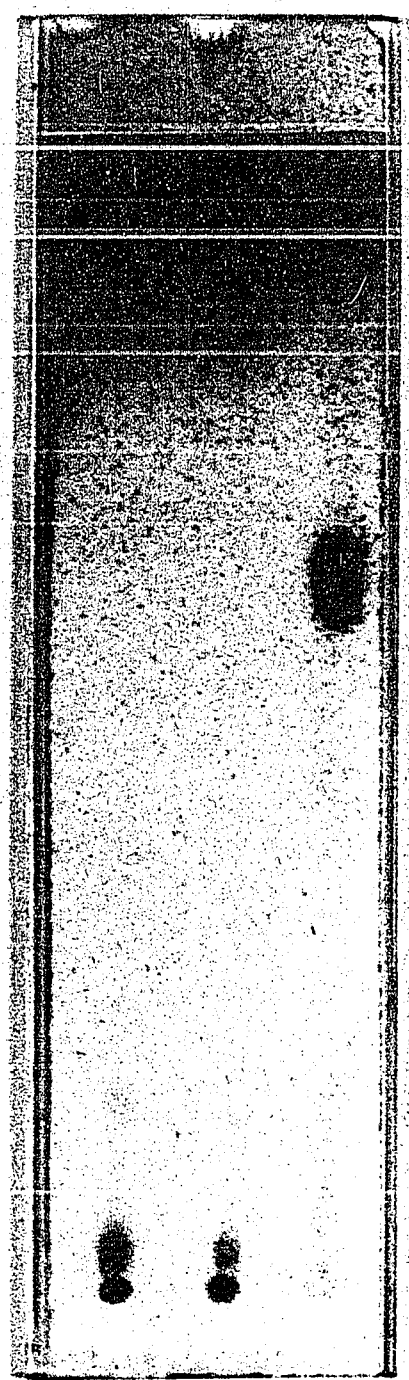
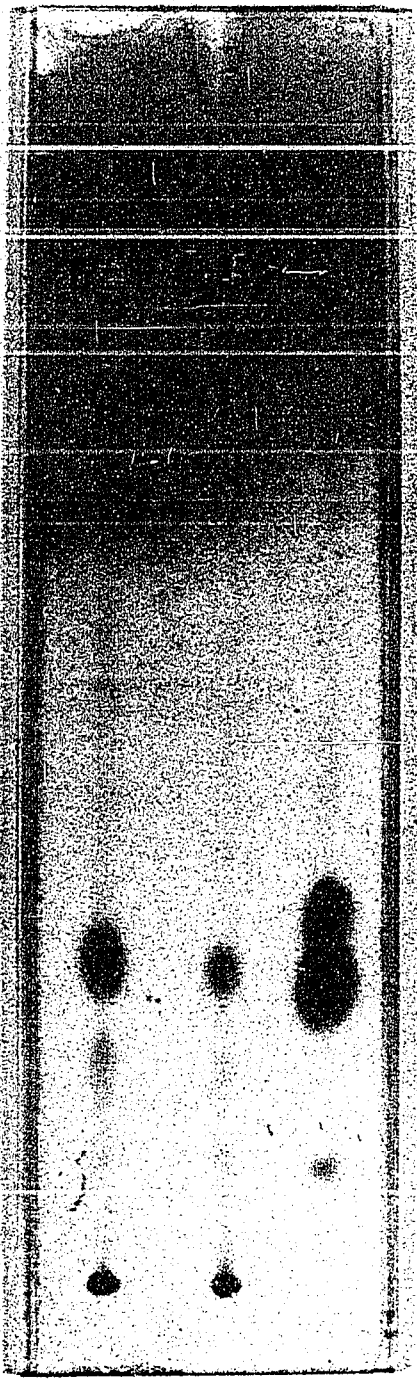
(ii) VHL sample

(iii) glycolipid

FIGURE 62

A

B



(i) (ii) (iii)

(i) (ii) (iii)

FIGURE 63

Two-dimensional thin-layer chromatoplate of VHA and VHL lipid samples

Solvent 1: chloroform-methanol-water

(65: 20: 3 by volume)

Solvent 2: chloroform-acetone-methanol-acetic acid-water

(10:4:2:2:1 by volume)

FIGURE 64

Two-dimensional thin-layer chromatoplate of phosphatidylethanolamine.

Solvent systems as in Figure 63.

FIGURE 63

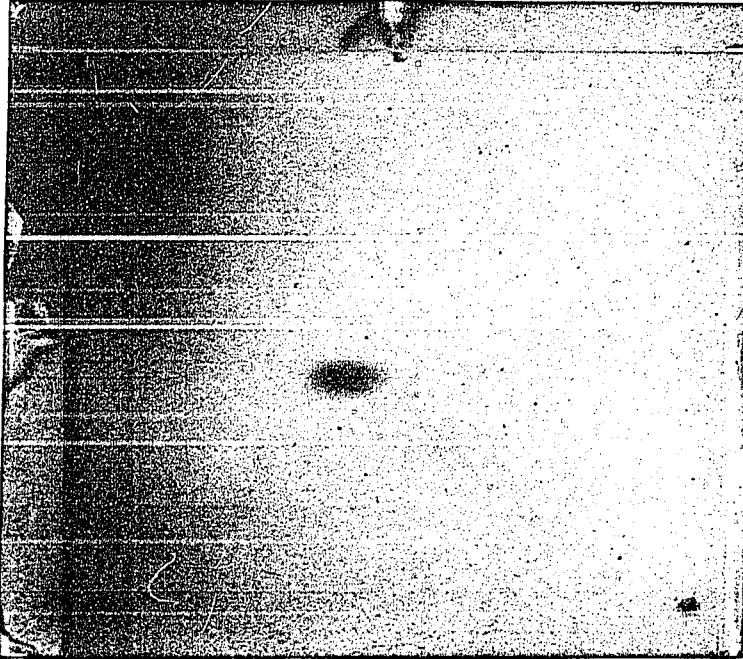
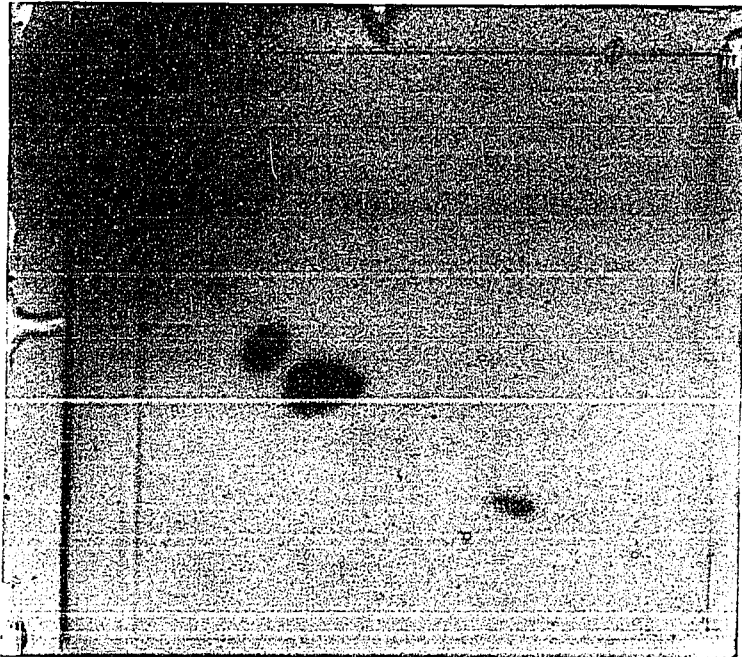


FIGURE 64



(iii) Sugar chromatography

The hydrolysed pattern material isolated from S. serpens VHA produced several spots on developed chromatograms. Two of these were identified by comparison with known standards but a component which ran with the solvent front, and a slowly migrating indistinct spot remained unknown. The colours and Rf values listed in Table 4 demonstrate the presence of the aldohexose, galactose, and the methylpentose, rhamnose, as monosaccharide constituents in the pattern material.

(iv) Isolated polysaccharide

Polysaccharide was extracted from isolated VHA pattern material by a method described by Kwapinski (1965). Figure 65 illustrates the globular nature of the white floccular precipitate produced by the extraction. In this shadowed preparation, the globules measured 60 to 90 Å.

(v) Amino acid analysis

Hydrolysates of isolated VHA pattern material were analysed with a Beckman Model 120C amino acid analyzer. Molar ratios of the constituent amino acids are listed in Table 5. Cystine and histidine were absent; diaminopimelic acid was not determined.

(7) Freeze-etched cells

The structure observed in cells prepared by the freeze-etching process can be considered as a close approach to the natural state of the structural component, as it occurs in the living organism. Figure 66 depicts the surface of a Spirillum serpens VHA cell prepared

TABLE 4CHROMATOGRAPHY OF SUGARS AND VHA PATTERN MATERIAL

<u>Spot Applied</u>	<u>Rf Value</u>	<u>Colour</u>
Arabinose standard	0.39	Pale orange
Fructose standard	0.32	Dark blue
Galactose standard	0.15	Green-brown
Glucose standard	0.28	Green-brown
Mannose standard	0.54	Yellow-brown
Rhamnose standard	0.87	Orange
Ribose standard	0.76	Pale pink
Xylose standard	0.72	Pink
	(0.87	Orange
	(
VHA pattern hydrolysate	(0.15	Green-brown
	(
	(0.05(?)	Green-brown

TABLE 5AMINO ACIDS IN ISOLATED VHA PATTERN MATERIAL

Amino Acids	μ moles	Molar Ratios
Alanine	1.3	3.42
Arginine	0.79	2.07
Aspartic Acid	1.4	3.68
Glutamic Acid	0.38	1.00
Glycine	1.3	3.42
Isoleucine	0.45	1.18
Leucine	0.78	2.05
Lysine	0.27	0.71
Methionine	0.04	0.10
Phenylalanine	0.46	1.21
Proline	0.16	0.42
Serine	0.56	1.42
Threonine	1.3	3.42
Tyrosine	0.01	0.02
Valine	0.67	1.76

FIGURE 65

Spirillum serpens VHA: polysaccharide extracted from isolated VHA pattern material. Shadowed preparation with tungsten oxide (reversed) X 110,000.

FIGURE 66

Spirillum serpens VHA: appearance of the pattern at the cell surface. Freeze-etching X 97,000.

FIGURE 65

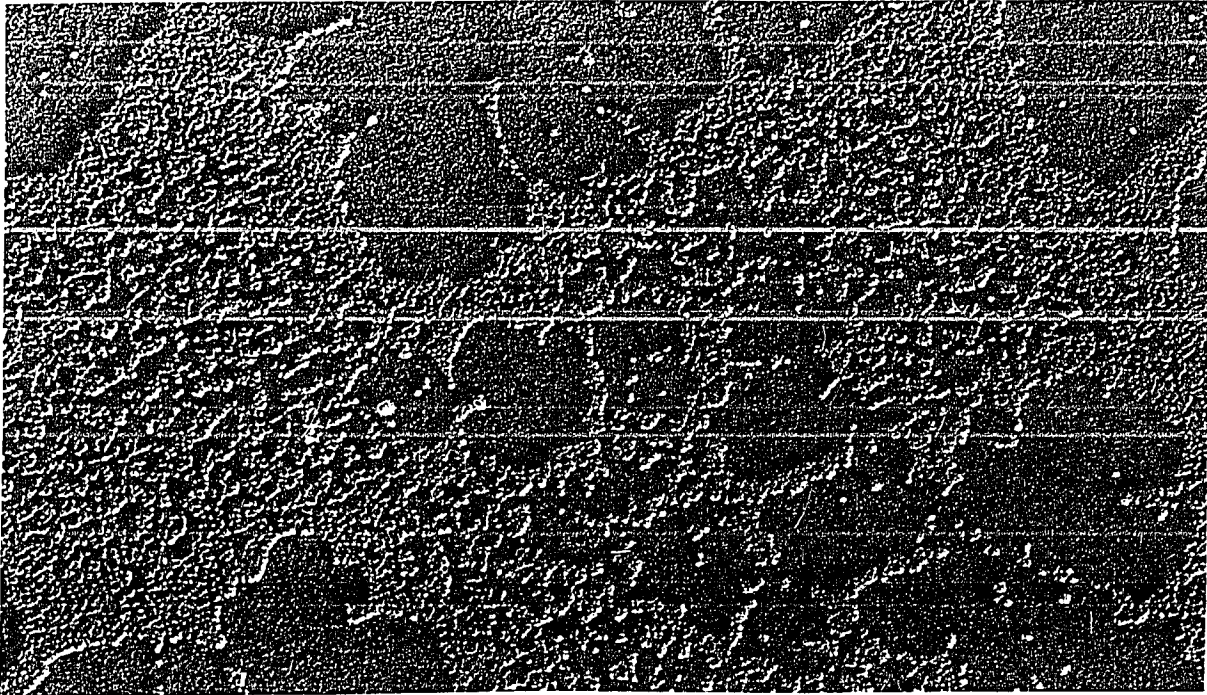
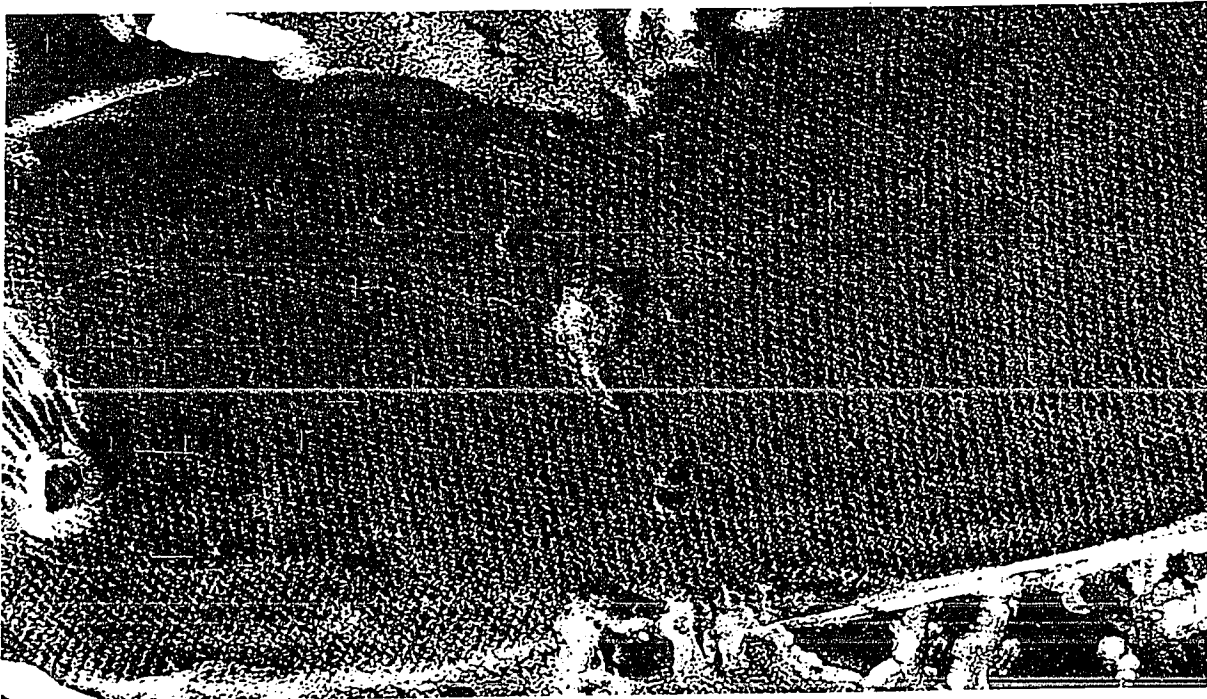


FIGURE 66



by the freeze-etching process. The regular hexagonal array of globular subunits in this specimen resembles the appearance of the surface pattern in other kinds of preparations.

(E) Lineola longa

Lineola longa was originally described as a Gram-negative, non-sporing, rod-shaped bacterium (Pringsheim and Robinow, 1947). However, an investigation by Bennett and Canale-Parola (1965) revealed that cultures of this organism became Gram-positive and produced spores after a period of subculturing on laboratory media. A brief look at the ultrastructure of this "taxonomic misfit" was undertaken to determine the nature of wall structure and division in this organism. The culture used for this study was obtained from Dr. C. F. Robinow of this Department who received it from Pringsheim's laboratory in 1963. After four years of subculturing on laboratory media, the culture has remained Gram-negative and non-sporing.

(1) Surface profile

A section of L. longa aerated in YPA broth at 37°C for six hours is shown in Figure 67. The culture was prefixed by addition of OsO₄ and processed in the usual manner using a one-half (1/2) dilution of VA buffer. Although the wall is a multilayered structure, the outermost layer is of uniform, medium electron density (112Å wide) and does not have the characteristic dense-light-dense profile of the triplet-layer observed in other Gram-negative bacteria. An electron-trans-

FIGURE 67

Lineola longa: section of an osmium-fixed cell revealing a multi-layered wall differing from the "typical Gram-negative" profile. Note the very thick, inner, electron-dense component and the absence of an outer triplet-layer. X 110,000.

FIGURE 68

Lineola longa: cell prefixed with glutaraldehyde showing disruption of the outer wall layer. Lead-stained section X 110,000.

FIGURE 67

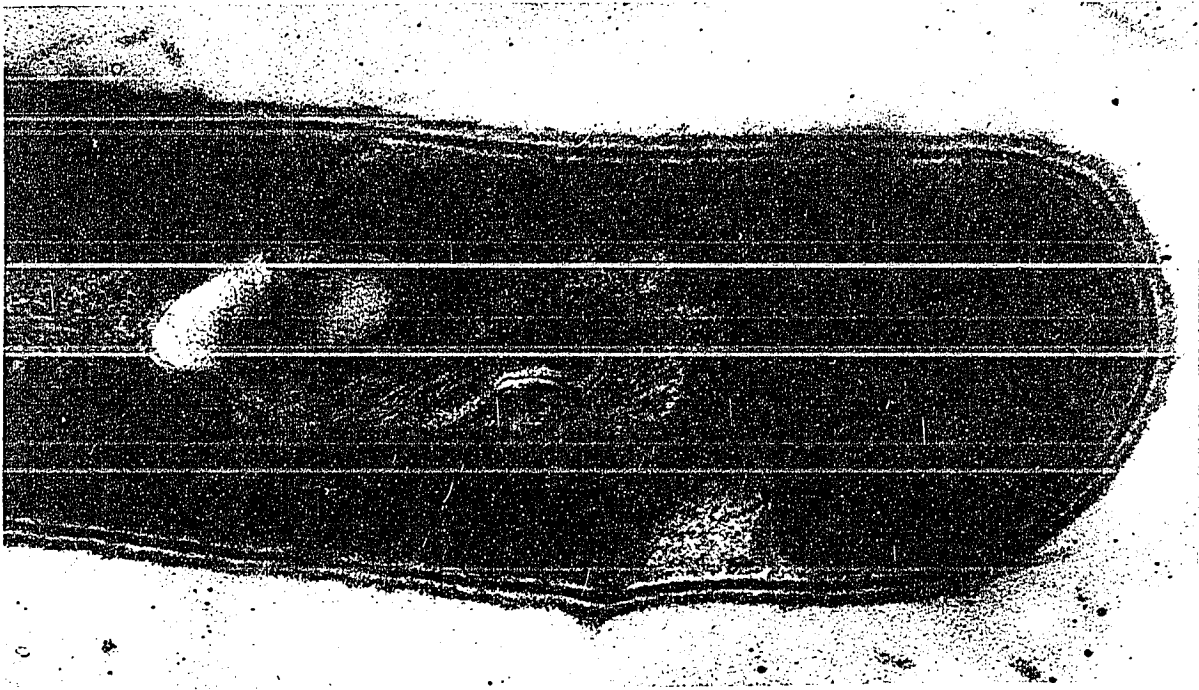


FIGURE 68



parent region ($40\overset{\circ}{\text{Å}}$ wide) separates this outer layer from a thick, electron dense element analogous to the rigid element in the wall profile of other species examined in this study. The width of the dense layer is of the order of $110\overset{\circ}{\text{Å}}$, much greater than that of the thin, rigid element of Spirillum serpens wall (40 to $45\overset{\circ}{\text{Å}}$). Another electron transparent zone ("gap substance") lies adjacent to the dense-light-dense profile of the cytoplasmic membrane. An imperfectly preserved membranous body is associated with the chromatin material and possibly represents a mesosomal structure.

Figure 68 shows a sectioned cell of L. longa aerated in YPA broth at 26°C for nine hours. This culture was prefixed with glutaraldehyde (6.5% v/v) followed by fixation in 0.05M phosphate buffer pH6.4 containing 1% (w/v) OsO_4 . Partial disruption of the wall resulted from this fixation procedure in contrast with the preservation of compact surface profiles in glutaraldehyde-fixed E. coli specimens (Figure 18). However, this picture of L. longa serves to indicate the rigid nature of the taut, electron-dense layer of the wall.

(2) Division

Division by septation is suggested by the section illustrated in Figure 68. This appears to be a late stage in the cytokinetic process as the dense septal element shows signs of splitting. Figure 69 demonstrates an early stage with inward projections of the inner dense wall layer contained within pockets of cytoplasmic membrane. Only the inner dense layer of the wall is involved in septum formation and in this

FIGURE 69

Lineola longa: section showing a nascent septum and associated mesosome in a dividing cell X 110,000.

FIGURE 70

Lineola longa: complex mesosomes in transverse cell sections X 78,000.

FIGURE 69

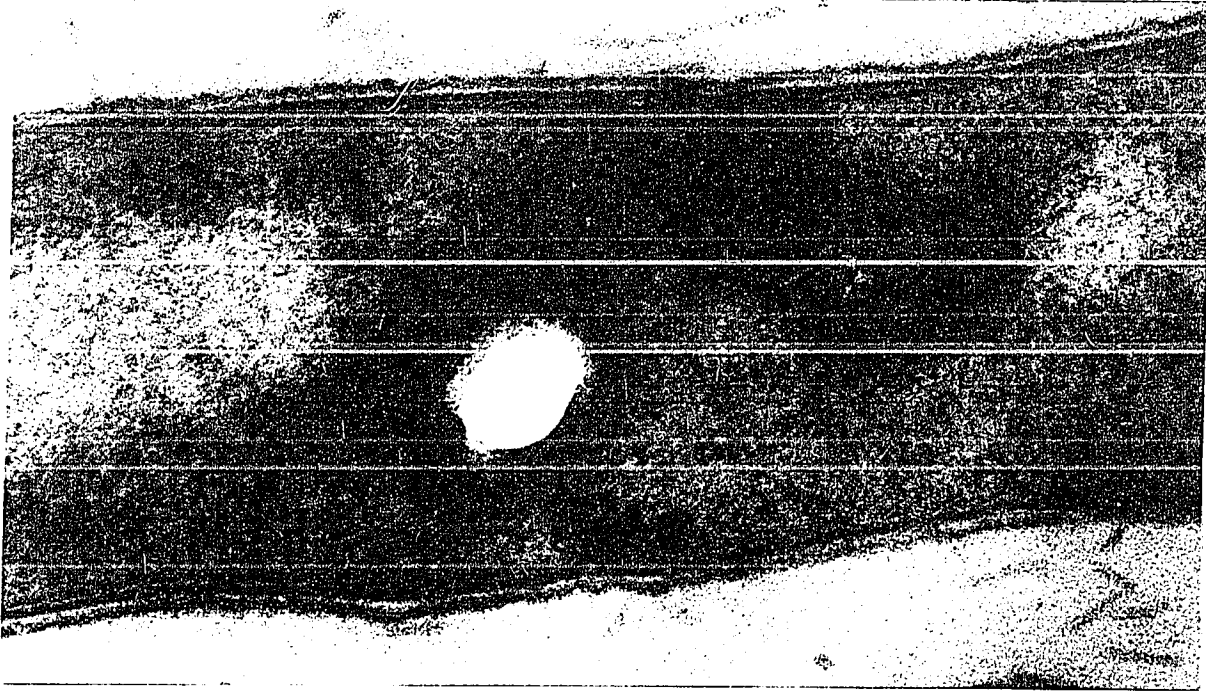


FIGURE 70



respect, the appearance of septation in L. longa resembles that in other Gram-negative bacteria.

The large complex membranous body lying immediately adjacent to one of the septal components in Figure 69 appears to be equivalent in structure and location to the mesosomes of Gram-positive bacteria. Other examples of mesosomes are shown in cross-sections of L. longa cells (Figure 70).

(3) Surface pattern

L. longa cells negatively stained with PTA produced preparations with little surface or internal detail; no evidence of a surface pattern was seen. However, when cells grown on YPA agar at 26°C for one to two days were shaken for one hour at room temperature in aqueous 0.01% (w/v) SLS then washed twice with distilled water prior to examination in the electron microscope, a regular pattern was observed. Figure 71 shows the intact pattern attached to a cell in a PTA preparation. Figure 72 depicts a shadowed specimen containing cell-free pattern material. Unlike the hexagonal arrangement in Spirillum serpens VHA, the globules (approximately 75Å diameter) form a rectangular array; centre-to-centre distance of the globules was estimated to be approximately 105Å. It would seem, then, that treatment with SLS followed by washing removes some surface material which normally masks this pattern in L. longa. A similar rectangular arrangement of globular elements has been described by Baddiley (1964) in what was thought to be the mucopeptide layer of Bacillus polymyxa walls, and

FIGURE 71

Lineola longa: spherical subunits arranged in a rectangular pattern at the surface of a cell washed twice after exposure to 0.01% SLS for 60 minutes. Phosphotungstate preparation X 83,000.

FIGURE 72

Lineola longa: cell-free pattern obtained from cells treated as in Figure 71. Note the globular subunits on one surface and the smooth, striated appearance of the other. Shadowed with tungsten oxide (reversed) X 110,000.

FIGURE 71

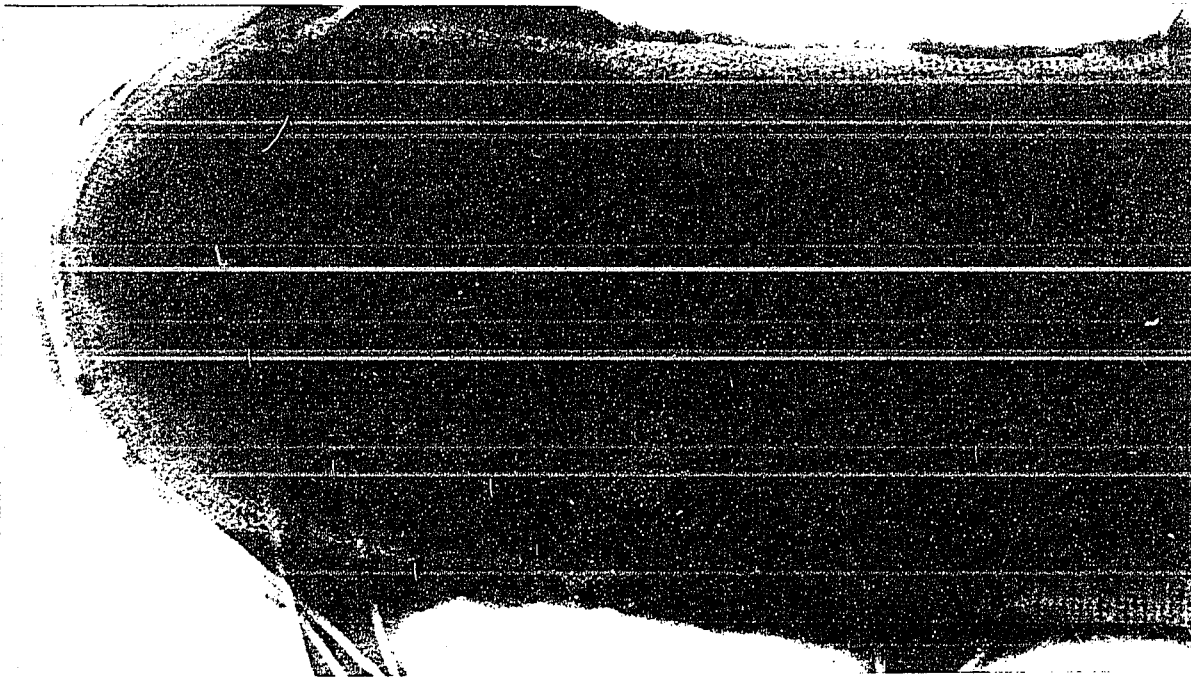
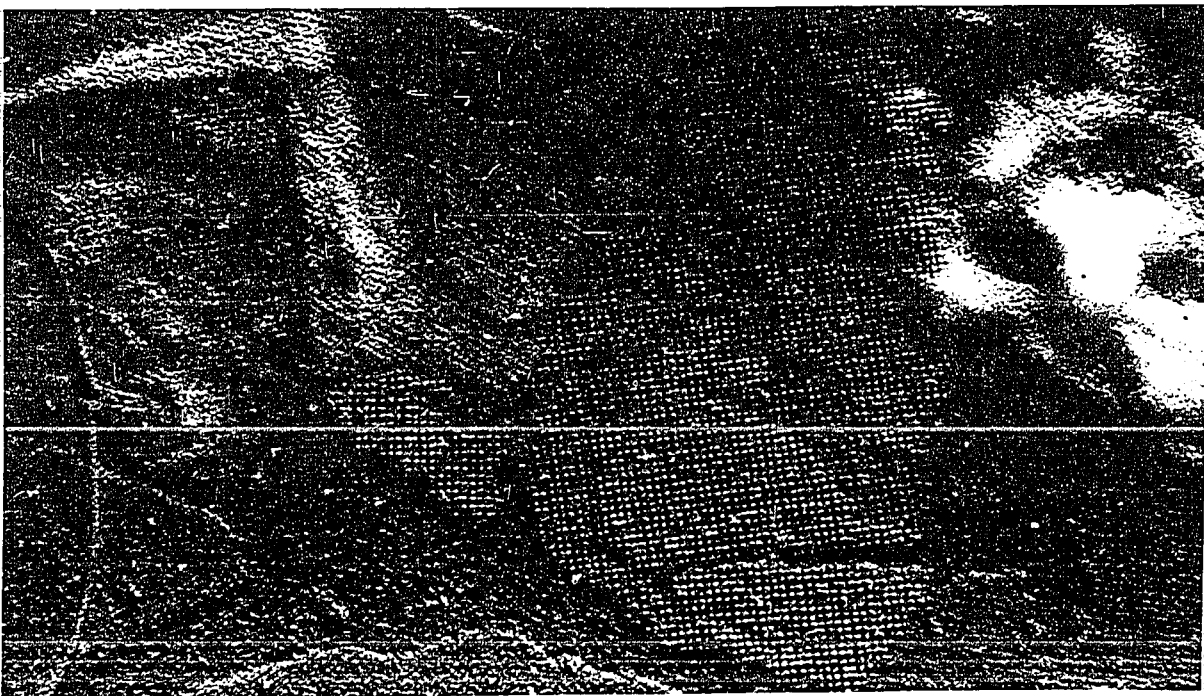


FIGURE 72



more recently by Nermut and Murray (1967).

DISCUSSION

(A) Surface Profiles

This study has demonstrated that the wall profiles observed in sections of Spirillum serpens, Escherichia coli, and ribbon-like gliding filaments of Simonsiella crassa and Alysiella filiiformis, are composed of two major structural components. These are the inner, electron-dense element and the outer triplet layer.

(i) The inner, dense layer

An inner, taut, dense layer has been established as a constituent of wall profiles in many Gram-negative bacteria (Murray, 1963a; Reyn et al., 1964). In 1964, Claus and Roth suggested that this layer might represent the mucopeptide element involved in rigidity. Experiments conducted in this study have shown that loss of cell shape and rigidity, effected by the combined action of lysozyme and EDTA, coincides with disappearance of the inner, dense layer from the wall profile. Thus, the taut, dense component lying between the triplet-layer and cytoplasmic membrane was identified as the rigid layer. This interpretation applied to all three types of Gram-negative bacteria included in this study. Support for this conclusion was provided by a similar loss of the same entity in penicillin-induced spheroplasts of E. coli. In addition to this

line of evidence, the ability of the taut, dense element to maintain cell rigidity when the triplet-layer was partially or completely disrupted also established the rigid nature of this component.

The question which arises from these observations is whether or not the rigid layer, so identified, is equivalent to the chemically defined mucopeptide moiety which represents the minimum constituent able to maintain rigidity and be dissolved by lysozyme. In the case of E. coli the answer is negative. Chemical analysis of the wall of E. coli has revealed that after removal of the non-covalently bound lipoprotein and lipopolysaccharide constituents, the underlying rigid structure ("R-layer") consists of mucopeptide with covalently attached lipid, protein and polysaccharide constituents (Leutgeb, Maas and Weidel, 1963). It is proposed, then, that in the sections of E. coli presented in this thesis, the unusually thick (65 to 70^oÅ) electron-dense rigid layer is equivalent to the chemically defined "R-layer." Only a thin, inner portion represents mucopeptide, the remainder probably being the covalently bound accessories. Evidence for this interpretation is indicated by the section of a dividing cell shown in Figure 35. This picture illustrates the newly synthesized septum in which the septal mucopeptide is continuous with only the innermost surface of the peripheral rigid layer. Furthermore, the conditions which produced spheroplasts of Spirillum serpens failed to do so with cells of E. coli. This, in itself, indicates a difference in the rigid layers of these two species, presumably in the accessibility of the mucopeptide moiety. This distinction in behaviour and structure

can be correlated with a difference in chemical composition. Martin and Frank (1962b) examined the residual walls of E. coli B and Spirillum after treatment with SLS. Spirillum was distinguished by the complete removal of all surface layers from the underlying smooth, rigid mucopeptide component. Thus, in the section of S. serpens shown in Figure 6 the rigid layer remaining after exposure to SLS should be equivalent to the mucopeptide moiety and it is this component that is digested by lysozyme. Corresponding elements in negatively-stained and shadowed preparations are depicted in Figures 9 and 10 respectively. The case of Simonsiella crassa and Alysiella filiformis is less certain but it seems reasonable to regard the rigid component of these species as mucopeptide on the basis of total involvement of the layer in septum formation.

Although most wall profiles observed in sections of E. coli possessed a thickened, rigid layer there were some instances in which the inner, dense element had the dimensions of the septal mucopeptide (Figures 16, 36). A satisfactory explanation of this discrepancy is not available at this time, but possibly some physiological and/or cultural factors as well as staining affinities may be involved.

(ii) The triplet-layer

The triplet-layer corresponds to the multilayered wall profile observed by Birch-Andersen, Maaløe and Sjöstrand (1953) and by Kellenberger and Ryter (1958) in sections of E. coli. Subsequent studies carried out by numerous investigators in this field indicate that the

triplet-layer is a feature common to all Gram-negative bacteria examined to date (Reyn et al., 1964). Presence of this component after destruction of bacterial shape and rigidity was demonstrated by Salton and Shafa (1958), thus characterizing its non-rigid nature. Sections of spheroplasts of Spirillum serpens, Escherichia coli and Simonsiella crassa, induced by the action of lysozyme and EDTA, were also shown to have the triplet-layer at their surface. From these observations, the dense-light-dense wall component can be readily identified as a non-rigid entity. However, the nature of this structure in terms of its chemical composition is much more difficult to assess.

On the basis that the triplet-layer is present in Gram-negative bacteria but is not found in Gram-positive species, it would be logical to regard this structure as being composed of chemical constituents peculiar to Gram-negative bacteria. Salton (1953) noted that Gram-negative species were distinguished by a higher lipid content and wider range of amino acids in their walls. In 1958, Weidel and Primosigh identified a lipoprotein component, soluble in 90% phenol, which could be separated from a phenol-insoluble lipopolysaccharide moiety in the wall of E. coli. The dense-light-dense profile of the triplet-layer, like that of the cytoplasmic membrane, has the "unit-membrane" configuration characteristic of many other membrane structures (Robertson, 1959). Thus, the lipid-containing components of Gram-negative bacterial walls might very well be accommodated by this "unit-membrane" wall entity, particularly in view of the fact that the model discussed by Robertson

can embody lipid, protein and polysaccharide constituents. Clarke and Lilly (1962) proposed such a scheme for Gram-negative bacterial surfaces in which a rigid component was thought to be sandwiched between two 'unit-membrane' elements, the cytoplasmic membrane and triplet-layer, with the lipopolysaccharide moiety of each membrane facing the middle rigid layer. Further chemical analysis of E. coli walls by Leutgeb, Maass and Weidel (1963) and by Martin and Frank (1962a) verified the presence of an outer lipoprotein layer, intermediate lipopolysaccharide component, and inner rigid element as the major wall constituents. The two outer components were found to be non-covalently attached to the rigid layer and were removed by treatment with SLS and phenol. Disappearance of the triplet-layer after exposure to SLS has been demonstrated in this study, and a similar effect was observed in Veillonella after treatment with phenol (Bladen and Mergenhagen, 1964; Mergenhagen, Bladen and Hsu, 1966). These results certainly implicate lipoprotein and lipopolysaccharide as components associated with the triplet-layer.

In 1965, Bayer and Anderson published a schematic wall model for E. coli which was based on their observations of negatively stained preparations of unfixed, frozen cells sectioned in a cryostat. The surface lipoprotein component, containing certain phage receptor sites, was depicted as an amorphous layer with irregular surface protrusions; the underlying lipopolysaccharide entity, in which other phage receptor sites are located, was partly exposed to the surface by means of a system of

channels. Endotoxins of enteric Gram-negative bacilli, composed of lipopolysaccharide associated with a protein or polypeptide moiety, were shown to be restricted to the cell wall by Ribí, Milner and Perrine (1959). In a subsequent study, Milner, et al, (1963) described the lipopolysaccharide layer of Gram-negative bacterial walls as the component containing the endotoxic somatic antigens. However, the ferritin-conjugated antibody study of somatic antigens by Shands (1965) revealed fibrillar strands of antigenic substance extending as far as 1500Å beyond the triplet-layer. It would seem, then, that the lipopolysaccharide, and possibly also the lipoprotein, constituents of Gram-negative bacterial walls are not strictly confined to specific strata in the wall profile, but tend to overlap and extrude beyond their location within the wall unit.

(iii) Compact profiles

The most commonly observed picture of the triplet-layer in sections of Gram-negative bacteria has been that of a loose, wavy component of the wall profile, even in such complex walls as those described by Maier and Murray (1965). This has been interpreted as a natural rather than artificial appearance by at least two groups of workers (Takagi and Ueyama, 1963; Ritchie, Keeler and Bryner, 1966) but most authors have been less certain or non-committal about this characteristic feature. An indication that it is an artifact is given by the closely applied strata in wall profiles of aldehyde-fixed specimens (Conti and Gettner, 1962; de Petris, 1965; Remsen and Lundgren, 1966). Figure 18 illustrates this effect in E. coli. Moreover, this study has shown the

occasional presence of compact profiles in osmium-fixed cells (Figures 14, 15 and 16). It is quite possible, then, that the loose, wavy appearance of the triplet-layer results from imperfect preservation and does not represent the true association of this entity in the wall structure.

The electron-transparent zone between the triplet-layer and dense, rigid component in wall profiles also presents a problem for interpretation. Opinion that this region represents a distinct layer of the wall has been expressed by several investigators (Takagi and Ueyama, 1963; de Petris, 1965; Mahoney and Edwards, 1966; Remsen and Lundgren, 1966). The width of this area is extremely variable in profiles containing a loose, wavy triplet-layer; only compact surfaces with a five-layered (dense-light-dense)-(light)-(dense) profile, such as those shown in Figures 14 and 16, indicate an ordered relationship between this intermediate component and the triplet-layer and rigid element of the wall.

(iv) Wall models

It is one thing to label certain images as layers in the wall structure, and another to relate these images to the chemical composition of Gram-negative bacterial walls. Martin (1963) provided elegant wall models of E. coli cells before and after conversion to spheroplasts by the action of penicillin and of lysozyme with EDTA. Composition of layers in these models was based on the results of biochemical dissection (Martin and Frank, 1962a). An outer lipoprotein,

an intermediate lipopolysaccharide, and an inner rigid layer (mucopеп-
tide with covalently bound protein granules) were depicted. Unfortunately,
the models were based on the triplet-layer alone, and did not take into
account the inner, dense layer of the wall profile.

Studies of a lysine-requiring E. coli mutant (Knox and Work,
1966; Work, Knox and Vesk, 1966) have provided some useful infor-
mation pertaining to the chemical composition of various layers in the
wall structure. Under lysine-limiting conditions the cells excrete a
complex consisting of 60% lipopolysaccharide, 26% phospholipid (mainly
phosphatidylethanolamine) and 10% protein (with all normal amino acids
except cystine and no diaminopimelic acid). Sections of cells excreting
the complex revealed surface blebs formed by outward protrusions of
the triplet-layer. Cell-free globules, apparently derived from the blebs,
became more numerous with increasing excretion of the complex. Thus,
the complex was thought to consist of the blebs and globules. The
sectioned material demonstrates that these structures are composed of
a homogeneous substance of medium electron-density bounded by the
triplet-layer. It seems likely that the chemical composition of the blebs
and globules represents that of the four outer layers, (dense-light-dense)
-(light), of the wall profile. It is proposed, then, that the intermediate
component located between the triplet-layer and rigid element is largely
lipopolysaccharide, while the triplet-layer embodies lipoprotein consti-
tuents.

An electron-transparent "gap substance" situated between the
rigid layer and the cytoplasmic membrane has been observed in sectioned

bacteria by many workers. Both de Petris (1965) and Remsen and Lundgren (1966) regarded this area as the rigid mucopolymer layer. Such an interpretation is in direct disagreement with the evidence provided in this thesis for identification of the inner, taut, electron-dense element as the structure containing the rigid mucopeptide component. (The wall model proposed by Remsen and Lundgren (1966) is particularly difficult to understand in that the inner electron-dense layer is depicted as a loose, wavy structure while the triplet-layer is illustrated as a taut, smooth entity). Other interpretations have implicated the "gap substance" as an entity associated with the cytoplasmic membrane (Fitz-James, 1960; Murray, 1960/1961, 1963c). It is proposed that this area does not represent a real structural layer of the wall. Rather it may be the site of unassembled wall constituents. The pronounced increase in the width of the "gap substance" in the lysine-requiring E. coli mutant described previously (Work, Knox and Vesk, 1966) supports this proposition. It is only logical that when wall material is being produced in excess (as indicated by the formation of blebs and globules) the site of synthesis and accumulation of the unassembled constituents should also appear enlarged.

A model of the surface profile of a "typical" Gram-negative bacterium, such as Spirillum serpens VHL, is shown in Figure 73. Models proposed by other investigators are also presented for purposes of comparison (Figures 74, 75). It is obvious that no single model can apply to all Gram-negative bacteria. There are many indications in the

FIGURE 73

Wall Models

Models proposed for "typical" Gram-negative bacterial surfaces.

A. Wavy Profile

B. Compact Profile

a: triplet-layer; mainly lipoprotein.

b: intermediate layer; mainly lipopolysaccharide.

c: rigid layer; mucopeptide and covalently-bound accessories when present.

d: "gap substance"; unassembled wall constituents and substances involved in their synthesis.

e: cytoplasmic membrane.

f: cytoplasm.

C.

It is possible that there is some degree of overlap of the lipopolysaccharide moiety. In addition, the presence of lipopolysaccharide as fibrils extending beyond the triplet-layer (Shands, 1965) and exposure of lipopolysaccharide phage receptor sites to the surface (Bayer and Anderson, 1965) indicate that this constituent is not strictly confined to a layer within the wall structure.

a': protein.

a'': lipid.

a''': protein and/or polysaccharide.

b-f: as in A., B.

FIGURE 73.

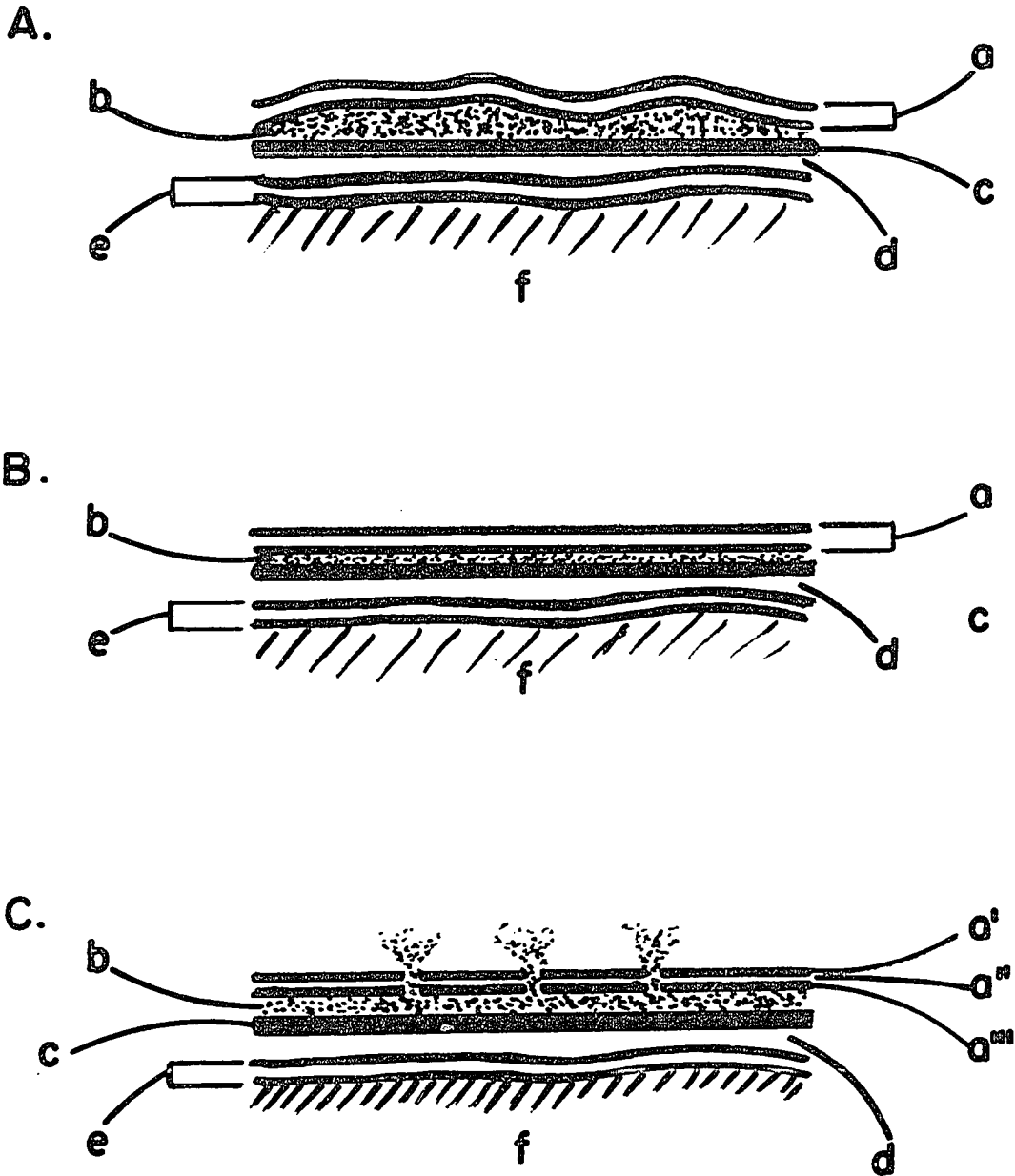


FIGURE 74

Wall Models

- A. Escherichia coli: from Kellenberger and Ryter (1958).
- CW: triple-layered cell wall; possibly polysaccharide coated on either side by proteins and/or lipidic groups.
- CM: cytoplasmic membrane.
- C: cytoplasm.
- B. Escherichia coli: from Martin (1963).
- CW: cell wall.
- LP: lipoprotein layer.
- LS: lipopolysaccharide layer.
- RL: rigid layer.
- PG: protein granula
- MP: mucopolymer.
- CM: cytoplasmic membrane
- C: cytoplasm.
- C. Escherichia coli: from Ogura (1963).
- F: filaments; possibly polysaccharide.
- O: outer layer of water-soluble mucopeptides and mucopolysaccharides.
- I: inner layer; mainly lipoprotein.
- CM: cytoplasmic membrane.

FIGURE 74.

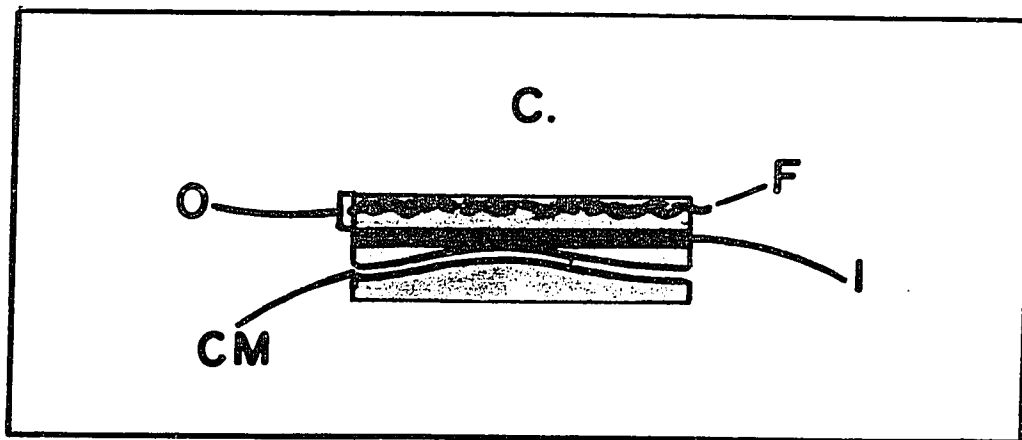
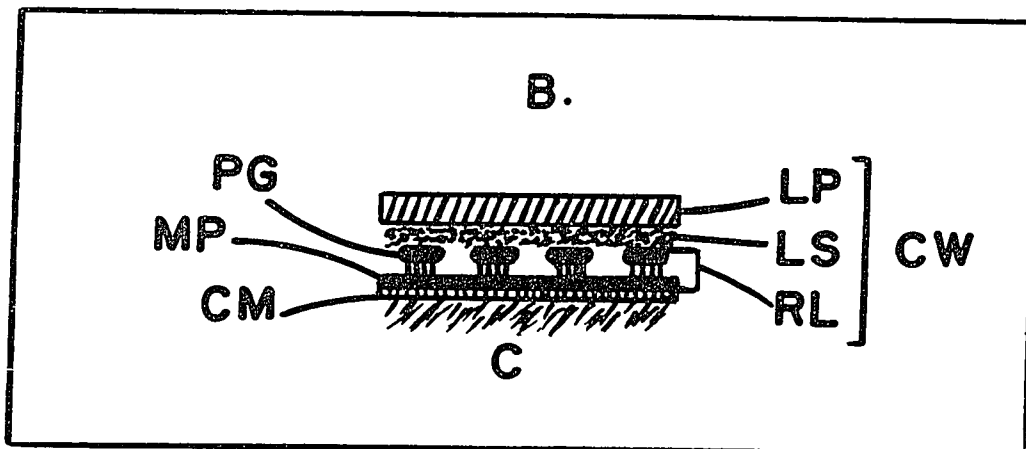
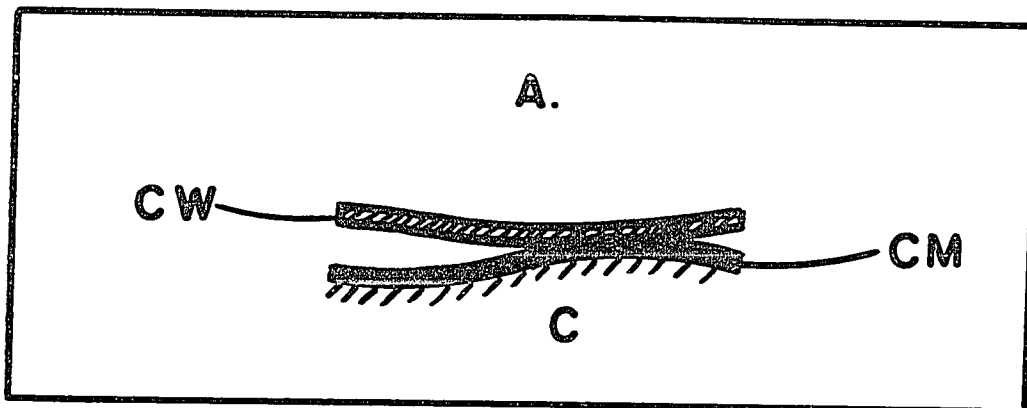


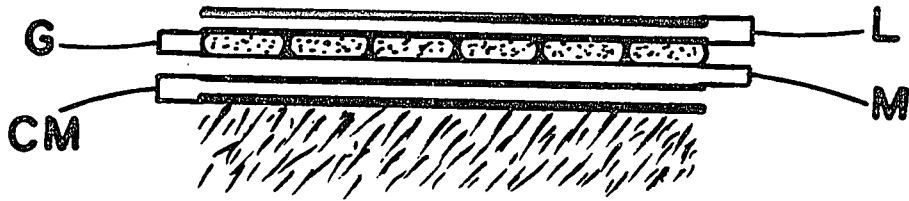
FIGURE 75

Wall Models

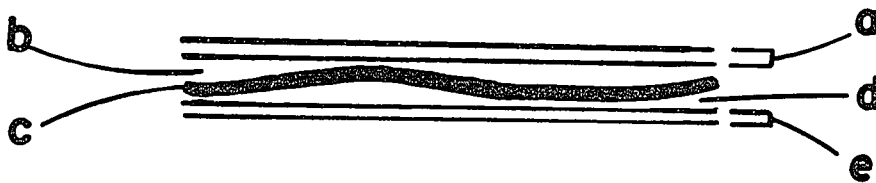
- A. Escherichia coli: from de Petris (1965).
- L: triplet-layer; possibly a complex mosaic structure of lipoprotein - lipopolysaccharide.
 - G: globular protein layer.
 - M: mucopolymer layer.
 - CM: cytoplasmic membrane.
- B. Ferrobacillus ferrooxidans: from Remsen and Lundgren (1966).
- a: lipoprotein layer.
 - b: lipopolysaccharide layer.
 - c: globular protein layer.
 - d: mucopolymer layer.
 - e: cytoplasmic membrane.

FIGURE 75.

A.



B.



literature that wall components, particularly the rigid layer, are constructed and behave quite differently in certain species such as marine bacteria (Buckmire and MacLeod, 1964) and treponemes (Pillot, Ryter and Ginger, 1966). Also, the brief study of Lineola longa has shown that this bacterium has a distinctive wall profile. However, because the wall model presented here is based on the structure and behaviour of walls in several bacteria, it may be representative of the "typical" Gram-negative bacterium.

(B) Division

(i) Septa

It is the common consensus of opinion that many Gram-negative bacteria, including E. coli and Spirillum serpens, divide by constriction while most Gram-positive species divide by septation. However, this study has demonstrated that incubation at 45°C consistently produced cells of E. coli and S. serpens dividing by septation. Moreover, the higher growth temperature was found to be statistically significant in determining the appearance of dividing S. serpens cells. Apparently, then, the cytokinetic mechanism of S. serpens is directly affected by the incubation temperature. Support for this view is found in the variety of temperature-dependent effects on mutants with division impairments (Plunkett, 1962; van de Putte et al, 1963, 1964; Ivanovics, 1964; Adler and Hardigree, 1965; O'Donovan, Kearney and Ingraham, 1965). Some of the effects induced by temperature consisted of abolition

of the division inhibition while others were themselves inhibitory, indicating that different types of mutation were involved. Among the temperature-sensitive E. coli mutants studied by Kohiyama, Cousin, Ryter and Jacob (1966) was one dividing with septa structurally similar to those described in this study. However, the regular appearance of septa in S. serpens and E. coli grown at 45°C cannot be explained in terms of selection of mutants by growth at this temperature. Rather, it indicates a selective effect on certain stage(s) of the division mechanism itself.

When cells of S. serpens and E. coli grown at routine temperatures (26°C, 30°C or 37°C) were fixed by the standard method of Kellenberger et al. (1958), relatively few cells appeared to be in the process of cytokinesis. Division, when observed in such sectioned material was always constrictive in appearance. However, when the hypertonicity of the standard veronal-acetate (VA) buffer was remedied by suitable dilution, dividing cells were observed more frequently, many of which possessed septa. These results suggest that septation is the normal division process in these bacteria.

If, indeed, septa are formed in bacteria which appear to divide by constriction, then the absence of septa can be explained in terms of their lability and inadequate preservation by commonly used methods of fixation. The labile, rather than static, nature of bacterial walls was emphasized by Weidel, Frank and Leutgeb (1963) who pointed out that the mucopeptide element is particularly susceptible to the action of autolytic enzymes. Weidel and Pelzer (1964) described a balanced co-ordination

of synthetic and degradative enzymes required for incorporating new units into the growing mucopeptide complex. An upset of this delicate balance and consequent degradation of labile mucopeptide could occur during fixation since fixatives do not cause immediate cessation of all enzymatic activities (Barnett, 1964). Furthermore, signs of plasmolysis, observed in S. serpens and E. coli cells suspended in the standard hypertonic fixative, reversed before fixation was complete. Thus, the structures which constitute septa in these cells are the very elements which are susceptible to degradation or disruption when "fixed" by standard procedures. This behaviour of septal mucopeptide and cytoplasmic membrane could, therefore, account for the appearance of constriction, rather than septation, in dividing Gram-negative bacteria.

Several kinds of Gram-negative bacteria are known in which division has always appeared as a septation process (Morita and Stave, 1963; Bladen and Mergenhagen, 1964; Maier and Murray, 1965). The ultrastructural study of stalked bacteria by Poindexter and Cohen-Bazire (1964) is of particular interest in that Asticcacaulis possessed septa while the closely related Caulobacter appeared to divide by constriction. Similarly, septa have been demonstrated in Spirillum volutans (Williams and Rittenberg, 1957) but are not found in Spirillum serpens under normal conditions of growth and fixation. It is proposed then that Gram-negative bacteria do divide by septation, the appearance of constriction being the outcome of imperfect preservation of septal components. In species whose septa are preserved under normal conditions of growth and

fixation, the mechanism involved in stabilizing the septa may be better developed than in "constrictive" species. This stabilization, which may be determined by certain kinds or numbers of cross-linkages in the mucopeptide structure, can be achieved in septa of S. serpens and E. coli by growth at 45°C. In all instances, the septa observed in Gram-negative bacteria consist of the innermost, rigid electron-dense wall layer (mucopeptide) and associated cytoplasmic membrane.

(ii) Mesosomes

Complex membranous bodies, termed mesosomes (Fitz-James, 1960) were assigned a role in division of Gram-positive bacteria (Ryter and Jacob, 1963, 1964). Similar structures were not observed in Gram-negative bacteria although examples of simple membranous elements were described in some species (Vanderwinkel and Murray, 1962). It is now known that certain Gram-negative species, such as the stalked bacteria, do possess mesosomes (Poindexter and Cohen-Bazire, 1964). Moreover, this study has demonstrated the presence of mesosome-like bodies associated with septa in bacteria which normally lack these structures. Absence of mesosomal membranes in bacteria exposed to normal conditions of growth and fixation could also be explained in terms of inadequate preservation when reorganization of membranes can occur. Thus, the presence of mesosomal structures can be considered as a feature of dividing Gram-negative bacteria. This view is supported by the effect of pantoyl lactone observed in this study. The stimulation of membrane production at the site of division (Figure 40) and the abolition

of certain division inhibitions by this reagent (van de Putte et al., 1963; Adler and Hardigree, 1965; Kantor and Deering, 1966) suggest that mesosomal membranes function in the division of Gram-negative bacteria.

Conclusion

Robinow (1945) considered septation and constriction to be essentially similar mechanisms differing in the time relationship of sequential stages common to both processes. The study of division presented in this thesis not only supports this view but indicates that constriction is really a type of septation that is particularly labile and subject to deformation when adequate preservation is not attained.

Invagination of the cytoplasmic membrane appears to be the initial step in the septation process and is accompanied by centripetal synthesis of septal mucopeptide. This mechanism, together with the association of mesosomal structures, is essentially the same process which operates in division of Gram-positive bacteria.

(C) Behaviour of the Surface Pattern

(i) Lability

Destruction of both cell-bound and cell-free pattern of S. serpens VHA by urea and by guanidine hydrochloride, agents known to denature proteins (Joly, 1965) suggests that non-covalent bonds (probably hydrogen and/or ionic) are involved in holding together the globular subunits as a network attached to the cell surface. The hexagonally arranged

globules of VHA pattern resemble those described in Halobacterium cutirubrum (Kushner and Bayley, 1963) in their disappearance at low pH values, and those observed in Rhodospirillum rubrum (Salton and Williams, 1954) in their resistance to trypsin. The latter authors suggested that the subunits might be protein in nature; the results of chemical analysis of VHA pattern contained in this study support this suggestion.

(ii) Temporary loss

Cells of S. serpens VHA grown in liquid culture were found to lose the hexagonal pattern but always reproduced the component upon transfer to fresh liquid or solid media. The absence of identifiable fragments or subunits of the pattern in culture supernatants in which this loss occurred suggests that extensive degradation of the pattern constituents takes place. The mechanism involved in the disappearance and reappearance of the surface hexagonal moiety is not known. However, it is suggested that further studies of S. serpens VHA liquid cultures, preferably in chemically defined media with antigenic analysis of the culture supernatants, might throw some light on the behaviour of this surface network.

(iii) Permanent loss

Observations of the pattern-less mutant, S. serpens VHL, indicate that permanent loss of the pattern does not impair the viability of the bacteria. Furthermore, the serological cross-reaction between VHA and VHL cells suggests that some surface component, possibly the

amorphous "backing" of the pattern is retained although the hexagonally arranged subunits seem to be no longer manufactured and assembled.

(iv) Lysis

VHA cells were lysed under conditions which failed to cause lysis of the pattern-less VHL cells. The lytic response by pattern-producing cells was elicited in aerated YPA broth cultures and appeared to be triggered by a depletion of specific divalent cations; an active lytic agent was not demonstrated. The role of cations in lysis was revealed by the inhibitory action of calcium and strontium whereas sodium and magnesium seemed to enhance the lytic effect. Both VHA and VHL cells failed to lyse when aerated in aqueous solutions of the metal-chelating agent EDTA. This observation indicated the involvement of active growth in the lytic behaviour of VHA cells.

These features can be compared with other examples of bacterial lysis involving metal cations. Calcium and strontium ions were assigned a role in stabilizing the rigid wall layer of a Gram-negative bacterium (Vincent and Humphrey, 1963) and these same ions were found to be specific in preventing lysis of VHA cells. Brown and Turner (1963) and Brown (1964) studied the stability of bacterial envelopes in relation to the ionic environment; the degree of tolerance to sodium chloride was shown to be significant with respect to envelope lysis which was inhibited by calcium chloride. These authors demonstrated that Spirillum serpens tolerated only 1% (0.2M) sodium chloride compared with 4% for Escherichia coli. Selective inhibition of multiplication of Pseudomonas aeruginosa

cells damaged by EDTA was abolished by divalent cations normally found in the cell wall (Asbell and Eagon, 1966). Furthermore, replacement of multivalent cations by sodium ions produced a weakened cell wall and osmotically fragile cells. Pseudomonas aeruginosa (Gray and Wilkinson, 1965a, b; Eagon and Carson, 1965; Asbell and Eagon, 1966) and marine bacteria (Buckmire and MacLeod, 1964; MacLeod, 1965) are well known examples in which metal ions are involved in maintenance of cellular integrity. Lysis of VHA cells could also be explained in terms of a loss of cellular integrity mediated by metal cations.

(v) Chemical composition

Chemical analysis of the aggregated cell-free pattern material produced in old agar cultures of S. serpens VHA revealed that it contained mainly protein and smaller amounts of lipid and polysaccharide. Electron microscopic examination of the isolated material showed the hexagonal arrangement of globular subunits associated with an amorphous "backing." The amorphous material which accumulated in old agar cultures of S. serpens VHL showed a structural similarity to the "backing" of VHA pattern and was found to consist mainly of lipid with smaller amounts of protein and polysaccharide. The phospholipids present in both VHA and VHL samples appeared to be identical and were identified as phosphatidyl-ethanolamine. The results of chemical analysis and microscopic examination suggest that the hexagonally arranged globular subunits of VHA pattern are composed of a protein containing a wide range of amino acids (but no cystine or histidine) and are associated with

an amorphous "backing" (mainly phospholipid) which seems to be equivalent to the material accumulated in old agar cultures of VHL. It is possible that this "backing" substance is in some way related to the heteropolymers (lipoprotein, lipopolysaccharide) which form structural elements of the cell wall.

A major problem, yet to be resolved, is whether or not the cell-free pattern isolated from VHA cultures represents the intact hexagonal network as it occurs at the cell surface. It is possible that the isolated cell-free material is an artificial product formed by the binding of individual globular subunits to extracellular aggregates of "backing". If this is not the case, the accumulated cell-free pattern may be the "slough" cast off as a new "skin" of pattern is synthesized and assembled at the cell surface.

Phosphatidyl ethanolamine has been identified as a major phospholipid constituent in walls of several Gram-negative bacteria (Kates, 1964; Asbell and Eagon, 1966; Osborn and Weiner, 1967) and in the wall-derived lipoglycopeptide excreted by a lysine-requiring E. coli mutant (Knox, Vesik and Work, 1966; Knox and Work, 1966; Taylor, Knox, and Work, 1966; Work, Knox and Vesik, 1966). The lipopolysaccharide-containing endotoxins of enteric Gram-negative bacteria have been the subject of intensive investigation by several groups of workers in recent years (Ribi, et al, 1959; Milner, et al, 1963; Shands, 1965). The morphological appearances of negatively stained endotoxin and lipopolysaccharide extracts (Mergenhagen, Bladen and Hsu, 1966; Rudbach, Anacker,

Haskins, Johnson, Milner and Ribí, 1966; Keeler, Ritchie, Bryner and Elmore, 1966; Shands, Graham and Nath, 1967) are not unlike the pictures of the amorphous cell-free aggregates shown in Figure 52 and Figure 59. Studies on the role of phospholipid in the biosynthesis of wall lipopolysaccharide (Rothfield and Takeshita, 1966; Osborn and Weiner, 1967) have implicated phosphatidylethanolamine as an active agent in determining the physicochemical state of the lipopolysaccharide. Hancock and Meadow (1967) found that most of the cell lipids were distributed in the wall lipoprotein and the cytoplasmic membrane of Pseudomonas aeruginosa. Four polar lipids (principally phosphatidylethanolamine) were identified and the authors concluded that lipid compositions of both structures were practically identical.

Asbell and Eagon (1966) suggested that the divalent cations involved in cellular integrity of Pseudomonas aeruginosa might be associated with the wall phospholipids (principally phosphatidylethanolamine). These authors speculated upon a physicochemical association of lipopolysaccharide and lipoprotein wall components with bonding by multivalent cations.

On the basis of the evidence presented above, the following explanation of the behaviour of S. serpens VHA pattern is proposed. It is assumed that continuing synthesis of pattern components operates in both liquid and solid cultures of S. serpens VHA. The loss of pattern from the cell surface and lack of accumulation of cell-free pattern in liquid cultures are in direct contrast with the behaviour in agar cultures.

Perhaps some physical or physicochemical factors are involved in this differential behaviour. Because the unassembled pattern components in liquid cultures are not observed as the distinct globular subunits which constitute the hexagonal array, it is thought that sub-microscopic constituents of the pattern are produced without further polymerisation. Depletion of certain divalent cations in broth cultures could then be the result of chelation by exposed groups or sites on the unassembled constituents; formation of globular subunits by the proper assembly of the constituents would eliminate this excessive metal-binding activity. The specificity of calcium and strontium in preventing lysis of VHA cells identifies these ions as those depleted from liquid media. Both calcium and strontium have been shown to occur in bacterial walls (Vincent and Humphrey, 1963) and several instances of cation-mediated cellular integrity have already been discussed. Since only growing VHA cells are lysed in a depleted cationic environment it seems likely that the lytic response is the outcome of competition for certain essential divalent cations. Perhaps the synthesis or stabilization of some structure vital to cellular integrity, such as occurs during cell division when new surface layers are manufactured at the division site, requires the participation of specific divalent metal cations. Lysis occurred only in aerated YPA broth cultures of S. serpens VHA; the organized pattern was lost in shaken and stationary broth cultures but lysis did not occur. It would seem, then, that conditions of growth by aeration favour the processes leading to depletion of certain cations with subsequent cell

lysis. Absence of lysis in aerated YPA broth cultures supplemented with calcium or strontium would indicate that the YPA medium is relatively low in content of these cations.

Finally, the accumulation of cell-free pattern in old agar cultures requires some explanation. It has been shown that the substance isolated from VHL cultures appears to be equivalent to the "backing" associated with the hexagonal array of VHA pattern both in ultrastructural appearance and in the presence of phosphatidylethanolamine. This phospholipid may be derived from the cytoplasmic membrane, perhaps functioning in the transport of components to be assembled at the cell surface. This kind of role was discussed by Osborn and Weiner (1967) in their study of lipopolysaccharide biosynthesis in Salmonella, and was also mentioned by Bayer and Anderson (1965) with respect to wall structure in E. coli. Shands (1965) demonstrated the presence of elongated strands of antigenic lipopolysaccharide extending beyond the cell surface. It is possible, then, that endotoxins of enteric Gram-negative bacteria, and the surface pattern of S. serpens VHA, may be formed by complexing with a phospholipid moiety involved in the transport of surface components across the cytoplasmic membrane. Excessive production of such a complex would then explain the cell-free accumulation of pattern in VHA cultures. Similarly, the "backing"-like substance isolated from VHL cultures could also result from over production of such a phospholipid moiety.

(D) A General Look at Bacterial Walls

The brief ultrastructural study of Lineola longa included in this thesis served to illustrate that bacteria are not always suitably classified by the Gram stain. With respect to wall structure, L. longa fits neither the "typical" Gram-positive nor the "typical" Gram-negative category. The Gram stain reaction and multilayered wall relate this organism to other Gram-negative bacteria. However, the absence of a triplet-layer and presence of an unusually thick, rigid layer in the wall are features which set this bacterium apart from the Gram-negative group. Multilayered walls are known in some Gram-positive species (Thornley, Horne and Glauert, 1965; Nermut and Murray, 1967). Despite the Gram stain reaction, L. longa possesses characteristics found in Gram-positive rather than Gram-negative bacteria. Furthermore, L. longa cultures have been shown to become Gram-positive and form spores (Bennett and Canale-Parola, 1965). It would, therefore, be of interest to see if some structural alteration of the wall profile is associated with the change in Gram stain reaction.

With respect to the structural appearance of sectioned walls, a bacterium may be considered as being either:

- (i) a "typical Gram-positive" in having a thick homogeneous rigid wall, such as Micrococcus lysodeikticus (Salton and Chapman, 1962),
- (ii) an "atypical Gram-positive" in having a multilayered wall with a relatively thick inner, rigid layer, such as Micrococcus radiodurans (Thornley et al, 1965),

- (iii) an "atypical Gram-negative" in having additional layers, such as the hexagonal pattern of Spirillum serpens VHA or complex outer layers of Beggiatoa (Morita and Stave, 1963; Maier and Murray, 1965) outside the basal Gram-negative wall structure, or
- (iv) a "typical Gram-negative" with a thin rigid layer and outer triplet-layer, such as Spirillum serpens VHL.

In the middle of this series could be placed Lineola longa and other species such as Micrococcus cryophilus (Mazanec, Kocur and Martinec, 1966) to represent transitional types.

The similarities and differences in the wall structures of bacteria are a reflection of their chemical composition. A mucopeptide component, fundamental to wall strength, is common to most, if not all, bacteria with the exception of Mycoplasma species. In "typical Gram-positive" bacteria the non-mucopeptide wall elements are incorporated with the mucopeptide to form a morphologically homogeneous structure; "atypical Gram-positive" species have additional non-mucopeptide components assembled as distinct outer layers. In Gram-negative bacterial walls the non-mucopeptide constituents, with the exception of those covalently attached to the mucopeptide, are organized as separate outer layers. The broad spectrum of wall structure in bacteria may explain the quantitative rather than qualitative difference between Gram-positive and Gram-negative species observed by Salton (1958, 1963), who also noted the range of lysozyme-sensitivity among bacteria. The accessibility of the mucopeptide substrate to lysozyme action would be expected

to vary according to the amount and/or kind of accessory wall components.

In considering the dispensability and essentiality of wall components it seems that the non-rigid elements of Gram-negative bacterial walls are not essential for maintenance of cellular integrity although their association with the rigid layer may have some indirect stabilizing effect. Weidel, Frank and Leutgeb (1963) remarked upon the ability of the outer wall layers to maintain cell shape in the absence of the rigid layer. Such cells, however, would disintegrate if the weak, non-covalent bonds of the residual wall were disrupted. The mucopeptide, together with any covalently bound constituents which may play some part in determining the rigidity of this structure, protect the cell against internal osmotic pressures and, in this respect, would appear to be indispensable. However, the variety of L-forms, particularly those of Proteus mirabilis studied by Martin (1964) indicates that "spheroplast L-forms" can manage with defective mucopeptides while "protoplast L-forms" apparently exist without cell walls. Perhaps these kinds of cells are protected by a cytoplasmic membrane which is associated with or modified by certain stabilizing entities other than those forming the cell wall structure.

The stabilization or "holding-together" of the mucopeptide required for maintaining rigidity may be accomplished in different ways by different bacteria. Specific cations have been assigned such a role in Pseudomonas aeruginosa (Gray and Wilkinson, 1965a, b; Eagon and Carson, 1965; Asbell and Eagon, 1966) and marine bacteria (Buckmire

and MacLeod, 1964; MacLeod, 1965). Perhaps this mechanism operates to some extent in other bacteria but varies in the degree and specificity of cationic involvement which may depend upon the ionic environment.

It is a well known fact that bacteria come in assorted shapes and sizes.

If shape, as well as rigidity is determined by the rigid layer, then the variety of bacterial forms is another indication of variation in the assembly of mucopeptide constituents.

Muramic acid, a characteristic component of mucopeptide, has been identified not only in bacteria, including actinomycetes and spirochaetes, but also in cyanophytes and rickettsiae (Ginger, 1963). The evolutionary implications arising from this distribution would identify the primitive predecessor of all these organisms as a protoplast protected by a wall composed of the simplest muramic acid-containing moiety able to provide rigidity. This would imply that Mycoplasma species, which lack cell walls, evolved separately from these organisms.

SUMMARY

(1) Electron microscopic examination of sectioned specimens of Spirillum serpens, Escherichia coli, Simonsiella crassa and Alysiella filiformis revealed a multilayered wall profile similar to that described in other Gram-negative bacteria. This profile was seen to consist of an inner, electron-dense component and an outer dense-light-dense triplet-layer. The inner component was identified as the rigid layer embodying the mucopeptide moiety which provides the mechanical strength of bacterial walls. The triplet-layer was shown to be a non-rigid element which remained intact in osmotically-sensitive spheroplasts. Removal of this outer component in Spirillum serpens was accomplished by treatment with sodium lauryl sulphate, an anionic detergent known to disrupt the lipoprotein layer of Gram-negative bacterial walls. An additional wall component was demonstrated in the surface profile of Spirillum serpens VHA and was identified as the hexagonal pattern produced by this organism.

The information derived from these studies was correlated with chemical data provided by other workers in this field. Schematic models were proposed to account for the structure, composition and behaviour of these walls and were compared with models of other investigators.

(2) Apparent differences in the mode of cell division were studied in relation to the role played by the surface layers. When growth temperatures and fixation procedures were varied, division by septation was observed in bacteria which appeared to divide by constriction under normal conditions of growth and fixation. Some unity in the processes of bacterial division was indicated by the consideration of constriction as an imperfectly preserved septation. In addition to this, the role of mesosome-like bodies in division of Gram-negative bacteria enabled a more satisfactory comparison to be made with the division mechanism of Gram-positive bacteria.

(3) A comparative study of Spirillum serpens VHA and its patternless mutant Spirillum serpens VHL demonstrated that cells producing the pattern component were lysed under certain growth conditions. Isolation and chemical analysis of the pattern material from S. serpens VHA and of an extracellular substance from S. serpens VHL proved useful in attempting an explanation of the nature of this lytic phenomenon.

(4) Electron microscopic studies of Lineola longa revealed that this organism is somewhat of a taxonomic misfit when compared with other Gram-negative bacteria. The consideration of L. longa as a transitional species within a continuous series of Gram-positive and Gram-negative bacteria was discussed in terms of wall structure. Finally, a generalized view of structure, composition and behaviour of bacterial walls was presented.

"Faith is a fine invention
For gentlemen who see;
But microscopes are prudent
In an emergency!"

..... Emily Dickinson ⁽¹⁾

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APPENDIX I

Routine Preparation for Sectioning

All steps for routine preparation of bacterial specimens for sectioning were carried out at room temperature unless stated otherwise. The procedure is essentially that described by Kellenberger, Ryter and Séchaud (1958). However, since a large portion of the thesis involves sectioned material, the details of various preparative steps are made available.

(1) Prefixing

Cells on agar were prefixed by flooding the agar surface with veronal-acetate buffer pH6.1 (VA buffer) containing 0.1% (w/v) osmium tetroxide (O_5O_4). Bacterial specimens already in suspension were prefixed by direct addition of 1% or 2% O_5O_4 to give 0.1% concentration. After centrifugation, the prefixed specimens were recovered for enrobing.

(2) Enrobing

The pellet of prefixed material was mixed with one or two drops of melted 2% (w/v) Noble agar (Difco) kept hot in a depression slide placed in a thermostatically controlled hot plate. The mixture was sucked up into the narrow stem of a pasteur pipette and set aside to cool and gel. The long, thin cylinder of agar was then removed and cut into several small discs.

(3) Fixing

Agar discs containing bacterial specimens were fixed in VA buffer containing 1% O_5O_4 and one to two drops per ml of a solution consisting of 1% (w/v) Bactotryptone (Difco) and 0.5% sodium chloride. Fixation periods varied from 4 to 18 hours.

(4) Staining

After removal of the fixative, uranyl acetate (0.5 to 1% w/v) dissolved in distilled water or in VA buffer was used to rinse and then stain the fixed specimens. Staining was carried out for 1 to 2 hours.

(5) Dehydrating

Specimens were dehydrated by passing the discs through an acetone series allowing 15 minutes in each of 30%, 50% and 75% (v/v) acetone and 30 minutes in each of 90% (v/v) and absolute acetone (stored over anhydrous copper sulphate).

(6) Embedding

The polyester embedding medium Vestopal W, was obtained from M. Jaeger, Vérenaz, Geneva. Discs were placed for 1 hour in each of 25%, 50% and 75% (v/v) Vestopal in absolute acetone mixtures. The final mixture (VIA) contained 1% (v/v) tertiary-butyl-perbenzoate (initiator) and 1% (v/v) cobaltnaphtenate (activator) in pure Vestopal. The discs were soaked in this mixture overnight then transferred to gelatin capsules (No. 4, Eli Lilly and Company) filled with fresh VIA mixture. Polymerization was allowed to proceed at 60°C to 65°C for three to four days.

(7) Sectioning

Thin sections were cut with glass knives mounted on Sorvall Porter-Blum Ultra-Microtomes (Models MTI and MT2). The sections were floated on the surface of distilled water and picked up on coated grids.